General introduction
### 1.1 The complex brain

The nervous system is one of the most complex organs able to perceive external stimuli, integrate them and regulate voluntary and involuntary processes in the body. The basic cellular entities of the brain are neurons and non-neuronal cells which form a complex network allowing communication between them. The human brain consumes about 20% of the entire body’s energy and has an average weight of 1.5 kg being comprised of 86 billion neurons and 85 billion non-neuronal cells (Magistretti and Allaman, 2015; Herculano-Houzel, 2009). The structure of individual neurons is highly important in defining the neuronal ability to receive and send information.

Dendrites are specialized to receive information and the combined signals from many dendrites are integrated in the soma. Axons, typically one per neuron, emanate from the soma and elongate to make connections with other neurons through specialized structures called synapses (Burns and Augustine, 1995). Action potentials (APs), generated at the axon hillock, are propagated along the axon to the presynaptic site of a synapse to send the information onto the receiving postsynaptic neuron (Figure 1). To understand the brain and its complexity, simple research questions, from a microscopic to macroscopic view, need to be answered and the findings merged together. Here I will introduce some fundamentals of molecular, cellular and physiological processes relevant for central nervous system functioning. In view of the high degree of evolutionary conservation of these processes, many insights derived from experiments in animal models are also important to understand human brain function.

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**Figure 1. Structure of three individual neurons.** Neurons (simplified) are characterised by a dendritic tree, a soma containing the nucleus and an axon. The synapse (dashed circle) is the contact point between the axon and the dendritic tree. Line with arrows indicates the direction of information transfer.
1.2 Neurotransmission

Neurotransmission refers to mechanisms that allow communication between neurons. Neurotransmission can occur via electrical or chemical synapses, which are the neuronal connection points that allow information transfer. Only a limited number of cell types employ electrical synapses. This thesis focuses on chemical synapses which are used throughout the nervous system. The major signalling components of chemical synapses are neurotransmitters. There is a number of different neurotransmitters, exerting different functions (Hyman, 2005). The neurotransmitters are stored in vesicles within the synapse. In response to specific triggering signal, the neurotransmitters are secreted into the synaptic cleft where they bind their cognate receptor on the surface of the postsynaptic cell. The final effect on the postsynaptic cell is determined by the activity of the receptor and downstream processes in the postsynaptic cell.

The two best characterised vesicle types are synaptic vesicles (SVs) and large dense core vesicles (LDCVs) (De Camilli and Jahn, 1990). SVs predominately contain classical neurotransmitters like glutamate or GABA and mediate fast synaptic neurotransmission. LDCVs contain neuropeptides, for example, Neuropeptide Y (NPY), monoamines and neurotrophins which are important for neuronal survival and synaptic plasticity (Gondré-Lewis et al., 2012; Park and Kim, 2009).

Several modes of neurotransmission have been identified that allow the release of SVs and LDCVs (Martin, 2003; Kaeser and Regehr, 2014). Secretion can occur in response to an action potential (AP-dependent) or in its absence (AP-independent) as detailed in the following paragraphs. Each secretory event involves the fusion of the vesicular membrane to the plasma membrane (PM) of the presynaptic neuron (Jahn and Fasshauer, 2012). The synaptic active zone defines the site of neurotransmitter release where several components (i.e. active zone proteins, secretory proteins, ion channels and SVs) concentrate and coordinate their action (Zhai et al., 2001). The molecular architecture of the active zone enables neurotransmitter secretion with fast kinetics, tight regulation and at close distance from the target receptors located on the postsynaptic side of the synaptic cleft. The most important aspects of this highly complex release machinery are detailed in the following paragraphs.

Cell biology of the synaptic release machinery

Prior to the formation of a synapse but also to maintain existing synapses by the delivery of new material, active zone proteins are transported in piccolo-bassoon transport vesicles (PTVs) (Shapira et al., 2003; Zhai et al., 2001), while synaptic vesicle-associated proteins are transported in synaptic vesicle precursors (SVPs) (Sytnyk et al., 2004; Ahmari et al., 2000). After transport from the soma along the axon to individual sites of each synapse (Bury and Sabo, 2011; Gondré-Lewis et al., 2012), SVs are filled with neurotransmitters by the activity of vesicular transporters (Edwards, 1992; Blakely and Edwards, 2012; Takamori et al., 2006). SVs accumulate at the synapse in vesicle pools which are defined based on the localization and/or maturation state of SVs (Alabi and Tsien, 2012). Interesting-
ly, observations from fluorescence imaging and correlative electron microscopy showed that SVs can be exchanged by transport between synapses in the same axon (Staras et al., 2010). Before vesicles are ready to fuse with the PM, they first need to undergo docking and priming steps (Banerjee et al., 1996). After priming, SVs are ready for release upon AP stimulation. The total number of release-ready vesicles together constitute the readily releasable pool (RRP). After the SV release event, the phospholipids and proteins are endocytosed and the SVs are retrieved, re-acidified, refilled with neurotransmitters and used for another round of fusion (Figure 2, right) (Rizzoli, 2014). A good measure of the size of the RRP is obtained by stimulation with hyperosmotic solution (Rosenmund and Stevens, 1996). This method has been extensively compared to depolarization-induced synaptic release parameters and is a widely accepted tool, even though the exact release mechanism is not elucidated.

**Synaptic plasticity**

The synapse is not a static structure: instead its chemical, structural and functional features change over time based on activity, a phenomenon referred to as plasticity. Synaptic plasticity is thought to be involved in memory formation. During synaptic plasticity, synapses can be strengthened or weakened, resulting in changes in the strength of neuronal connections (Ho et al., 2011; Citri and Malenka, 2008). More drastically, synapses can be completely disassembled or newly generated. Several processes (both pre- and postsynaptic) contribute to synaptic plasticity. In the presynapse, one fundamental mechanism of plasticity leading to long- and short-term plasticity is the change in SV release probability, which is reached by altering trafficking, docking, and fusion processes (Citri and Malenka, 2008; Park and Kim, 2009; Zucker and Regehr, 2002). Other possible ways include the lateral axonal transport of SVs in adjacent presynaptic boutons which may be involved in resizing of SV pools and, thus, could contribute to the regulation of the release probability (Darcy et al., 2006; Staras et al., 2010). The time course of short-term plasticity is on the order of milliseconds to several minutes and is triggered by local and relatively fast processes, such as the accumulation of presynaptic Ca$^{2+}$ due to neuronal activity, the depletion of readily-releasable vesicles or biochemical processes changing the SV number or release probability. Postsynaptic mechanisms also contribute to synaptic plasticity and to overall brain function, but these processes are considered beyond the scope of this thesis.

**1.3 Evoked neurotransmission**

AP-dependent neurotransmission also called evoked neurotransmission is well characterised in terms of its time course and the pathways involved (Südhof, 2012). AP-dependent neurotransmission is an extremely fast type of secretion which lasts for hundreds of microseconds through a combination of electrical and chemical signals, resulting in the synchronized fusion of many SVs from a single cell or even from a single synapse (Sabatini and Regehr, 1996; Meinrenken et al., 2003). Like many other types of regulated membrane fusion, it depends on a core machinery named the SNARE complex (abbreviated from soluble NSF at-
Attachment protein receptor, where NSF is the N-ethylmaleimide-sensitive factor. The SNARE complex contains a four-helical bundle which contains two coiled coil domains of SNAP25, one of syntaxin-1 and one of synaptobrevin-2, also named VAMP2 (Sutton et al., 1998). SNAP25 and Syntaxin-1 are associated with the target membrane and, hence, named t-SNAREs, whereas synaptobrevin is anchored in the vesicle membrane and is named a v-SNARE (Söllner et al., 1993). Other types of regulated secretion employ other combinations of SNARE proteins, but the structural principles appear to be widely conserved (Fasshauer et al., 1998).

Assembly of the neuronal SNARE complex is sufficient for membrane fusion in cell-free assays (Weber et al., 1998) but additional components are essential to provide the exquisite speed and tight regulation that is characteristic for neurotransmitter secretion from living synapses. To support extremely fast exocytosis, vesicles in the RRP are already tethered, docked and primed to achieve a state where spontaneous secretion is inhibited until a triggering signal occurs (Figure 2). SNARE-accessory proteins such as Munc18 (Verhage et al., 2000; Gerber et al., 2008), Munc13 (Augustin et al., 1999), RIM (Han et al., 2015), RIM-binding proteins (Liu et al., 2011), synaptotagmins (Geppert et al., 1994; Südhof, 2002) and complexin (Tang et al., 2006; Reim et al., 2001) critically contribute to neu-

Figure 2. Time course and synaptic events of Ca²⁺-evoked neurotransmission. Schematic representation of time course of AP-evoked neurotransmission deducted from pre- and postsynaptic patch-clamp recordings at the calyx of Held. The presynaptic AP triggers Ca²⁺ currents and SV release which induces a postsynaptic EPSC and consequent AP. The time bar applies to all traces (left panel) (Reproduced with permission from Sudhof 2004; Meinrenken, Borst, and Sakmann 2003). The cartoon (right panel) shows the main proteins involved in the process. At the presynaptic site, SVs are acidified, filled with neurotransmitters and stored. The active zone is the region on the synaptic PM containing all the material required for the Ca²⁺-triggered fusion of the PM and SV membrane. Docking implies SVs that are tethered to the active zone. Primed SVs, by definition, have undergone all the steps required for SV fusion and are ready to release neurotransmitters upon Ca²⁺ entry. After the fusion, the SV membrane and its bound proteins are retrieved by endocytosis and SVs are regenerated for a new cycle of release. The main endocytic pathway is mediated by clathrin. (Reproduced with permission from Rossetto, Pirazzini, and Montecucco 2014).
rotransmitter release by controlling the gradual assembly of the SNARE complex and orchestrating a metastable primed state, providing inhibitory control in absence of a release trigger and positioning a Ca\textsuperscript{2+} sensor protein in the proximity of Ca\textsuperscript{2+} channels at the active zone. A tripartite interface containing complexin, the Ca\textsuperscript{2+} sensor synaptotagmin-1 (syt1) and the SNARE complex has been structurally resolved which may represent the primed state (Zhou et al., 2017). Consistent with this idea, syt1 and complexin together inhibit spontaneous neurotransmitter release by binding the SNARE complex but activate Ca\textsuperscript{2+}-induced release (Maximov et al., 2009; Lai et al., 2014; Tang et al., 2006). After the fusion event, the vesicle-anchored protein synaptophysin (previously named p38) is implicated in SV recycling by endocytosis (Kwon and Chapman, 2011; Navone et al., 1986). Subsequently, NSF and alpha-SNAP disassemble the SNARE complex to recycle the SNAREs for another round of fusion (Figure 2, right) (Burgalossi et al., 2010).

Synaptobrevin-2, synaptophysin and synapsin, the latter reversibly associated with the synaptic vesicle membrane (Huttner et al., 1983), are commonly used as synaptic markers. This list of SNARE-accessory proteins is by far not complete, and several other synaptic proteins may regulate the assembly of SNARE complexes. Two soluble proteins called tomosyn (Fujita et al., 1998) and amisyn (Scales et al., 2002) have a C-terminal SNARE motif that can compete with synaptobrevin in the synaptic SNARE complex. The tomosyns will be introduced to detail in section 1.4.

After extensive studies in neuronal secretion, it has become clear that the core mechanism is widely shared with other types of membrane fusion (Martens and McMahon, 2008), including spontaneous vesicle fusion (Schneggenburger and Rosenmund, 2015; Kaeser and Regehr, 2014). The next section will focus on the process that triggers the secretory response.

The role of Ca\textsuperscript{2+} in evoked neurotransmission

The coordinated action of several ions is extremely important for evoked neurotransmission. Na\textsuperscript{+} and K\textsuperscript{+} ions are necessary for cell depolarization and hyperpolarization (respectively involved in the generation and suppression of an AP). When the membrane potential exceeds the threshold for AP generation, the AP travels from the axon initial segment along the axon, reaches the synapses and leads to a synchronized and brief opening of the voltage-gated Ca\textsuperscript{2+} channels (VGCC) in the presynaptic terminal. This induces a sharp increase of the presynaptic Ca\textsuperscript{2+} concentration, which has been calculated to rise from 50 nM to 500 nM (Neher and Sakaba, 2008; Maravall et al., 2000; Helmchen et al., 1997; Müller et al., 2007). This results in the activation of Ca\textsuperscript{2+} sensor proteins, quickly followed by the formation of a fusion pore connecting the SV lumen and the extracellular environment, leading to the release of neurotransmitter. The action of neurotransmitters in the postsynaptic terminal generates an excitatory postsynaptic current (EPSC) which can develop an AP in the postsynaptic neuron and which can be measured by a technique called whole cell patch-clamp electrophysiology (Figure 2, left) (Sabatini and Regehr, 1996; Südhof, 2012).
Ca\textsuperscript{2+} sensors are proteins that recognize alterations in Ca\textsuperscript{2+} concentration and couple these signals to downstream processes such as SVs release. The Ca\textsuperscript{2+} sensor family includes proteins with different Ca\textsuperscript{2+} affinities that trigger various and independent downstream pathways (Plattner and Verkhratsky, 2015; Clapham, 2007; Berridge et al., 2000). In neurons, the two major Ca\textsuperscript{2+} sensor proteins for fast neurotransmitter release are synaptotagmin-1 (Geppert et al., 1994) and synaptotagmin-2 (Pang et al., 2006b), each expressed in different brain regions (Pang et al., 2006a). In addition, synaptotagmin-9 can also support fast neurotransmission upon overexpression (Xu et al., 2007).

In resting neurons, there is an uneven distribution in the Ca\textsuperscript{2+} concentration between the extracellular (1–3 mM) (Jones and Keep, 1988; Egelman and Montague, 1999) and some intracellular compartments (order of µM) (Solovyova et al., 2002), compared to the low amount of Ca\textsuperscript{2+} (50–100 nM) in the cytosol (Neher and Sakaba, 2008; Maravall et al., 2000; Helmchen et al., 1997; Müller et al., 2007). The steep gradient is the driving force for the entry of Ca\textsuperscript{2+} ions through VGCCs in the PM, which open for sub-millisecond periods upon AP-induced depolarization of the synaptic membrane (Chao and Yang, 2019). The absence of extracellular Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{e}) or the impairment of VGCC completely abolish the EPSC and the Ca\textsuperscript{2+} entry evoked from a single AP (Seabrook et al., 1989; Ermolyuk et al., 2013). Ca\textsuperscript{2+} is also stored in intracellular compartments such as the endoplasmic reticulum (ER) and mitochondria. Through inositol trisphosphate receptors (IP\textsubscript{3}Rs) and ryanodine receptors (RyRs), which are ligand-activated ion channels located in intracellular membranes, Ca\textsuperscript{2+} can be released from intracellular stores to increase the cytosolic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) and trigger specific cellular pathways such as Ca\textsuperscript{2+} induced Ca\textsuperscript{2+} release (CICR). CICR is a mechanism by which an increase of [Ca\textsuperscript{2+}]\textsubscript{i} can evoke a larger release of Ca\textsuperscript{2+} from the ER (Figure 3) (Verkhratsky and Shmigol, 1996; Berridge, 1998). Intracellular Ca\textsuperscript{2+} stores are implicated in evoked neurotransmission but they are not essential. Activation of RyRs by the agonistic effect of caffeine shows a positive effect on field EPSC amplitude at the hippocampal mossy fiber synapse (Shimizu et al., 2008). Conversely, RyR blockade by antagonistic concentrations of ryanodine or by mutations in the orthologous gene unc-68 impairs EPSCs at the C. elegans neuromuscular junction (NMJ) (Liu et al., 2005).

1.4 Tomosyn

Tomosyn (syntaxin binding protein) is a protein involved in neurotransmission. Tomosyn (tomo = friend in Japanese, syn = syntaxin), has been first identified by its linkage to syntaxin in a protein pull-down assay of rat brain (Fujita et al., 1998). In the mouse genome, Stxbp5 (tomasyn-1) and Stxbp5l (tomasyn-2) are paralogues on chromosome 10 and 16, respectively, and present 77% amino acid sequence similarity. Splice variants of the two genes generate at least seven isoforms (b-tomosyn-1, m-tomosyn-1, s-tomosyn-1, xb-tomosyn-2, b-tomosyn-2, m-tomosyn-2 and s-tomosyn-2) (Groffen et al., 2005). Out of the three tomosyn-1 splice variants, the biggest variant is expressed in brain, heart, lung and kidney while the smaller two are brain specific (Yokoyama et al., 1999).
The fruit fly *Drosophila melanogaster* and the nematode *C. elegans* both have a single orthologous Tomosyn gene. In *C. elegans*, tom-1 shares ≈33% amino acid identity with isoforms of mammalian tomosyn-1 and tomosyn-2 (Dybbs et al., 2005; Gracheva et al., 2006). The fly and worm also contain a paralogous gene named Lethal (2) giant larvae (Lgl), a tumor suppressor in *Drosophila melanogaster*, which is represented in mammals by two separate genes mgl-1 and mgl-2. In the yeast *S. cerevisiae*, two genes Sro7 and Sro77 are the ancestors of the...
mammalian tomosyn/Mgl family (Lehman et al., 1999; Wirtz-Peitz and Knoblich, 2006; Groffen et al., 2005).

Tomosyn localization

Tomosyn-1 is widely expressed in the adult brain while tomosyn-2 is more restricted to the hippocampus (CA2 region), medial habenular nucleus and the internal granular layer of the cerebellar cortex. During development, tomosyn-1 is stably expressed and tomosyn-2 expression increases with development, along with syntaxin-1 and Munc18-1 (Groffen et al. 2005; Barak et al. 2010). Tomosyn-1 is also expressed in endocrine systems such as pancreatic β-cells (Cheviet et al., 2006; Zhang et al., 2006) and chromaffin cells (Yizhar et al., 2004; Gladycheva et al., 2007). Despite their high similarity, these differences in expression pattern and splice variants might reflect functional differences between tomosyn-1 and tomosyn-2 (Williams et al., 2011).

Subcellular localization of tomosyn has mainly been assessed with biochemical and immunolabeling experiments as detailed below. Tomosyn is not homogeneously distributed but presents a cytoplasmic distribution with a varying degree of punctate accumulation (Barak et al., 2010; Cazares et al., 2016). Although tomosyn does not contain a transmembrane domain, it targets the PM through an interaction with syntaxin-1A in PC12 and chromaffin cells (Williams et al. 2011; Gladycheva et al. 2007). Moreover, tomosyn associates with DCVs in C. elegans (Gracheva et al., 2007b) and it is linked to secretory granules in insulin-secreting cell lines INS-1E and MIN6 (Cheviet et al., 2006; Cazares et al., 2016). Tomosyn takes part in the synaptic vesicle proteome (Burré and Volknandt, 2007) since it was found linked to synaptic vesicle fractions isolated from rat brain (Fujita et al., 1998; Takamori et al., 2006). However, the mechanism mediating secretory vesicle association is still unknown. Tomosyn colocalizes with presynaptic markers in superior cervical ganglion (SCG) neurons (Baba et al., 2005) and C. elegans motor neurons (Gracheva et al. 2006). In hippocampal neurons, tomosyn colocalizes with the synaptic vesicle marker synaptophysin (Cazares et al., 2016) but also to a lesser extent with postsynaptic markers. In particular, tomosyn-2 is detected in dendritic structures (Barak et al., 2010).

Most of the tomosyn localization studies were performed on fixed preparations. Given the various tomosyn interactors and posttranslational modifications, as detailed below, it is conceivable that vesicle association of tomosyn may be dynamically regulated. Hence, the analysis in living neurons could reveal novel tomosyn localizations, which may be important to understand tomosyn activity.

Functional properties of Tomosyn-1 and -2

The established role of tomosyn is to negatively regulate secretion. This has been deduced from studies performed in different systems (for references see below). Tomosyn-1 has been more extensively studied than tomosyn-2.

In PC12 cells and chromaffin cells, vesicle secretion is reduced by tomosyn-1 overexpression (Fujita et al., 1998; Gladycheva et al., 2007; Yizhar et al., 2004).
Furthermore, overexpression of tomosyn-1 in chromaffin cells prevents the immobilization of the newly arriving vesicles at the PM and reduces the secretion of vesicle which is partially relieved elevated Ca$^{2+}$ concentrations (Yizhar et al., 2004; Yizhar and Ashery, 2008). Overexpression of either tomosyn-1 or tomosyn-2 in PC12 cells similarly inhibits the potassium-evoked secretion of co-transfected growth hormone (Williams et al. 2011).

In pancreatic β-cells, overexpression of tomosyn-1 and tomosyn-2 inhibits insulin secretion in a Ca$^{2+}$-dependent manner (Bhatnagar et al., 2011; Zhang et al., 2006), although these observations are seemingly at odds with another study where overexpression of tomosyn-1 in INS-1E cells does not interfere with the release of cotransfected human growth hormone (hGH) which is, instead, reduced to 50% by the siRNA for tomosyn (Cheviet et al., 2006).

Tomosyn has been investigated in NMJ of C. elegans, Drosophila and Tomosyn-2-deficient mice. In the C. elegans NMJ, overexpression of wildtype tomosyn (tom-1) decreases acetylcholine release. Conversely, tom-1 mutant nematodes exhibit an increased EPSC charge transfer and an accumulation of SVs at the PM which are dispersed throughout the terminal (Gracheva et al. 2006). In both cases, spontaneous release properties are unaffected. The increase of charge transfer in the tom-1 mutant is larger in low extracellular Ca$^{2+}$ (0.5 mM) than high Ca$^{2+}$ (5 mM). At low Ca$^{2+}$ concentrations, the amplitude is bigger for mutant than wildtype (Gracheva et al. 2006). This suggests that tomosyn’s inhibitory effect on secretion might be activity dependent. Also, the number of presynaptic LDCVs is reduced in tom-1 mutant nematodes. Instead, overexpressing tomosyn in C. elegans leads to an accumulation of LDCV (Gracheva et al., 2007b). These effects of tom-1 may reflect differences in LDCV release but also in LDCV biogenesis or trafficking to the synapse. At the glutamatergic Drosophila NMJ, neuronal tomosyn knockdown enhances the extrajunctional charge transfer, decreases paired-pulse ratio and prolongs the decay time of spontaneous release events (Chen et al. 2011). In the same study, the neuronal knockdown of tomosyn strongly affected late memory (i.e. 3h after training) in an aversive odor memory task. This effect was attributed to a cAMP / PKA mediated pathway that, besides tomosyn, also requires synapsin (Chen et al. 2011), suggesting that tomosyn might be involved in synaptic plasticity. Tomosyn-2-deficient mice show mild impairments in motor performance. At the NMJ, tomosyn-2-deficient mice have an enhanced frequency of spontaneous acetylcholine release and faster depression of muscle motor endplate potentials during repetitive stimulation (Geerts et al., 2015). Thus, also studies in mammalian neurons indicate that tomosyn might be involved in synaptic plasticity.

In rat SCG neurons, the presynaptic overexpression of tomosyn-1 increases EPSC failure and decreases EPSC amplitude. However, tomosyn-1 knockdown by siRNA evokes the same effect (Baba et al., 2005). Overexpression of tomosyn in mouse dentate gyrus neurons results in a disruption in hippocampus-dependent spatial learning and memory with a concomitant reduction in paired-pulse facilitation but no effect on EPSC amplitude (Barak et al., 2013). Tomosyn-1-de-
ficient mice are born at a lower than expected frequency, but born homozygous null mice appear healthy (Sakisaka et al., 2008). Hippocampal mossy fiber synapses of these mice show an enhanced but not prolonged synaptic transmission and the paired-pulse facilitation is decreased suggesting an increase in the initial release probability. Conversely after tetanic stimulation (100 Hz for 1s), the lack of tomosyn decreases LTP in the initial phase (i.e. 0–20 min) while the late phase of LTP is unaffected (Sakisaka et al., 2008). Tomosyn-1 was also proposed to play an important function in growth cone extension. It prevents the fusion of plasmalemmal precursor vesicles at the palm of the growth cones, thus promoting transport to the leading edges of growth cones (Sakisaka et al., 2004).

In summary, tomosyn is a negative regulator of evoked neurotransmitter release in several secretory cell types and its function is activity dependent. Moreover, several observations suggest that tomosyn is involved in synaptic plasticity. Other tomosyn functions associated with synaptic vesicles have been found, which prompt a more detailed analysis of the role of tomosyn in the mobility of synaptic vesicles.

**Tomosyn protein domains and molecular mechanism of action**

The main molecular mechanism of tomosyn as inhibitor of vesicle release is to compete with the v-SNARE VAMP II for t-SNARE-binding (Fujita et al., 1998; Yamamoto et al., 2010a). To comprehend tomosyn function, it is essential to understand the protein structural domains (Figure 4). The main identified domains are: 1) The large N-terminal domain comprising two seven-bladed β-propellers in which each blade is formed by a WD40 repeat motif (40 amino acids which terminate with Tryptophan (W) and aspartate (D) residues) (Hattendorf et al., 2007). 2) The β-propeller structures are interrupted by three loops named loop 1, 2 and 3 which are absent from yeast Sro7 but conserved between rat tomosyn-1 and -2 (Williams et al., 2011). 3) The C-terminal coiled-coil (CC) domain (40 amino acids) is homologous to the synaptobrevin SNARE domain, and therefore also called C-terminal SNARE domain or VAMP-like domain (VLD) (Bielopolski et al., 2014; Masuda et al., 1998). 4) Finally, the tail domain positioned between the β-propellers and the CC domain supports intramolecular conformational changes which

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**Figure 4. Tomosyn functional domains.** Homology model based on the crystal structure of yeast Sro7 (Hattendorf et al., 2007). It shows the N-terminal (purple), the β-propeller structure containing the WD40 repeats (grey and purple) from which the 3 loops (yellow, red, cyan) extend and the tail domain (green) which has been predicted to form a coiled-coil structure. The VAMP-like domain (VLD) is not shown (Reproduced with permission from A. L. Williams et al. 2011).
may regulate the SNARE-binding activity of the CC domain.

The N-terminal WD40 domain is one of the domains that interact with SNAP25 and syntaxin, enhances the oligomerization of VAMP2/SNAP25/syntaxin-1 in vitro and reduces neurotransmission since the EPSC amplitude is increased in tomosyn-deficient mice (Sakisaka et al., 2008). It is not very clear how the oligomerization of VAMP2/SNAP25/syntaxin-1 in vitro impairs neurotransmitter release. Complexin seems not to be involved since it does not bind to the tomosyn-1/ SNAP25/ syntaxin-1A complex (Pobbati et al., 2004). The N-terminal WD40 domain interacts directly with syt1 in a Ca\(^{2+}\)-dependent manner (Yamamoto et al., 2010b).

The CC-domain binds syntaxin-1 and SNAP25 forming a four-helical bundle and inhibits the formation of the fusogenic SNARE complex by competing with VAMPII (Hatsuzawa et al., 2003; Pobbati et al., 2004; Sakisaka et al., 2008; Masuda et al., 1998). The CC-domain alone is sufficient to inhibit fusion of SNARE-liposomes (Yamamoto et al., 2010a). In contrast, the CC-domain alone is not sufficient to induce inhibition of release as determined by membrane capacitance measurement in chromaffin cells (Yizhar et al., 2007). Similarly in PC12 cells, the expression of full length tomosyn or tomosyn without CC-domain inhibits the potassium-induced release of human growth hormone (Gladycheva et al., 2007).

In m-tomosyn-1, loop 1 and loop 3 are necessary for the inhibition of potassium-induced growth hormone secretion in PC12 cells (Williams et al. 2011). Loop 2 seems not to be involved in this inhibition. Loop 2 is also subjected to splicing and contain several sites for posttranslational modification as detailed below. Loop 1, 2 and 3 are not involved in syntaxin-1A binding.

The tail domain is a conserved domain which presents an intrinsic regulatory function for tomosyn activity. It can interact with the CC-domain, reducing its inhibitory function on SNARE complex formation (Yamamoto et al., 2009). Alternatively, it can also interact with the WD40 domain, leaving the inhibitory function of CC domain active.

A better understanding of the functional activity of each domain and their regulation would give a better knowledge of the neuronal pathways in which tomosyn is involved.

**Tomosyn interactors**

Other tomosyn interactors besides syntaxin-1 and SNAP25 have been found. Syt1 has been shown to copurify together with tomosyn in the same complex with syntaxin-1 and SNAP25 (Fujita et al., 1998). *In vitro*, tomosyn N-terminal domain binds directly to syt1 in a Ca\(^{2+}\)-enhanced manner decreasing the release of ACh in SCG neurons (Yamamoto et al., 2010b). This interaction is likely to reduce synaptotagmin’s ability to induce membrane curvature and promote SNARE-mediated membrane fusion.

In yeast, Sro7p and Sro77p associate with secretory vesicles through an inter-
action with Sec4p (Rab GTPase), which is important in vesicle tethering / docking (Rossi et al., 2015; Watson et al., 2015). The interaction with Rab GTPase is conserved in rat tomosyn-1 which binds Rab3A, but not Rim1/2, actin or synaptophysin (Cazares et al., 2016). Moreover, there is evidence that Rab3A makes a bridge between tomosyn-1 and synapsin-1 under the control of cyclin-dependent kinase 5 (Cdk5). Cdk5 phosphorylates tomosyn-1 regulating the SV pool sizes and distribution during homeostatic synaptic plasticity induced by chronic silencing of network activity (Cazares et al., 2016). Interestingly, Cdk5 regulates hippocampal plasticity and learning and memory formation (Lai et al., 2012; Sananbenesi et al., 2007).

In yeast, Sro7p binds Myosin-V through the N-terminal 13 amino acids of Sro7p (Rossi and Brennwald, 2011). In Drosophila, Lgl associates with Myosin-II using the C-terminal domain (Betschinger et al., 2005). In rats, syntaxin-1A binds Myosin-Va in a Ca\(^{2+}\)-dependent manner (Watanabe et al., 2005). In view of the data from yeast and flies, it is conceivable that tomosyn might function as a bridge in this interaction recruiting Myosin-V containing vesicles to the PM, although, there is no evidence for this hypothesis in mammalian neurons.

In conclusion, the various conserved interactions of tomosyn make it feasible that its function extends beyond secretion inhibition to include regulation of vesicle trafficking.

**Tomosyn posttranslational modifications**

Two main posttranslational modifications of tomosyn have been described. S724 present on loop 2 domain of tomosyn-m1 can be phosphorylated by cAMP-dependent protein kinase (PKA) (Baba et al., 2005). It is well known that PKA contributes to overall brain function by regulating synaptic plasticity (Nguyen and Woo, 2003). Tomosyn phosphorylation reduces syntaxin-1 interaction and increases SNARE complex formation in vitro (Baba et al., 2005). All of this translates in an upregulation of the neurotransmitter release by PKA-induced increase of RRP size in SCG neurons to maintain a pool of readily releasable synaptic vesicles during repetitive stimulation.

SUMOylation is the covalent binding of small ubiquitin-like modifier (SUMO) peptides to target proteins. K730, also present on loop 2 of m-tomosyn-1, is SUMOylated by SUMO-2/3 through the SUMO E3 ligase PIAS-gamma (Geerts et al., 2014; Williams et al., 2011). SUMOylation of tomosyn-1 reduces its inhibitory activity without affecting syntaxin binding in PC12 cells (Williams et al., 2011).

Members of the Rho GTPase family are primary regulators of actin cytoskeleton organization. The Rho-associated serine/ threonine kinase (ROCK) increases the binding affinity between syntaxin-1 and tomosyn, therefore, reducing the availability of functional SNARE complexes and preventing the fusion of plasmalemmal vesicle at the tip of the growth cone (Sakisaka et al., 2004). The target phosphorylation site is located in syntaxin-1 at residue S14 and consistent with this, the effect of ROCK phosphorylation can be mimicked by a S14D mutant of...
syntaxin (Sakisaka et al., 2004). The RhoA/ROCK-dependent phosphorylation of syntaxin could regulate the specific location where tomosyn should inhibit vesicle fusion during neurite outgrowth. The same pathway increases tomosyn association with plasma membrane-bound syntaxin-1A in chromaffin cells in response to lysophosphatidic acid (Gladycheva et al., 2007).

Thus, posttranslational modification of tomosyn and syntaxin provide mechanisms to modulate the inhibitory activity of tomosyn in a dynamic and activity-dependent manner. The discovery of novel modifications and a more extended analysis of the known posttranslational modification could fully elucidate the tomosyn dependent regulation of vesicle transports and secretion.

1.5 Spontaneous neurotransmission.

Genetic blockade of AP-evoked neurotransmission does not completely abolish SV release. This is illustrated by the phenotype of syt1 null mice where evoked synchronous neurotransmission is abolished, but at least two release components remain intact: asynchronous (still dependent on AP but with slower kinetics) and spontaneous release (independent of AP) (Geppert et al., 1994). The physiological role and the mechanism of spontaneous release are still under investigation (Kaeeser and Regehr, 2014). In most neurons, a characteristic feature of spontaneous release is the fusion of a single SV (quantal release) which evokes a miniature excitatory postsynaptic current (mEPSC) in the postsynaptic neuron (Fatt and Katz, 1950, 1952). In some neuronal subtypes including cerebellar Purkinje cells, multivesicular release events occur spontaneously (Llano et al., 2000). The term ‘spontaneous’, refers to the absence of AP stimulation (Fatt and Katz, 1950) but does not exclude the presence of other triggering mechanisms (Glitsch, 2008), such as by the stochastic opening of a single VGCC (Ermolyuk et al., 2013; Williams and Smith, 2018) (other potential mechanisms will be discussed in the next sections). Alternatively, SV release events may arise by chance with low stochastic probabilities in the absence of any triggering signal. To avoid confusion, we will use the term AP-independent release to define all such release events.

Function of AP-independent neurotransmission

The physiological function of AP-independent neurotransmission is less clear the AP-dependent neurotransmission. AP-dependent neurotransmission is the primary source responsible for transferring information, whereas AP-independent neurotransmission might serve diverse neuronal functions such as synaptogenesis, maturation (Huntwork and Littleton, 2007) and maintenance of synapses (McKinney et al., 1999). It has been also shown that spontaneous release is involved in regulating dendritic protein translation (Sutton et al., 2004) and influencing neuronal spiking patterns (Carter and Regehr, 2002). Spontaneous release contributes to the regulation of homeostatic plasticity (Garcia-Berenguiain et al., 2016) which involves both pre- and postsynaptic mechanisms and gene translation (Turrigiano, 2012; Lazarevic et al., 2013). Alteration in homeostatic plasticity has been associated with neurological disorders and intellectual disabil-
Chapter 1

Ities such us Kleefstra syndrome (Benevento et al., 2016). Spontaneous release seems to be involved in homeostasis at neuronal and network level in different developmental stages and brain functions. Therefore, a better understanding of the mechanisms regulating spontaneous release would give more insights about neurological disorders and, on the longer term, the possibility to find a therapy for them.

Mechanism of AP-independent neurotransmission

Similar to the high dependency of Ca\(^{2+}\) in AP-evoked neurotransmission, there are many indications that spontaneous release is also regulated by Ca\(^{2+}\) (Seabrook et al., 1989; Koester and Sakmann, 2000; Dunlap et al., 1995; Lin and Scheller, 2000). Various lines of evidence support the idea that Ca\(^{2+}\) arising from extracellular and intracellular Ca\(^{2+}\) stores, and proteins known to regulate evoked release, are also involved in spontaneous release (Glitsch, 2008; Williams and Smith, 2018). A role of Ca\(^{2+}\) is also suggested by the existence of Ca\(^{2+}\) sensor proteins that mediate spontaneous, but not synchronous release (Groffen et al., 2010).

Role of Ca\(^{2+}\) in AP-independent neurotransmission

The role of Ca\(^{2+}\) in spontaneous neurotransmission has often been studied by either changing \([\text{Ca}^{2+}]_{e}\) or interfering with Ca\(^{2+}\) channels and assessing the properties of mEPSCs. Buffering cytoplasmic Ca\(^{2+}\) using fast or slow or Ca\(^{2+}\) chelators, for instance EGTA (ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid) or BAPTA (1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid), reduces mEPSC frequency approximately two-fold in hippocampal and pyramidal neurons in layer II (Ermolyuk et al., 2013; Simkus and Stricker, 2002).

It is widely documented that an increase of \([\text{Ca}^{2+}]_{e}\) leads to a higher mEPSC frequency; however, a complete removal of \([\text{Ca}^{2+}]_{e}\) does not fully abolish mEPSC (Xu et al., 2009; Groffen et al., 2010; Vyleta and Smith, 2011). The involvement of VGCC may differ between neuronal subtypes. In neocortical neurons, VGCCs seem not to be involved in the increase mEPSC frequency after increase of \([\text{Ca}^{2+}]_{e}\) (Vyleta and Smith, 2011). Other studies in hippocampal synapses showed that the combined pharmacological blockade of several VGCC types decreases mEPSC to ≈50% with a large contribution of R-type channels, which are less important in synchronous SV release (Ermolyuk et al., 2013).

In addition to VGCCs, intracellular Ca\(^{2+}\) stores also contribute to the regulation of spontaneous neurotransmission, involving the CICR mechanism. This mechanism depends on RyR and IP\(_3\)R, which are well known for their vital contractile functions in skeletal and cardiac muscle cells. Members of both gene families are also widely expressed in the brain, and multiple are associated with neurological disorders (Stutzmann and Mattson, 2011). Simkus and Stricker (2002) show that the inhibition of either RyRs through ryanodine or IP\(_3\)Rs by 2-aminoethoxydiphenyl borate (2APB) decrease mEPSC frequency in cortical pyramidal cells (Simkus and Stricker, 2002). Furthermore, caffeine activation of RyRs induces an
increase in mEPSC frequency (Simkus and Stricker, 2002; Xu et al., 2009).

All of this suggests that Ca\(^{2+}\), VGCC, RyRs, IP\(_3\)R are partially involved in quantal neurotransmission, although a substantial part of the spontaneous release is still maintained in their absence. In summary, it is clear that Ca\(^{2+}\) has an important role in spontaneous release; however, more research is needed to discover the trigger/s of the cytoplasmic Ca\(^{2+}\) signals and the molecular mechanisms of the Ca\(^{2+}\) sensor/s that couple these signals to quantal release events.

**Role of proteins in spontaneous neurotransmission**

Some proteins implicated in evoked neurotransmission have similar functions in spontaneous release. Other proteins regulate spontaneous and evoked release with different features. In detail, the main SNARE proteins are important for both spontaneous and evoked neurotransmission. Clostridial neurotoxins, such as botulinum and tetanus toxins, prevent the AP-dependent release and reduce the mEPSC frequency by cleaving SNAP-25, VAMPII or syntaxin-1 in several neuronal preparations (de Wit et al., 2006a; Beske et al., 2016; Kim et al., 1984; Neale et al., 1999). The genetic manipulation of SNARE proteins also affects evoked and spontaneous release. In Drosophila, synaptobrevin is more important for evoked than spontaneous neurotransmission (Deitcher et al., 1998). The neurons of SNAP-25-deficient mice have a loss of evoked exocytosis and reduced spontaneous release (Bronk et al., 2007; Delgado-Martínez et al., 2007). Syntaxin double KO mice show a reduction in evoked neurotransmission and in the frequency of spontaneous release (Zhou et al., 2013; Vardar et al., 2016). Although there is a 'core machinery' that is shared by evoked and spontaneous SV release, the latter is not abolished by the lack of one SNARE protein. One possibility is that other SNARE isoforms might be involved in the remaining spontaneous release. It is also possible that components other than SNARE proteins might differ. Hence, it will be valuable to identify these components more clearly in the future. Most obvious examples of such components are Ca\(^{2+}\) sensors, molecules involved in Ca\(^{2+}\) signalling and molecules determining the spatial organization of Ca\(^{2+}\)/secretion coupling.

Munc13 and Munc18 are important SNARE-regulatory proteins for SVs release for both evoked and spontaneous neurotransmission. KO neurons for either Munc13-1/2 or Munc18-1 present neither evoked nor spontaneous SV release (Varoqueaux et al., 2002, 2005; Verhage et al., 2000). Other regulatory proteins such us syt1 and complexin affect evoked and spontaneous release in different ways. In mice, loss of syt1 abolishes the evoked-synchronous neurotransmission but increases the rate of spontaneous release indicating that synaptotagmin inhibits ('clamps') spontaneous release. Syt1 might inhibit the activity of a second, more sensitive Ca\(^{2+}\) sensor for spontaneous release (Geppert et al., 1994; Liu et al., 2009; Chapman, 2008; Xu et al., 2009). The depletion of complexin impairs evoked release and increases the rate of spontaneous release, again indicating a clamping role (Maximov et al., 2009; Lai et al., 2014; Jorquera et al., 2012). Doc2 proteins have been shown to be involved specifically in spontaneous release. In hippocampal neurons, the loss of Doc2a and Doc2b does not affect evoked
release but drastically reduces the rate of spontaneous release to 50%. Therefore, Doc2 proteins are considered the Ca\(^{2+}\) sensor for spontaneous release in particular Doc2b, although, this is still a debate in the field (Groffen et al., 2010; Pang et al., 2011).

The complexity of protein function in modulating spontaneous and/or evoked release makes it difficult to define the molecular pathways regulating spontaneous neurotransmitter release. To better understand the mechanism triggering spontaneous release, we chose to investigate the possibility that small-local Ca\(^{2+}\) increases trigger spontaneous SV release, similar to the triggering mechanism for synchronous release by AP-evoked Ca\(^{2+}\) influx. If small-local Ca\(^{2+}\) increases trigger spontaneous SV release, then a specific Ca\(^{2+}\) sensor with the corresponding location and Ca\(^{2+}\) affinity could be involved in sensing those Ca\(^{2+}\) fluctuations.

1.6 Neuronal Ca\(^{2+}\)

Ca\(^{2+}\) ions act as signalling molecules in many pathways of many cell types. Ca\(^{2+}\) binds to many biological structures with varying affinities and binding kinetics and has a widespread range of action. For instance, it interacts with amino acids through carboxylate oxygen atoms changing the protein charge and, in turn, the activity of the bound target proteins (Jaiswal, 2001). Changes in [Ca\(^{2+}\)], in time and in space, trigger divergent cellular pathways such as gene expression, energy production, cytoskeletal dynamics, apoptosis and more (Plattner and Verkhratsky, 2015; Clapham, 2007; Berridge et al., 2000). Different Ca\(^{2+}\)-binding proteins detect [Ca\(^{2+}\)] with different Ca\(^{2+}\) affinities and trigger unique downstream pathways (Plattner and Verkhratsky, 2015; Clapham, 2007; Berridge et al., 2000). In excitable cells, such as muscle cells and neurons, Ca\(^{2+}\) is the main trigger for the contraction and neurotransmission mechanisms, respectively (Dulhunty, 2006; Neher and Sakaba, 2008).

The [Ca\(^{2+}\)], is highly dynamic and strongly depends on neuronal activity. As previously illustrated in Figure 3, neurons maintain low cytoplasmic [Ca\(^{2+}\)] in the 50-100 nM range (Neher and Sakaba, 2008; Maravall et al., 2000; Helmchen et al., 1997; Müller et al., 2007; Scott and Rusakov, 2006) while intracellular Ca\(^{2+}\) stores such as the ER (Solovyova et al., 2002) and mitochondria (Ivannikov and Macleod, 2013) and, especially the extracellular space contain much higher Ca\(^{2+}\) concentrations (Jones and Keep, 1988; Egelman and Montague, 1999). To maintain these steep concentration gradients, low [Ca\(^{2+}\)] is maintained through pumps, such as the sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) on the ER and the plasma membrane Ca\(^{2+}\)-ATPase (PMCA) on the PM; through exchangers on the PM and intracellular membranes, such as the Na\(^{+}\)/Ca\(^{2+}\) exchanger (NCX) (Brini et al., 2014). In addition, intracellular Ca\(^{2+}\) is chelated away by Ca\(^{2+}\) buffers such as calbindin, calretinin and parvalbumin (Baimbridge et al., 1992). The low [Ca\(^{2+}\)] during the resting state may suddenly increase after activation of voltage-gated or ligand-gated Ca\(^{2+}\) channels, such as VGCCs, IP\(_3\)R, RyRs and many others (Figure 3) (Plattner and Verkhratsky, 2015). The dynamic nature of the intracellular Ca\(^{2+}\) signal imposes important challenges for the experimental measurement of Ca\(^{2+}\) on a biologically meaningful timescale.
Techniques to visualise and analyse Ca\textsuperscript{2+} fluctuations

One of the techniques used to measure the spatial and temporal parameters of intracellular Ca\textsuperscript{2+} dynamics is live fluorescence microscopy. Detailed knowledge of intracellular Ca\textsuperscript{2+} dynamics has been acquired thanks to the development of fluorescent Ca\textsuperscript{2+} indicator probes with a range of Ca\textsuperscript{2+} affinities and concomitant improvements in sensitive live imaging techniques (Grienberger and Konnerth, 2012). The research questions linked to Ca\textsuperscript{2+} pathways are widespread in science and require different tissues or cell types, Ca\textsuperscript{2+} indicators and imaging hardware.

Ca\textsuperscript{2+} indicators

Ca\textsuperscript{2+} indicators, also called Ca\textsuperscript{2+} sensors or Ca\textsuperscript{2+} reporters, have been widely used to examine the role of Ca\textsuperscript{2+} in several cellular pathways and in different cell types. Since the discovery of the bioluminescent Ca\textsuperscript{2+} indicators Aequorin (SHI-MOMURA et al., 1962), a lot of effort has been made to improve and discover new classes of Ca\textsuperscript{2+} indicators. In general two main classes of Ca\textsuperscript{2+} indicators are distinguished: chemical probes and genetically encoded-Ca\textsuperscript{2+} indicators (GECIs) (Takahashi et al., 1999; Whitaker, 2010).

**Chemical Ca\textsuperscript{2+} indicators**

The chemical indicators are based on Ca\textsuperscript{2+} chelator compounds in which Ca\textsuperscript{2+} binding causes intramolecular rearrangements that affect the fluorescent properties of the compound. Different Ca\textsuperscript{2+} indicators differ for their emission spectrum upon Ca\textsuperscript{2+} binding. Quin2 yields blue emission; Fluo3, calcium green and Oregon Green BAPTA-1 (OGB-1) emit in the green part of the spectrum; calcium orange, calcium crimson and rhod 2 in the yellow/orange part; Fura red in the red part of the spectrum. A few indicators are suitable for quantitative Ca\textsuperscript{2+} measurement using ratiometric measurements, including Indo-1 and Fura-2. Many Ca\textsuperscript{2+} indicators can be loaded into cells in primary cultures or tissue slices using cell permeant AM-ester forms that are converted in the cytoplasm to cell-impermeant compounds. Dextran conjugates allow more stability of the compound in the cytosol, decreasing the rate of extrusion of the probe from the cells (Takahashi et al., 1999). OGB-1 is excited at 488 nm and has a good photostability. It has been widely used in the neuroscience field to study several cellular Ca\textsuperscript{2+} pathways, such as evoked neurotransmission (DiGregorio and Vergara, 1997).

**Genetically encoded Ca\textsuperscript{2+} indicators**

GECIs are a more recent class of protein-based Ca\textsuperscript{2+} indicators (Miyawaki et al., 1997). GECIs can be expressed in primary cells and slices using viral infection and electroporation. GECIs have been stably expressed in different tissues of genetic animal models (Dana et al., 2014; Kotlikoff, 2007; Zariwala et al., 2012). The emitted light is derived from a single fluorophore or from two fluorophores by Föster resonant energy transfer (FRET). One of the most promising classes of single-fluorophore Ca\textsuperscript{2+} indicators is the GCaMP family. GCaMPs consist of a circularly permuted green fluorescent protein (cpGFP) fused at the C-terminal to the Ca\textsuperscript{2+} binding protein calmodulin and at the N-terminal to the calmodulin
binding peptide M13 from myosin light chain kinase. Four Ca\(^{2+}\) ions (two for calmodulin and two for M13) induce a structural rearrangement of GCaMPs, causing a conformational change in cpGFP and leading to an increase in its fluorescence (Figure 5) (Nagai et al., 2001).

The GCaMP family was further optimized by altering properties such as Ca\(^{2+}\) affinity, brightness, and it is currently used for \textit{in vitro} and \textit{in vivo} Ca\(^{2+}\) analysis (Akerboom et al., 2012; Horikawa, 2015; Mao et al., 2008; Chen et al., 2013) The GECI family offers the advantage to be targeted in different neuronal populations or in specific subcellular compartments (Dreosti and Lagnado, 2010; Mao et al., 2008; Dana et al., 2014). In 2012, Zariwala and colleagues produced a transgenic GCaMP3 mouse with a Cre-dependent strategy expressing GCaMP3 in retina, cortex and cerebellum, thus allowing Ca\(^{2+}\) analysis also during development (Zariwala et al., 2012). To increase the flexibility for Ca\(^{2+}\) imaging, several variants of genetically encoded Ca\(^{2+}\) indicators for optical imaging (GECOs) have been created to vary the performance and spectral properties of the Ca\(^{2+}\) indicators. For instance, R-GECO is a “red shifted” Ca\(^{2+}\) indicator (Yamada and Mikoshiba, 2012) which was further developed to be targeted to intracellular compartments (Walker et al., 2013) or to present characteristic spectral properties, such us REX-GECO and jREX-GECO1 which feature a long Stokes shift (Molina et al., 2019; Wu et al., 2014).

Several members of the GCaMP family have been compared to each other and to chemical Ca\(^{2+}\) indicators (OGB-1) in their ability to detect the Ca\(^{2+}\) response following an AP in neurons (Yamada and Mikoshiba, 2012). These analyses showed that GCaMP6s is equally sensitive as OGB-1 and, GCaMP6f is less sensitive but with a faster kinetics than OGB-1 and GCaMP6s (Chen et al., 2013).

Live Ca\(^{2+}\) imaging

Temporal fluctuations in the [Ca\(^{2+}\)], in living cells can be studied by labelling cells with specific Ca\(^{2+}\) indicators and recording images during live fluorescence microscopy (Takahashi et al., 1999; Grienberger and Konnerth, 2012). Other than Ca\(^{2+}\) indicators, imaging hardware is an important factor for the detection of [Ca\(^{2+}\)] \_ fluctuations. Temporal resolution and light sensitivity of the imaging hardware are essential parameters which must match the features of the studied [Ca\(^{2+}\)] dy-
namics. For fast imaging (framerates above 10 Hz) and low signal-to-noise ratios, high temporal resolution and light sensitivity are extremely necessary. Therefore, the microscopic objectives need to have a high numerical aperture, which is a reason to opt for high-quality oil-immersion lenses. Moreover, the availability of vacuum-cooled electron multiplying CCD (EM-CCD) cameras allows the amplification of weak signal events (down to single photons) to a signal level that is well above the noise level of the camera. There is an important trade-off between the speed of image acquisition and the imaging sensitivity. At lower framerates more photons are collected, producing a better signal-to-noise ratio. On the other hand, the image capture time should be short enough to allow sufficient sampling of signal over the duration of a \( \mathrm{Ca^{2+}} \) fluctuation event (Takahashi et al., 1999; Wilt et al., 2009).

**Ca\( ^{2+} \) image analysis**

\( \mathrm{Ca^{2+}} \) imaging of living systems produces complex datasets and many tools have been developed to analyse live \( \mathrm{Ca^{2+}} \) imaging data. A common strategy involves four basic steps. First, to analyse \( \mathrm{Ca^{2+}} \) imaging data, regions of interest (ROIs) are defined on the biological structure to visualise the intensity signal over time which is expressed in arbitrary units (AU) and depends on the acquisition parameters. Second, to compensate for uneven indicator concentration, the intensity signal of each time point is normalized to a baseline signal (\( F_0 \)) producing a time-dependent \( \Delta F/F_0 \) (Bootman et al., 2013). The \( F_0 \) to choose may vary between experiments: it can be static or it can be a constant moving window. Third, biological events are selected and, fourth, parameters calculated on the raw or \( \Delta F/F_0 \) trace.

Especially for fast or long time-lapse movies, manual processing of \( \mathrm{Ca^{2+}} \) imaging analysis becomes very laborious and human decision-making may be biased by a long time spent on the analysis of individual cells (Watters et al., 2014; Rueckl et al., 2017). Many algorithms have been developed to speed up the data analysis processing, including ROI definitions (Steele and Steele, 2014; Cheng et al., 1999; Ellefsen et al., 2014; Hamed et al., 2015; Tomek et al., 2013; Prada et al., 2018; Patel et al., 2015; Jang and Nam, 2015). Images are filtered to reduce noise using a Gaussian filter for the spatial and/or temporal dimension. To automatically calculate ROIs, a threshold based on data features is applied to the images and pixels above threshold are selected. ROIs for each frame can be connected in the spatial and temporal dimension using several methods such as connected-component analysis, spatio-temporal independent component analysis (ICA) (Mukamel et al., 2009; Patel et al., 2015), non-negative matrix factorization (NMF) (Maruyama et al., 2014) and constrained non-negative matrix factorization and deconvolution (Pnevmatikakis et al., 2016).

Among the many computational tools available to perform a specific analysis of \( \mathrm{Ca^{2+}} \) imaging data, as far as we know, there is no program which allows visual inspection of automatically detected ROIs, therefore, allowing a fully controlled \( \mathrm{Ca^{2+}} \) analysis. Considering the complexity and the versatility of the \( \mathrm{Ca^{2+}} \) signals (as detailed below), a further computational requirement is the ability to detect
and categories different Ca\(^{2+}\) kinetics. A Ca\(^{2+}\) analysis tool with all these features could potentially be applied to many research questions using a highly standardized procedure.

**Ca\(^{2+}\) transients**

Many attempts have been made to reveal the Ca\(^{2+}\) signalling pathway underlying spontaneous vesicle release. As detailed above, many experiments assessed the properties of mEPSC and mIPSC in response to changes in cytoplasmic Ca\(^{2+}\) due to release from extracellular and intracellular stores (Ermolyuk et al., 2013; Simkus and Stricker, 2002; Xu et al., 2009; Groffen et al., 2010; Vyleta and Smith, 2011; Llano et al., 2000; Liu et al., 2018). However with this methodology, the Ca\(^{2+}\) fluctuations are not directly assessed. Ca\(^{2+}\) fluctuations might be local or global and, in case of local Ca\(^{2+}\) signalling, it should be induced by one or a cluster of Ca\(^{2+}\) channels (Augustine et al., 2003). With imaging methodology, AP-dependent Ca\(^{2+}\) influx is extensively documented (Akerboom et al., 2012; Chen et al., 2013; Ermolyuk et al., 2013). An AP generates a Ca\(^{2+}\) influx which is initially local, mainly localized at the presynapse and subsequently diffuses away to generate a global Ca\(^{2+}\) rise. Koester and Sakmann (2000) combined two-photon microscopy and whole-cell recordings of the presynaptic pyramidal neurons of cortical layer 2/3 of juvenile rats loaded with OGB-1. They showed that the Ca\(^{2+}\) contribution to a single AP in the synaptic bouton is two-fold higher than in the axonal segment (Koester and Sakmann, 2000). Direct imaging of the Ca\(^{2+}\) events related to spontaneous SV release in single cells has not been performed yet, although several types of Ca\(^{2+}\) events occur spontaneously in absence of AP as described in the following paragraph.

**Spontaneous Ca\(^{2+}\) transients (SCTs)**

Besides AP-evoked Ca\(^{2+}\) transients which are well-documented in neurons (Koester and Sakmann, 2000; Yamada and Mikoshiba, 2012), various types of Ca\(^{2+}\) rises have been reported in resting cells. In general, Ca\(^{2+}\) sparks, Ca\(^{2+}\) puffs and Ca\(^{2+}\) syntillas are terms to define Ca\(^{2+}\) transients which occur in confined subcellular compartments. All of them have specific features, for example, Ca\(^{2+}\) sparks are local Ca\(^{2+}\) transients that are mainly observed in muscle cells and they are defined as “an event and its fluorescent recording of Ca\(^{2+}\) release from a single Ca\(^{2+}\) release unit, which refers to a cluster of Ca\(^{2+}\) release channels, either RyRs or IP\(^{3}\)Rs or a mixture, in the ER/SR membrane” (Cheng and Lederer, 2008). Other than in muscle cells, Ca\(^{2+}\) sparks have been recorded using Fluo-3-AM at 330 Hz in PC12 cells, with a frequency of ≈0.02 Hz, and in hippocampal cells with a much lower frequency. These events were classified as elementary when a Ca\(^{2+}\) event had a minimum amplitude of two times the standard deviation of the noise and a total lifetime of 260 ms (Koizumi et al., 1999). DRG neurons, loaded with fluo-4-AM, showed also individual Ca\(^{2+}\) sparks that are confined in time and space and exhibit an amplitude of approximately 0.3 ΔF/F\(_{0}\) and a release duration of 42 ms (Ouyang et al., 2005). Ca\(^{2+}\) puffs have been studied using fluo-8 in SH-SY5Y neuroblastoma cells (Wiltgen et al., 2014). Specifically, Ca\(^{2+}\) syntillas are defined as Ca\(^{2+}\) sparks in a neuronal presynaptic terminal and have
been recorded in mouse nerve terminals from hypothalamic neurons with a sampling frequency of 200 Hz using fluo-3 (De Crescenzo et al., 2004). These Ca\(^{2+}\) transients occurred spontaneously at a holding potential -80 mV and showed a frequency of 0.31 Hz. They are independent of extracellular Ca\(^{2+}\) but depend on RyRs.

Finally, spontaneous Ca\(^{2+}\) transients (SCTs), which have been observed in neurons in absence of AP, were also shown to involve RyR function. SCTs were initially observed in Purkinje cells loaded with fluo-4, in presence of TTX and low agonistic concentration of ryanodine (5-10 μM), showing a SCTs frequency of 0.019 Hz (Llano et al., 2000). SCTs were also recorded in hippocampal slice culture, using OGB-1-AM and a sampling rate of 84 ms, in presence of TTX, 1 μM nicotine and 10 mM Ca\(^{2+}\) in the extracellular solution (Emptage et al., 2001). This system detected a SCT frequency of 0.66 Hz, which is dramatically reduced by the antagonistic effect of 20 μM ryanodine. In 2015, SCTs were studied in hippocampal primary culture, in presence of TTX, loaded with fluo-4-AM and recorded at 10 frames per second. These STCs showed a frequency of 0.32 ± 0.04 min\(^{-1}\) per ROI which depend on [Ca\(^{2+}\)]\(_e\), NMDARs and RyRs (Reese and Kavalali, 2015).

The recurrent role of intracellular Ca\(^{2+}\) channels in Ca\(^{2+}\) sparks, puffs, syntillas and SCTs suggests a shared mechanism for these types of events. Spontaneous Ca\(^{2+}\) transients can also originate from the spontaneous opening of VGCC at resting membrane potential, as demonstrated in cultured hippocampal neurons (Ermolyuk et al., 2013). The exact Ca\(^{2+}\) source and the role of Ca\(^{2+}\) fluctuations in triggering spontaneous release events are currently not clear. Of the same importance, SCTs might activate specific neuronal pathways which have not been elucidated yet. Therefore, more investigations on the triggers and the consequences of SCTs are necessary to unravel their physiological role.

**Techniques to visualise synaptic vesicle release and Ca\(^{2+}\) events**

Several methods are used to assess the different properties of SV release (Dreosti and Lagnado, 2010; Kavalali and Jorgensen, 2014). On the level of single cells, electrophysiology is one of the most used. It provides a temporal resolution of tens of microseconds and is based on measuring the ion current through postsynaptic neurotransmitter receptors. However, this approach does not provide information about the location of the active synapses in the recorded cell (Clements and Bekkers, 1997). Another method involves the optical measurement of synaptic fusion events using probes with specific features such as pH-sensitive probes, FM-dyes, voltage-sensitive dyes and proteins (Dreosti and Lagnado, 2010). One of the most used fluorescent reporters of SV exocytosis and recycling is based on pHluorin linked to synaptophysin, commonly named sypHy (Miesenböck et al., 1998; Granseth et al., 2006). pHluorin is a pH-sensitive mutant of green fluorescent protein that is normally quenched in the acidic pH <6 that occurs in the SV lumen. At neutral pH (∼ 7.4) however, such as in the extracellular space, it emits fluorescence (Miesenböck et al., 1998). Therefore, SV fusion causes a sudden increase in pHluorin fluorescence, thus allowing the optical detection of vesicle
release events by live fluorescence microscopy.

A method to simultaneously measure localized presynaptic Ca\(^{2+}\) and the release of SVs combines the expression in the same neuron of a red-shifted pH-sensitive vesicle exocytosis reporter, for example, VGLUT-mOrange2 or sypHTomato, and a green-emitting GECI, for example, syGCaMP. These probes have been used to assess the Ca\(^{2+}\) and SV fusion properties in response to AP stimulations (Li, 2011; Li and Tsien, 2012). Jackson and Burrone, in 2016, performed a more detailed approach. They fused the red-shifted calcium indicator R-GECO1 (Zhao et al., 2011) and sypHy, in a unique probe sypHy-RGECO and assessed it in AP-evoked stimulation showing that the Ca\(^{2+}\) -dependence of evoked release varies between synapses of the same cell and, hence, the sensitivity of evoked neurotransmitter release to Ca\(^{2+}\) may be synapse specific (Jackson and Burrone, 2016).

Similar combined measurements with Ca\(^{2+}\) and pH indicators could provide valuable information about the relationship between Ca\(^{2+}\) and spontaneous SV release, but to the best of our knowledge this issue has not yet been investigated. Interestingly, this would allow investigating the spatial and temporal properties of the two events.

1.7 Thesis Aims

As detailed above, vesicle secretion is a highly regulated cellular pathway which requires the combined activity of many players, mainly ions and proteins, to coordinate efficient neurotransmission. Both AP-dependent and AP-independent mechanisms regulate SV release and many studies have addressed the function of regulatory proteins and Ca\(^{2+}\) in the two modes of neurotransmission. One of the regulatory proteins, tomosyn, controls neurotransmission by inhibiting evoked SV release through its association with SNARE proteins. Tomosyn is composed of multiple domains with specific roles. Further tomosyn features have been discovered: it has an activity-dependent function and is involved in synaptic plasticity. The Ca\(^{2+}\)-dependence of AP-independent neurotransmission is widely confirmed and the involved molecular pathway is largely resolved. For spontaneous release, a regulatory role for Ca\(^{2+}\) is suggested by many observations but the molecular mechanism of Ca\(^{2+}\)-induced release is mostly unclear. Neurons show SCTs which occur independent from APs, are spatially localized and are short-lived in the order of ms. The physiological role of SCTs is not currently well understood.

This thesis had two main aims. The first aim was to characterise SCTs and study their role in spontaneous SV release. To this purpose an automatic program to analyse Ca\(^{2+}\) imaging data was developed; several Ca\(^{2+}\) indicators (OGB-1, GCaMP6s, GCaMP6f, JRex) were compared for the characterisation of SCTs; SCTs and mEPSCs were correlated in time in the same neuron; and a new imaging tool was developed to simultaneously detect Ca\(^{2+}\) and synaptic fusion event in the same location. The second aim was to study tomosyn localization and novel interactors to unravel further tomosyn functions.
To study SCTs, it was important to develop a program which would satisfy our requirements for the SCTs analysis, and furthermore would be able to be extended by others for different purposes. Chapter 2 explains the methodology used to automatically detect ROIs representing Ca\(^{2+}\) fluctuation events in time-lapse imaging data, to assess and to categorise Ca\(^{2+}\) events and to extrapolate Ca\(^{2+}\) event parameters. The program is named SICT: supervised inspection of fast Ca\(^{2+}\) transients. SICT was able to detect low signal-to-noise-ratio Ca\(^{2+}\) events and detected more events compared to the manual analysis reducing the time of analysis. SICT presents a user-friendly interface which helps to visualise and select true Ca\(^{2+}\) events. Four human observers validated the selection event step. SICT detected an increase in frequency and duration of SCTs in the presence of caffeine in primary hippocampal neurons.

Chapter 3 describes the characterisation of SCTs and their role in spontaneous release in primary hippocampal neurons. GCaMP6f was the best probe to analyse SCTs compared to OGB-1 and GCaMP6s. SCTs were enriched in synaptic compartments and independent of an AP. To focus on the presynaptic function of SCTs, GCaMP6f was fused to synaptophysin, which anchors the resulting syG-CaMP6f specifically to SVs. Interestingly, several presynaptic spontaneous Ca\(^{2+}\) events (SCEs) were observed with different kinetics than SCTs. The simultaneous measurement of presynaptic SCEs and mEPSCs did not show a specific time correlation between the two events, although, the increase of \([\text{Ca}^{2+}]_e\) led to an upregulation of the frequency of both SCEs and mEPSC. A new fluorescent probe sypHyJReX was created to enable the detection of Ca\(^{2+}\) signals and secretion events for the same synaptic vesicle. Preliminary data show that sypHyJReX detected spontaneous Ca\(^{2+}\) events that were immediately followed by vesicle fusion events in the same synapse.

Chapter 4 focuses on tomosyn localization in live neurons. Tomosyn showed both a cytosolic and punctate distribution in wildtype hippocampal neurons. Tomosyn puncta colocalised with various presynaptic and vesicular markers and, moved often together with SV marker synapsin and the LDCV marker NPY. The velocity of the puncta was reduced by field stimulation. The tomosyn structure does not present a membrane-binding domain and it might bind to vesicles through interaction with vesicular proteins. Overexpressing several tomosyn mutants in primary hippocampal neurons showed a consistent vesicular binding indicating that tomosyn might bind vesicles by redundant mechanisms. Tomosyn immunoprecipitated together with syt1 (Fujita et al., 1998; Yamamoto et al., 2010b), but it still bound vesicles in its absence. The targeting of tomosyn to vesicles might be important to localize tomosyn close to vesicular release sites, to regulate vesicular release parameters and/or to control the delivery and pan-synaptic sharing of vesicles.

Chapter 5 presents a summary of the main finding in this thesis. Furthermore, the results are discussed with the current literature to give a general perspective with further pieces of evidence on the role of Ca\(^{2+}\) in spontaneous release. Finally, an updated model of tomosyn function is provided.