Chapter 5

General discussion
5.1 Research background, thesis aims and main findings

As extensively detailed in chapter 1, evoked neurotransmission is a tightly regulated mechanism which leads to the release of SVs upon depolarization of the synapse by an AP. Tomosyn contributes to the regulation of synaptic secretion by negatively affecting the release probability in naive neurons and positively enhancing the synaptic strength during sustained activity (Geerts et al., 2015; Sakisaka et al., 2008). In addition, SVs are released in the absence of AP depolarization, a process defined as spontaneous release. It has also recently been shown that neurons undergo spontaneous Ca\(^{2+}\) fluctuations, SCTs, which are independent of APs. Previous studies show that SCTs are dependent on extracellular and intracellular Ca\(^{2+}\) stores (Llano et al., 2000; Emptage et al., 2001; Reese and Kavalali, 2015). However, the molecular mechanism and function of SCTs are not fully elucidated yet.

To characterise SCTs in primary hippocampal neurons, it is important to consider that the manual analysis of SCTs is time-demanding and subject to bias. Despite the availability of many programs to analyse Ca\(^{2+}\) imaging data (Patel et al., 2015; Jang and Nam, 2015; Steele and Steele, 2014), none was fully suitable for our purpose for which we needed sufficient sensitivity to detect small-amplitude signals, automated generation of small regions of interest, handling of very large datasets, visual event representation and tools to categorise and quantitatively describe different event types. Hence, we developed SICT, a program which automatically detects ROIs containing Ca\(^{2+}\) events in imaging data with low signal-to-noise ratios. SICT is described in chapter 2. SICT was applied to speed up and standardize the SCT analysis but, also, to facilitate the analysis of Ca\(^{2+}\) events for various other purposes, such as complex Ca\(^{2+}\) events and AP-evoked Ca\(^{2+}\) influx. For SCT detection, a trade-off exists between sensitivity (avoiding false negatives) and accuracy (avoiding false positive hits). We opted for high sensitivity and chose to include a significant amount of false positive events in the analysis. These events can be sorted for properties that predict the likelihood to represent a biological signal, and quickly inspected in a graphical user interface. This method allows the spatial and temporal visualisation of the ROIs in both raw Ca\(^{2+}\) imaging data and the calculated \(\Delta F/F_0\) signal. Synthetic data and human observer comparison were used to validate SICT. Using the same dataset, SICT detected more events than an entirely manual analysis and reduced the manual labour to less than 10% of time. The intensity signal was different between SICT and manual analysis. This difference was dependent on the ROI area defined by the two methods. As an example application, we used SICT to show that caffeine increases the frequency and duration of SCTs. The availability of SICT is valuable to speed up future investigation of spontaneous Ca\(^{2+}\) signals in living cells. The open source code allows future fine-tuning of the software, which will likely be relevant to implement future discoveries of event types or optimized Ca\(^{2+}\) reporters.

In chapter 3, we characterised SCTs in primary hippocampal neurons. The peak durations of SCTs were strongly dependent on the probe used. GCaMP6f was the best Ca\(^{2+}\) indicator to detect SCTs compared to OGB-1, GCaMP6s and JRex.
in our experimental settings. SCTs were detected in absence of AP by clamping neurons at resting potential (-70 mV) or by using TTX. They were enriched in presynaptic compartments. Other types of spontaneous Ca\(^{2+}\) events (SCEs) were also observed, showing different kinetic waveforms of the fluorescent signal. The importance of Ca\(^{2+}\) influx for AP-evoked SV release is well established. The Ca\(^{2+}\)-dependence of spontaneous SV release cannot be ascribed to a single defined mechanism for Ca\(^{2+}\) elevation, because inhibiting intracellular Ca\(^{2+}\) influxes from several Ca\(^{2+}\) sources does not lead to a complete blockage of the SV spontaneous release (Ermolyuk et al., 2013; Simkus and Stricker, 2002). This suggests that multiple pathways exist. Aiming to clarify this debate, we simultaneously recorded SCEs and spontaneous SV release events from the same neuron using two parallel approaches which would examine both the temporal and spatial correlation of the two events. First, SCTs or SCEs and mEPSCs were recorded in the same neurons. An increased SCE and mEPSC frequency were detected after increasing [Ca\(^{2+}\)]. However, no strict temporal correlation linking the two events was found. Using a complementary approach, we developed a new fluorescent probe sypHyJReX which detects Ca\(^{2+}\) signals and secretion events for the same synaptic vesicle. SypHyJReX was validated to report both signals with good optical separation of the dual emission channels. The single-molecule nature of the GECI and the simultaneous imaging of both channels on a single EM-CCD chip provides an excellent tool to investigate Ca\(^{2+}\)-secretion coupling of spontaneous release. Interestingly, preliminary sypHyJReX data showed that SCEs co-occurred with spontaneous vesicle fusion events in the same synapse. However, further studies need to be done to finally assess how scarce or abundant such coupled events are in various types of neurons.

In chapter 4, we aimed to investigate the mechanism by which the SNARE-binding protein tomosyn regulates secretion. Tomosyn is mainly thought to function as an inhibitor of secretion in endocrine cells and individual tomosyn domains exert specific functions (Fujita et al., 1998; Yamamoto et al., 2010a; Hatsuzawa et al., 2003; Pobbati et al., 2004; Sakisaka et al., 2008; Gladycheva et al., 2007; Yizhar et al., 2007; Masuda et al., 1998; Yamamoto et al., 2009; Williams et al., 2011). Tomosyn does not contain a transmembrane domain. Nevertheless, it accumulated in mobile puncta in both axons and dendrites of hippocampal neurons. It co-localized and co-migrated with both SV and LDCV markers. Tomosyn puncta showed slightly reduced mobility after high-frequency field stimulation. A third type of tomosyn puncta did not co-migrate with SV or LDCV markers, suggesting additional involvement of another organelle. Different tomosyn fragments were sufficient for vesicular targeting, even in absence of the known interactor of tomosyn, syt1. This suggests that the tomosyn-vesicle interaction involves multiple parallel interactions. Our immunoprecipitation and MS approaches on mouse brain synaptosomes confirmed the previously established tomosyn-1 interaction with syntaxin-1A, SNAP25 and syt1, but not Rab3A or novel interactors. These results challenge the working hypothesis of tomosyn as soluble inhibitor of SNARE complex. This idea could relate to previous studies where tomosyn enhanced synaptic release during sustained activity (Geerts et al., 2015; Sakisaka-
ka et al., 2008). It is not clear in which stage of the secretory process tomosyn is involved. To clarify this point, more functional studies on the role and modes of tomosyn association to SVs are needed.

### 5.2 Mechanism of spontaneous release

**SCEs and spontaneous SV release**

Ca\(^{2+}\)-dependence of spontaneous SV release is a highly debated topic. The mechanism of spontaneous SV release may differ from the classical pathway that triggers evoked SV release (Ramirez and Kavalali, 2011; Kaeser and Regehr, 2014; Smith et al., 2012). In contrast to AP-dependent neurotransmission, which is well known to depend on Ca\(^{2+}\) influx, spontaneous SV release is only partly abolished by interfering with Ca\(^{2+}\) pathways such as by intracellular administration of BAPTA. Assuming that this treatment results in a complete blockade of Ca\(^{2+}\) signalling, the remaining fraction of spontaneous release must originate from a truly Ca\(^{2+}\)-independent mechanisms (Kaeser and Regehr, 2014; Vyleta and Smith, 2011; Simkus and Stricker, 2002). At the same time, a major fraction of spontaneous release events is Ca\(^{2+}\) dependent. To study this Ca\(^{2+}\) dependence, mEPSCs are generally counted before/after altering [Ca\(^{2+}\)]\(_i\) by influencing intracellular or extracellular Ca\(^{2+}\) concentration or perturbing the function of Ca\(^{2+}\) channels or Ca\(^{2+}\) sensors. Instead of electrophysiological detection of mEPSCs, optical detection of spontaneous SV release is also possible with fluorescent reporters for vesicle fusion (usually based on pHluorin) or FM dyes (Jackson and Burrone, 2016). An increase in mEPSC frequency is commonly observed in literature after increasing [Ca\(^{2+}\)]\(_e\) or applying caffeine (Vyleta and Smith, 2011; Sharma and Vijayaraghavan, 2003; Simkus and Stricker, 2002; Shimizu et al., 2008). In line with these observations, in our study, the increase of [Ca\(^{2+}\)]\(_e\) up to 10 mM boosted both mEPSC and SCE frequency in the same neuron and also more global [Ca\(^{2+}\)] increases (chapter 3). The SCE frequency is likely to affect the global [Ca\(^{2+}\)]\(_i\), because altered supply of Ca\(^{2+}\) ions into the cytosol will reset the balance with energy-consuming Ca\(^{2+}\) clearance. This effect is clearly visible in the Fura-2 experiment. Local and more global [Ca\(^{2+}\)] increases probably both contribute to enhancing spontaneous SV release. Our data provide support for the prominent involvement of longer lasting or complex Ca\(^{2+}\) elevations than for fast SCTs. This confirms the general notion that the more Ca\(^{2+}\) is around, the more spontaneous release is triggered.

SCEs include multiple waveforms: fast isolated Ca\(^{2+}\) transients (i.e. SCT), Ca\(^{2+}\) transients of longer duration; stepwise Ca\(^{2+}\) increase; waves; and waveforms with multiple peaks (chapter 2). Prada and colleagues measured the global hippocampal Ca\(^{2+}\) activity with OGB-1 and detected a reduction in the number of Ca\(^{2+}\) events after using TTX, CNQX, and APV (Prada et al., 2018). Interestingly, these SCEs are defined “to appear in an unpredictable spatiotemporal pattern” with smaller activity, more localised and not uniformly shaped like the typical Ca\(^{2+}\) spikes with fast onset and slow decay of naive cells. The Ca\(^{2+}\) signal waveform of SCTs is very different from that of other SCEs, suggesting that they originate from different signalling mechanisms. The large variety of waveforms of the longer
lasting SCEs may imply additional complexity or an interplay between multiple physiological processes. Further studies need to assess the requirement for different types of Ca\(^{2+}\) channels, the role of Ca\(^{2+}\) buffers in the surrounding space of the Ca\(^{2+}\) event and the effects of Ca\(^{2+}\) clearance mechanisms on Ca\(^{2+}\) homeostasis. Moreover, it would be interesting to evaluate if the different Ca\(^{2+}\) kinetics might trigger independent different neuronal pathways.

It has been observed that spontaneous SV release induces postsynaptic Ca\(^{2+}\) increase through the NMDAR (Leitz and Kavalali, 2014). Hippocampal neurons expressing a Ca\(^{2+}\) indicator linked to PSD95 show spontaneous postsynaptic Ca\(^{2+}\) transients which represent a minor contribution compared to the SCTs detected with a globally expressed Ca\(^{2+}\) indicator, suggesting that the majority of SCTs do not take place in the dendrites (Reese and Kavalali, 2015). In our hands, the presynaptic Ca\(^{2+}\) indicator syGCaMP6f was more effective at detecting SCTs than the global variant and, moreover, several SCE waveforms were observed. SCTs might have different roles based on their neuronal localization, such as presynapse and postsynapse. For future investigations, syGCaMP6f is a valuable tool to study presynaptic SCEs.

We aimed to evaluate if spontaneous presynaptic Ca\(^{2+}\) increases trigger spontaneous SV release. No temporal correlation was found between SCTs or SCEs and mEPSCs, at least, performed at 4 mM [Ca\(^{2+}\)]\(_e\) (chapter 3). As discussed below, methodological aspects could have contributed to this outcome. Although mEPSCs and SCEs/SCTs did not co-occur at the same time, sypHyJReX preliminary data shows that spontaneous SV fusion events occur in concomitance with Ca\(^{2+}\) events at the same subcellular compartment. In Purkinje cells, SCTs show an increase in frequency during RyR stimulation which also induces an increase in mIPSC frequency (Llano et al., 2000). This is in line with the increase of presynaptic SCT frequency induced by caffeine in our experiments (chapter 2). It has recently been shown that excitatory and inhibitory synapses have different spontaneous SV release properties (Courtney et al., 2018; Tsintsadze et al., 2017; Liu et al., 2018). For example, EGTA or Cd\(^{2+}\) inhibit the mEPSC frequency of cholinergic synapses at C. elegans NMJs more than the mIPSC frequency at GABAergic synapses (Liu et al., 2018). In contrast, Cd\(^{2+}\) reduces the spontaneous release of GABA but not glutamate in mouse neocortical neurons (Tsintsadze et al., 2017), in particular when mIPSC are measured in Doc2b-KO but not in syt1-KO (Courtney et al., 2018). In our primary hippocampal neuronal cultures, excitatory neurons were the main neuronal population and only a minor presence of inhibitory neurons was found. Unfortunately, we cannot discriminate between excitatory and inhibitory neurons with sypHyJReX analysis as we did for the temporal correlation where only excitatory neurons were analysed.

While our current results were obtained with an [Ca\(^{2+}\)]\(_e\) of 4 mM, it would also be interesting to study the temporal correlation between SCTs / SCEs and mEPSC under different conditions. Suitable tools to increase the frequency of SCEs are caffeine, ryanodine or nicotine (Emptage et al., 2001). Alternatively, compounds acting on Ca\(^{2+}\) extrusion can be exploited to selectively increase global [Ca\(^{2+}\)]\(_i\).
(e.g. the PMCA inhibitor vanadate or the more selective caloxins). Such a pharmacological approach may yield a shortlist of candidate proteins in Ca\(^{2+}\) signalling, which can subsequently be identified by genetic approaches. The same experiment could be also performed in GABAergic mouse neurons. Furthermore, syphHyJReX could be selectively expressed in either glutamatergic or GABAergic neurons, using specific promoters, to assess differences in the molecular mechanism of Ca\(^{2+}\)-coupled spontaneous SV release by altering Ca\(^{2+}\) pathways and Ca\(^{2+}\) sensors.

**Differences in the Ca\(^{2+}\)-dependence of spontaneous and AP-evoked SV release**

Many efforts have been made to quantify the exact [Ca\(^{2+}\)]\(_i\) at rest and in several conditions. It is important to emphasize that the Ca\(^{2+}\) signal caused by an AP is extremely short-lived, rising and falling quickly after the opening and closure of the VGCC. As a result of the three-dimensional diffusion of Ca\(^{2+}\) ions from the channel pore into the surrounding cytoplasm, the [Ca\(^{2+}\)]\(_i\) is also very inhomogeneous in the spatial dimension. This spatiotemporal heterogeneity, as well as the presence of endogenous Ca\(^{2+}\) buffers, makes it challenging to estimate the actual Ca\(^{2+}\) concentrations reached in living cells.

Despite these challenges, good estimations have been made in living neurons and synapses. For this purpose, most studies use ratiometric Ca\(^{2+}\) dyes. In most neurons the resting [Ca\(^{2+}\)]\(_i\) has been calculated to range around 50 nM which rises to \(\approx 500\) nM after a single AP (Neher and Sakaba, 2008) (Figure 1). For example, the resting [Ca\(^{2+}\)]\(_i\) in the proximal apical dendrite in CA1 pyramidal neurons in rat hippocampal slices is \(\approx 40\) nM which increases to \(\approx 250\) nM after single AP (Maravall et al., 2000). Similarly in the calyx of Held, a resting [Ca\(^{2+}\)]\(_i\) of \(\approx 40\) nM rises to \(\approx 450\) nM after single AP (Helmchen et al., 1997; Müller et al., 2007).

![Figure 1. Ca\(^{2+}\)-dependent SV release rate.](https://example.com) **Figure 1. Ca\(^{2+}\)-dependent SV release rate.** The intracellular dose-response curve of the Ca\(^{2+}\) involvement in SV release measured at the calyx of Held (Lou et al., 2005), showing the release rate at [Ca\(^{2+}\)]\(_i\) at rest, after a single AP and 100 Hz train stimulation (Reproduced from Neher & Sakaba 2008, with permission from Elsevier).
Thus, on average a single AP increases the $[\text{Ca}^{2+}]$ approximately 5-10 fold. If different $[\text{Ca}^{2+}]$ regulate spontaneous and AP-dependent SV release then smaller $[\text{Ca}^{2+}]$ changes up to 5 times the resting $[\text{Ca}^{2+}]$ might specifically trigger spontaneous SV release.

In our experiments, we aimed to monitor $\text{Ca}^{2+}$ fluctuations with a high temporal resolution. This precludes excitation wavelength switching, and thus ratiometric $\text{Ca}^{2+}$ imaging with Fura-2. Emission ratiometry with Indo-1 suffered from high bleaching rates and was therefore not suitable (data not shown). Reliable detection of SCTs was achieved with GCaMP6f, providing estimates of their frequency, kinetics and location within the neuron. In our hippocampal primary neurons, the $\text{Ca}^{2+}$ signal peak intensity evoked from single APs was 1.4-fold bigger compared to SCTs when syGCaMP6f was used and approximately four times bigger when OGB-1 was used; although the sample size was low for syGCaMP6f (chapter 3). These values might be underestimated because, as detailed below, lower amplitude SCTs/SCEs might have been missed which would lead to underestimation of the SCT frequency and overestimation of the SCT amplitude.

Interestingly at the frog NMJ, in the presence of TTX, the frequency of individual SV release events is gradually increased up to 100-fold after increasing the concentration of KCl, which elevates the resting $[\text{Ca}^{2+}]$ from $\approx 100 \text{ nM}$ to 1–5 $\mu\text{M}$ $[\text{Ca}^{2+}]$. A sharp increase in frequency is already detectable at around $\approx 500 \text{ nM}$ $[\text{Ca}^{2+}]$ (Angleson and Betz, 2001). The $\text{Ca}^{2+}$ chelators EGTA and BAPTA strongly reduce but do not completely abolish mEPSCs (Angleson and Betz, 2001; Simkus and Stricker, 2002). This might be explained by the inability of the $\text{Ca}^{2+}$ chelators to buffer the free $\text{Ca}^{2+}$ concentration to zero or SNARE-dependent membrane fusion without the contribution of $\text{Ca}^{2+}$-sensors. Alternatively, it cannot be completely excluded that the remaining mEPSCs arise from a different $\text{Ca}^{2+}$-independent mechanism. Although EGTA and BAPTA present different binding kinetics, the $K_d$ for both $\text{Ca}^{2+}$ chelators is in the order of 100 nM (depending on pH and temperature). Therefore, it cannot be concluded with certainty that SV release at lower $[\text{Ca}^{2+}](< 100 \text{ nM})$ is $\text{Ca}^{2+}$-independent (Angleson and Betz, 2001). The plasma membrane recruitment of the $\text{Ca}^{2+}$ sensor protein Doc2b, participating in spontaneous release, is responsive to $[\text{Ca}^{2+}]$ changes between 100 nM and 1 $\mu\text{M}$ with half-maximal activation at of 175 nM as measured in chromaffin cells (Groffen et al., 2006). The fluctuations in intracellular $\text{Ca}^{2+}$ will also be shaped by the presence of cytoplasmic $\text{Ca}^{2+}$ buffering proteins such as parvalbumin ($K_d$ 9 nM) and calbindin ($K_d$ 393 nM) (Fairless et al., 2019).

Taken together, the regulation of the spontaneous SV release rate likely depends on $[\text{Ca}^{2+}]$, fluctuations in the range of close to rest is regulated by a $\text{Ca}^{2+}$-dependent mechanism different from evoked release 50–500 nM. To trigger spontaneous SV release, the resting $[\text{Ca}^{2+}]$ should rise to 1–10 times and maybe a $\text{Ca}^{2+}$ sensor, different from the one involved in evoked release, could be involved in this process. Increasing the affinity of the $\text{Ca}^{2+}$ chelators and the sensitivity of SCE detection would help a more detailed analysis of the molecular mechanism underlying spontaneous SV release at resting $[\text{Ca}^{2+}]$. Finally, truly $\text{Ca}^{2+}$ independent
release could arise from the activity of SNARE proteins alone, as demonstrated by experiments with synthetic liposomes where SNARE proteins are minimal-ly sufficient to induce membrane fusion (Weber et al., 1998), even though the inclusion of Munc13, Munc18, syt1 and complexin greatly enhance the speed and Ca\(^{2+}\) dependence of this process (Malsam et al., 2012; Ma et al., 2013). To summarise all possible relations between SCEs, global [Ca\(^{2+}\)], and spontaneous SV release, a hypothetical model is depicted in Figure 2.

**Figure 2. Ca\(^{2+}\)-dependence of spontaneous SV release.** The cartoon model shows a presynaptic terminal with SVs, Ca\(^{2+}\) channels at the PM and at intracellular membranes. Besides the classical pathway for AP-evoked SV release, four additional pathways could lead to spontaneous SV release. First, spontaneous opening (‘flickering’) or ligand-induced opening of a single channel or channel cluster on the PM may give rise to SCEs and cause spontaneous SV release. As a second possibility, SCEs may originate from the ligand-induced opening of Ca\(^{2+}\) channels on intracellular Ca\(^{2+}\) stores. Third, global Ca\(^{2+}\) rises may result from an altered balance between Ca\(^{2+}\) influx and Ca\(^{2+}\) clearance causing an increased spontaneous release rate. Finally, the persistence of spontaneous release in presence of BAPTA suggests that a truly Ca\(^{2+}\)-independent mechanism also exists. The type of synapses, for example, glutamatergic and GABAergic, and the individual synapse composition of Ca\(^{2+}\) buffers, inhibitors of SV release, Ca\(^{2+}\) sensors, channels and SNARE isoforms may determine the ability of a synapse to release SV AP-dependently or -independently. Moreover, different [Ca\(^{2+}\)]\(_i\) could selectively release SVs from different pools. If multiple pathways are responsible for triggering spontaneous SV release then it is conceivable that several functional roles could be attributed to this process, ranging from the maintenance and homeostasis of synapses during early stages of neuronal development to synaptic plasticity in mature systems. Further research is necessary to clarify these hypotheses and draw a complete picture of the mechanisms and roles of spontaneous SV release.

**The temporal correlation between mEPSCs and SCEs**

Given the different types of spontaneous Ca\(^{2+}\) kinetics observed in hippocampal neurons and the many divergent functions of Ca\(^{2+}\), it is highly complex to dissect
the physiological role of SCEs. The SCTs, which form a distinct subtype of events characterised by fast kinetics (order of ms), are the single most abundant class of SCEs observed in neurons (chapter 2). However, no specific temporal coupling was detected between mEPSCs and SCTs (chapter 3). The same analysis for SCEs instead of SCTs yielded the same result. In both cases, the $[\text{Ca}^{2+}]_e$ was 4 mM. To consider the possibility that an existing temporal coupling may remain undetected, four methodological limitations may be noted. The first aspect involves the microscopic field of view which was smaller than the entire neuronal structure. This might lead to underestimate the number of SCEs per neuron. In contrast, using electrophysiology in autaptic neurons, it is possible to record all mEPSCs of a neuron (Bekkers and Stevens, 1991; Mennerick et al., 1995; Kimura et al., 1997). As a second limitation, a further underestimation of SCEs may be due to SICT, which has been measured to detect SCTs with a detection sensitivity of 89%. This detection sensitivity was calculated from simulated data in which synthetic signals were mixed at various ratios with experimentally acquired biological noise. Therefore, we cannot ensure that all SCEs are detected (chapter 2). The third issue concerns the neuronal Ca$^{2+}$ event detection sensitivity of the used Ca$^{2+}$ indicator. GCaMP6f, which was used for the temporal correlation, binds Ca$^{2+}$ with a $K_d$ of 290-375 nM (Badura et al., 2014; Chen et al., 2013). Hence, Ca$^{2+}$ events of a small increment (< 300 nM) may remain undetected (chapter 2 and 3). All of these three technical limitations may mask a hypothetical correlation between SCTs/SCEs and mEPSC, the latter of which occurs in higher numbers of events.

As a fourth limitation, the presynaptic Ca$^{2+}$ signal evoked by single APs, which occurs prior to vesicle release, and therefore also prior to the EPSC peak, had a delay of 50-150 ms relative to the EPSC peak, although the data were acquired at exactly the same time (chapter 2). A slightly longer delay of $\approx$320 ms is detected between AP and the Ca$^{2+}$ event measured with OGB-1 (Prada et al., 2018). This delay may vary and be due to the properties of the Ca$^{2+}$ signal, of the Ca$^{2+}$ indicators, their subcellular localization and the detection parameters. Based on these results a time range of $\pm$ 250 ms and $\pm$ 1 s was applied to investigate the potential temporal coupling of mEPSCs and SCEs. However, it can be debated if the same delay is to be expected for spontaneous and evoked SV release. It is also possible that SCEs and mEPSCs are linked by a mechanism that exhibits a variable time dependence, for example depending on the location of the Ca$^{2+}$ event and the Ca$^{2+}$ diffusion distance versus a responsive SV and sensor. The comparison of the signal peak widths measured with OGB-1, GCaMP6 and JRex suggests that the used Ca$^{2+}$ indicator contributes importantly to the delay in the Ca$^{2+}$ response.

5.3 Tomosyn

How tomosyn associates to secretory vesicles

Tomosyn has been shown to associate with vesicles. In insulin-secreting INS-1 cells, tomosyn associates with LDCVs (Trexler et al., 2016). Using hippocampal neurons, we showed that tomosyn co-localized and co-migrated with SVs and LDCVs markers. Moreover, tomosyn resided close to vesicle-like structures at
presynaptic zones. Tomosyn interactors were detected using mouse brain synaptosomes followed by mass spectrometry (MS) and confirmed by reverse IP-MS analysis (chapter 4). The previously established interactions with the t-SNAREs, SNAP25 and syntaxin-1A, and with syt1 were confirmed (Fujita et al., 1998; Yamamoto et al., 2010b).

The interaction with SNAP25 and syntaxin-1A involves the C-terminal CC domain of tomosyn which can engage in a stable four-helical bundle (Hatsuzawa et al., 2003; Masuda et al., 1998; Fasshauer et al., 1998). Expression in PC12 cells of syntaxin-1A alters the tomosyn distribution to the PM (Williams et al., 2011) and the expression of a tomosyn mutant lacking the C-terminal domain impairs tomosyn relocation to the PM (Bielopolski et al., 2014). Syntaxin-1A is also detected on the SV proteome (Takamori et al., 2006) and moves between synaptic and extrasynaptic regions in primary cultures of rat spinal cord neurons (Ribrault et al., 2011). Based on our results, SNARE interactions are not essential for tomosyn vesicular targeting since various tomosyn fragments carrying C-terminal deletions co-migrated normally with SVs and LDCVs (chapter 4). PKA-dependent tomosyn phosphorylation (Baba et al., 2005) and ROCK-dependent syntaxin-1A phosphorylation (Sakisaka et al., 2004) regulate syntaxin-1A/tomosyn binding, respectively by reducing and enhancing the interaction. Thus, post-translational modifications can theoretically increase the possible number of tomosyn binding partners and, therefore, its functions in different pathways and cell types. Recently, it has been found that in activated mast cells, PKC-gamma phosphorylates tomosyn which, in turn, inhibits ß-hexosaminidase release. Interestingly, mast cell activation leads to tomosyn translocation from the t-SNAREs STX4 to associate with the v-SNARE STX3 (Madera-Salcedo et al., 2018).

The N-terminal WD40 domain is sufficient for tomosyn association to SVs in C. elegans (McEwen et al., 2006), as we also observed in hippocampal neurons (chapter 4). Moreover in a Ca²⁺-dependent manner, the tomosyn WD40 domain binds syt1 and inhibits SNARE complex-mediated membrane fusion (Yamamoto et al. 2010). Although the tomosyn/ syt1 interaction was confirmed, further analysis in living neurons showed that both the WD40 and CC domains bind vesicles in the absence of syt1 (chapter 4), suggesting the involvement of additional SV interactions.

Further candidates that link tomosyn to vesicles are the small GTPase Sec4p, yeast homologous of the mammalian Rab3A (Rossi and Brennwald, 2011; Cazares et al., 2016), and the actin-binding motor protein Myo2p, yeast homologs of the mammalian Myosin-Va (Watson et al., 2015). Both Rab3A and Myosin-Va are identified as presynaptic vesicle-associated proteins in rat central neurons (Takamori et al., 2006). The N-terminal domain of Sro7p, yeast tomosyn ortholog (Hattendorf et al., 2007), interacts with Sec4p-GTP, to coordinate tethering and priming of vesicles at fusion sites (Rossi et al., 2015; Watson et al., 2015; Rossi et al., 2018). In hippocampal neurons, tomosyn interacts with Rab3A-GTP but not Rim1/2, actin or synaptophysin (Cazares et al., 2016). Rab3A functions as a bridge between tomosyn and synapsin (Cazares et al., 2016). Additional evi-
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dence shows that the amino acids upstream of the N-terminal domain of Sro7p bind the actin-binding motor protein Myo2p during polarized exocytosis (Rossi and Brennwald, 2011). Since tomosyn binding is dependent on the GTP bound state of Rab3A or Sec4p (Cazares et al., 2016; Rossi et al., 2018), the interaction might be transient and, therefore, remain undetected in our experiments. It is possible that the protein extraction procedure, i.e. from whole cell lysate to synaptosome fraction, could selectively detect specific tomosyn interactors, but prevent others. In support of this idea, the expression of tomosyn C-terminal domain showed a reduced co-migration with synapsin puncta but not NPY puncta indicating that tomosyn binding modes might differ between SVs and DCVs (chapter 4). We also did not detect an interaction with Myosin-Va. However, in the proteome screening where syt1 and Rab3A were immunoprecipitated, Myosin-Va was detected as an interactor. Myo18a (Isoform 6 of Unconventional myosin-XVIIIa) was detected as tomosyn-2 interactor and Myl2 (Myosin regulatory light chain 2) was detected as tomosyn-1 interactor. Myl2 was also detected as part of Rab3a interactors. However, a reverse IP experiment for Myo18a, Myl2 and Myosin-Va was not performed to confirm these bindings. Furthermore, tomosyn-2 was also found as interactor of Rab3A, although Rab3A was not present as tomosyn-2 interactor (unpublished data).

Given the strong evolutionary conservation of the overall domain structure and the function of tomosyn, it is very well conceivable that tomosyn interaction with Myosin-like and Rab-like proteins might be relevant in mammalian system too, even though firm evidence is currently lacking. In summary, tomosyn may associate with secretory vesicles using multiple distinct interactors depending on the type of vesicles, cellular pathway and/or cell types. The functional role of this association is discussed in the next paragraph.

Functions of tomosyn vesicle targeting

The canonical role of tomosyn is to interact with the PM t-SNARE proteins to inhibit vesicle fusion. Additional tomosyn functions can be attributed to tomosyn vesicle association. Tomosyn vesicle association through the interaction between tomosyn and syt1 occurs in a Ca$^{2+}$-dependent manner and inhibits the syt1-mediated step of Ca$^{2+}$-dependent neurotransmitter release as it has been shown in vitro in a liposome fusion assay and, in vivo, at the SCG presynapses injected with the N-terminal fragment of tomosyn-1 and/or the cytoplasmic domain of syt1 (Yamamoto et al., 2010b).

Vesicular association of tomosyn is conserved through evolution. The binding of the yeast ortholog Sro7p to Myo2p (Rossi and Brennwald, 2011) and to Sec4p-GTP (Watson et al., 2015) link Sro7p to vesicles and suggest a role in secretory organelle trafficking. Myo2p is important to localize Sro7p in the bud of growing cells and at sites of cell division during cytokinesis (Rossi and Brennwald, 2011). Myosin-Va, the mammalian homolog of Myo2p, contributes to vesicle motility. In hippocampal neurons, Myosin-Va regulates the retrograde axonal movement of DCVs (Bittins et al., 2010), local SV transport (Bridgman, 1999) and SV tethering at the plasma membrane during stimulation (Maschi et al., 2018). Rab proteins,
including Sec4-GTP (Rab3A-GTP), are involved in tethering vesicles to target membranes which is a process thought to occur prior to SNARE-mediated fusion (Binotti et al., 2016). For example, Rab3A-GTP function is required for the correct assembly and the anterograde transport of vesicles (Szodorai et al., 2009). Interestingly, Myo2 interacts directly with Sec4 and the disruption of this interaction results in compromised growth and the accumulation of secretory vesicles (Jin et al., 2011). In rat hippocampal synapses, inhibition of Myosin-Va causes a vesicle docking defect during sustained activity (Maschi et al., 2018). Similarly, Rab3A deletion reduced the number of docked vesicles at the active zone after stimulation, without affecting the total number of vesicles (Leenders et al., 2001). Tom-1 C. elegans null mutants have an increased number of vesicles contacting the plasma membrane distributed throughout the terminal (Gracheva et al., 2006), suggesting that tomosyn controls the spatial distribution of vesicles. Tomosyn-overexpressing bovine chromaffin cells show normal numbers of docked vesicles although the secretion is decreased (Yizhar et al., 2004). Contrarily, overexpression of the yeast tomosyn ortholog Sro7 drives the formation of post-Golgi vesicle clusters (Rossi and Brennwald, 2011). Moreover, the impairment of the Sro7p / Sec4-GTP binding blocks Sro7p’s ability to tether vesicles in vitro (Rossi et al., 2018). In insulin-secreting INS-1 cells, tomosyn is tightly associated with LDCVs until fusion, when it diffuses away (Trexler et al., 2016). Interestingly, tomosyn localizes at the palm of growth cones and directs the fusion of plasmalemmal vesicles to the tip of the growth cone during neurite extension (Sakisaka et al., 2004). In our experiments, mobile tomosyn puncta moved bidirectionally with various speeds. The puncta merged and departed from mobile and non-mobile structures identified to be positive for synapsin or NPY (chapter 4). In conclusion, all these observations support a possible role of tomosyn in regulating vesicle cluster formation and vesicle targeting.

Transport of vesicle clusters within presynaptic terminals is a possible mechanism involved in synaptic plasticity induced by repetitive stimulations (Darcy et al., 2006; Staras et al., 2010). In our live imaging study, tomosyn puncta were repeatedly observed to split up into two or more sub-structures, indicating that the original structure was a moving vesicle cluster (Staras et al., 2010). We did not detect an effect of overexpressed tomosyn on the velocity of vesicles; however, field stimulation slightly reduced the velocity of tomosyn puncta (chapter 4). Previous studies have also reported a reduction in vesicle speed during neuronal activity (de Wit et al., 2006b). A study in tomosyn-2 null mice indicated that tomosyn-2 inhibits spontaneous acetylcholine release in resting NMJs but positively affects excitatory postsynaptic potentials after repetitive firing (Geerts et al., 2015). Very likely, repetitive activity is accompanied by posttranslational modifications of tomosyn and its interactors, as has been shown for PKA and ROCK (González-Forero et al., 2012; Baba et al., 2005; Sakisaka et al., 2004), suggesting the possibility that these modifications have a marked effect on tomosyn activity. In conclusion, tomosyn function is likely activity dependent and might not regulate motor proteins but could contribute to vesicle clustering, traffic and delivery to target locations. Thus, the several tomosyn posttranslational modifi-
cations, binding partners and dynamic localization suggest that the function of tomosyn may differ between cells and cellular conditions.

Combining all these considerations, we hypothesize that tomosyn may regulate the clustering and transport of secretory vesicles from their biogenesis to their end point by interacting with several SV proteins and with motor proteins. Tomosyn could be involved in refilling the synapses with SVs to sustain repetitive neuronal activity preventing the depletion of SVs in the synaptic terminal and this is suggested by the following facts. First, tomosyn null synapses show an increase of docked vesicles; second, the velocity of tomosyn puncta is reduced after stimulation; third, tomosyn inhibits syt1 Ca\(^{2+}\)-dependent SV exocytosis and; fourth, tomosyn enhances sustained activity.

5.4 Conclusion

Previous studies have emphasized the challenge in understanding the Ca\(^{2+}\)-dependence of spontaneous SV release in neurons and several theories have been proposed. However, a direct link between cytoplasmic Ca\(^{2+}\) influx and SV release in terms of space and/or time is still missing. Part of this thesis aimed to characterise SCEs with a focus on SCTs, specifically at the presynapse. The SICT method significantly speeds up the analysis of Ca\(^{2+}\) events. SCTs were affected by caffeine and SCEs were influenced by [Ca\(^{2+}\)]\(_{e}\) together with mEPSCs. A temporal correlation analysis between mEPSC and SCEs did not demonstrate a tight coupling of the two events and preliminary data with the dual channel sypHyJRex clearly shows that spontaneous SV fusion events can occur in concomitance with SCEs in the same puncta. This apparent discrepancy may be due to the methodology used or the types of neuron analysed. To resolve these important questions, various approaches are proposed as future directions (see below).

The second topic of this thesis was to examine how tomosyn regulates synaptic strength. In the current literature, the canonical tomosyn role is to regulate SNARE protein function, presumably by competing with VAMPII for SNARE complex formation. The association of tomosyn with vesicular organelles may support various mechanisms other than simple competitive inhibition, including regulating the transport of proteins to sites of release with the goal to inhibit off-site secretion or to capture vesicles during synaptic depletion. However, the molecular mechanism that targets tomosyn to vesicular organelles is still not completely clear.

5.5 Future directions

Additional research is needed to improve our knowledge on the Ca\(^{2+}\)-dependence of spontaneous SV release. With the tools we developed, we can further address this issue. First, to exclude any temporal relation between Ca\(^{2+}\) and spontaneous SV release, the temporal correlation between SCTs/ SCEs and miniature postsynaptic currents should be repeated at higher [Ca\(^{2+}\)]\(_{e}\), after Cd\(^{2+}\), caffeine and ryanodine for glutamatergic neurons, but also for GABAergic neurons. Under these conditions, a hypothetical coupling between SCEs and spontaneous SV release may become detectable, and furthermore, these experiments could
give an indication of the Ca\textsuperscript{2+} source involved. Moreover, the study of GABAergic neurons may add information to the hypothesis that the Ca\textsuperscript{2+}-dependent regulation of spontaneous release differs between glutamatergic and GABAergic neurons. Second, to increase the Ca\textsuperscript{2+} detection sensitivity of sypHyJReX, the acquisition parameters can be modified, i.e. slower imaging rate and the ratio of pHluorin and JReX in sypHyJReX may be changed from 2:1 to 1:2. Alternatively, a new fusion protein sypHTomatoGCaMP6f could be made by fusing the Ca\textsuperscript{2+} sensor, GCaMP6f, and the fusion reporter, pHtomato, if the detection sensitivity of sypHyJReX cannot be improved sufficiently. Moreover, future experiments are required to extend sypHyJReX preliminary data to a quantitative dataset and, thus, to clearly state which percentage of spontaneous SV fusion events co-occurs with SCEs. Furthermore, using sypHyJReX and interfering with Ca\textsuperscript{2+} pathways as mentioned above, more insight can be gained about the Ca\textsuperscript{2+} source, the Ca\textsuperscript{2+} sensor and the molecular mechanism of spontaneous SV release.

To gain understanding of the complex neuronal Ca\textsuperscript{2+} pathways, it will be important to standardise the analysis of the Ca\textsuperscript{2+} events and produce more sensitive tools to discriminate between different types of Ca\textsuperscript{2+} events to more completely comprehend their physiological role.

Additional research also is required to better understand the mechanisms and implications of vesicular tomosyn targeting. Given the activity-dependent function of tomosyn in neurons, it would be interesting to examine tomosyn’s vesicular interaction and localization in several activity states and, possibly, at SV resolution using superresolution microscopy. The autapse model is well suited to perform such studies. For acute double knockout of tomosyn-1 and -2, use of the Cre-lox system would be ideal. Both mouse lines, as well as lentiviral overexpression vectors, are now available to test functional rescue by a series of tomosyn mutants.