CHAPTER SIX:

DISCUSSION

In this thesis, I have used a broad range of advanced and often developmental fluorescence microscopy techniques to investigate distribution and behaviour of the single voltage-gated calcium channel molecules at the cell plasma membrane. Both diffraction-limited methods and super resolution microscopy techniques were used together to address a range of questions.

My project was focused on N-type calcium channels and effect on their spatial distribution and mobility in relation to presence or absence of the synaptic protein interaction site.

6.1 Impact and the future research

Previous research[75,169] showed that secretory vesicles move between the membrane SNAREs clusters and are preferably docked and fused in the areas with lower density of tSNAREs. It has been also suggested that vesicle fusion is influenced by calcium channel distribution[213]. It is believed that the interaction of syntaxin-1A with the synprint motif at the cytoplasmic linker of the I-II loop of N-type calcium channel α_1 subunit has an important role in exocytosis[27] but is not the only interaction needed for vesicle fusion[61]. Synaptotagmin I also apparently interact through this motif[61]. My results suggest that the synprint site has an influence on the N-type calcium channel cluster patterning and behaviour. The findings from nearest neighbour analysis identify zero, or a very low number of syntaxin-1A molecules in close proximity to the N-type calcium channel cluster. These outcomes are supported by previous research[61] suggesting that N-type calcium channels may undergo many interactions with different SNARE proteins during exocytosis but the relation with syntaxin-1A through the synaptic protein interaction site might not be the crucial. My results show the new point of view on syntaxin-1A and synprint site interaction. Previous research was based on techniques like patch-clamp[52] or binding assays[227,55] but the interaction has not been observed under the microscope previously. The data obtained demonstrated that contrary to what was expected there was a very low number of syntaxin-1A molecules in close proximity (below 25 nm) to the N-type calcium channels (figure 6.1).

The same experiments could be done for full-length and splice variant of N-type calcium channel and secretory vesicles but also with other proteins taking part in exocytosis (e.g. SNAP-25, synaptotagmin I), to indicate the difference in secretion due to the presence of synprint site.

One of the limitations of the PALM method is the lack of good photoactivatable green fluorescent protein that could be used for a multi-colour single molecule imaging.

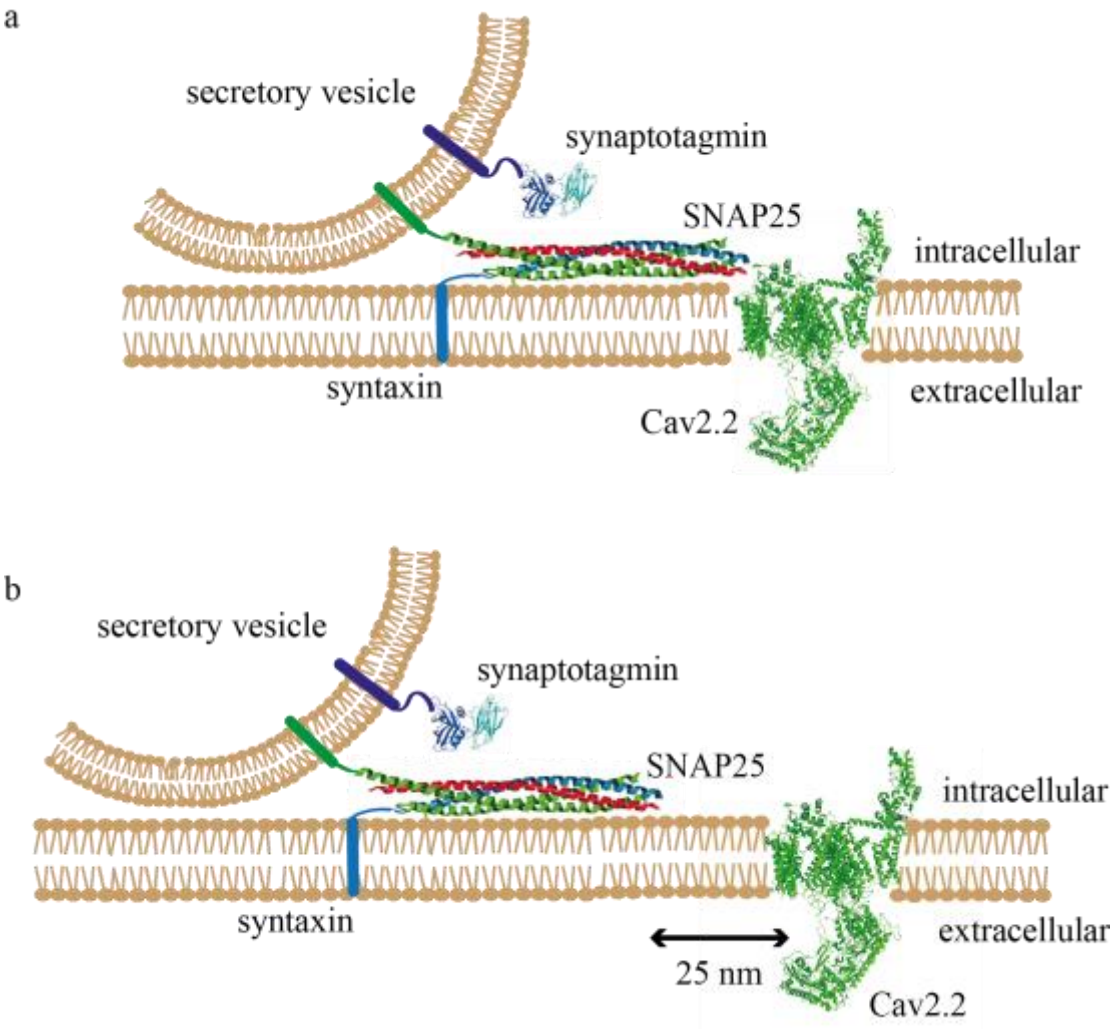

Figure 6.1 Model of the molecular architecture of syntaxin-1A relative to N-type calcium channels. (a) Current model represents the interaction between syntaxin-1A and synprint motif at the N-type calcium channel (b)The findings identify zero, or a very low number of syntaxin-1A molecules in close proximity (below 25 nm) to the N-type calcium channels.

The results from the single N-type calcium channels established the different mobility in regard to the occurrence of the synprint motif. By summarising all results together, it can be assumed that the N-type calcium channels without synprint motif are more mobile because they form smaller clusters with fewer numbers of molecules. To gain better understanding, their movement different techniques might be tested. Fluorescence correlation spectroscopy (FCS) or fluorescence recovery after photo-bleaching (FRAP) are

powerful methods that provide information about protein dynamics in the cell membrane. The mobility of calcium channels could be also tested in regard to the SNARE proteins distribution.

At the end of my PhD the research on calcium-mediated exocytosis has been published[217]. This report suggests that the calcium signal needed for the vesicle fusion is directly dependent on synaptotagmin I. Future research could focus on calcium channel distribution in regard to this synaptic vesicle membrane protein and also check the influence of calcium activity on the calcium channel movement.

Improvement and further investigation of calcium activity for different type of channels is needed; however, my preliminary data suggest that calcium channels can be distinguished based on opening time. These results together with the developing living laser method would be an asset for future pharmacological drugs testing. Overcoming the limitations of camera speed and fluorescent intensity of chemical calcium indicators would allow for even more accurate results.

6.2 Summary

Calcium ions take part in the most important cellular signalling pathways. Secretion of hormones or neuronal synaptic transmission is triggered by calcium signal. There are numerous regulatory pathways connected to the N-type calcium channels[207]. The deeper understanding of the exocytosis and its regulation by calcium signalling is crucial.

Due to the limit of the spatial resolution, the nanoscale distribution of voltage-gated calcium channels has not been well explored.

The results presented in this thesis putted together can create a model of the distribution and behaviour of N-type calcium channels at the secretory cells membrane. It is recommended that the methods used can be more widely employed to investigate the distribution and function of calcium, as well as other ion, channel types in neurones, and other cell types.

A more detailed understanding about the distribution and behaviour of voltage-gated calcium channels may have a huge impact on the development of new therapeutic drugs and biologics for many diseases like diabetes or neuropathic pain.