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Establishment of a novel lipid and content mixing assay to resolve the regulatory effect of Munc18-1 and CpxII on SNARE-mediated membrane fusion of reconstituted VAMP2/Syt1 SUVs and isolated rat brain synaptic vesicles.

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The aim of this thesis was to develop and establish a liposomal content mixing assay to study SNARE-mediated membrane fusion. In this thesis t-SNARE GUVs, instead of the widely used t-SNARE SUVs, were used, since effects of CpxII and other regulatory proteins are only resolvable in the presence of a flat target membrane which mimics the presynaptic membrane.

In the first part of this thesis a novel liposomal content mixing assay is presented, which is capable of depicting key characteristics of SNARE-mediated membrane fusion. The *in vitro* assay is based on reconstituted VAMP2/Syt1 SUVs and t-SNARE GUVs with encapsulated complementary oligonucleotides. T-SNARE GUVs were used to mimic the flat presynaptic membrane. The oligonucleotides were labelled with the fluorophore Cy3 or respectively Cy5 so that upon content mixing an increase in FRET-efficiency was measurable.

In the second part of this work a content mixing based on encapsulated self-quenched Sulforhodamine B was established using t-SNARE GUVs instead of the t-SNARE SUVs previously presented by other authors. In parallel the VAMP2/Syt1 SUVs were reconstituted with self-quenched amounts of the lipid bound fluorophore DiO. The here presented assay allows for the first time to measure lipid (DiO) and content (Sulforhodamine B) mixing simultaneously using t-SNARE GUVs as presynatpic membrane mimic.

By applying the assay, it was demonstrated that helix 12 of Munc18-1 domain 3a is essential for its exocytic (stimulatory) function. The Munc18-1 L348R mutant is still capable of binding monomeric Syntaxin1 (chaperone function), indicating that the overall fold is not affected. However the mutant fails to stimulate SNAREpin zippering as shown with fusion assays. The loss of function can be seen measuring lipid and content mixing. This finding suggests, as postulated by Parisotto et al., that helix 12 (residues 335-358) in domain 3a of Munc18-1 can adapt a confirmation with an extended helix (residues 295-358), which interacts with the C-terminal region of the SNARE motif and the linker region of VAMP2 to facilitate SNAREpin zippering. Hereby, the N-terminal part of Munc18-1 helix 12 (residues 327-348) most likely is positioned antiparallel to VAMP2 (residues 75-96).

Lastly rat brain synaptic vesicles were isolated, purified and loaded with self-quenched concentrations of Sulforhodamine B by a freeze-thaw cycle. In the t-SNARE GUV content mixing assay the isolated and purified rat brain synaptic vesicles were shown to be constitutively active and fusion rates were not influenced by physiological Ca^{2+}-concentrations of 100 µM or less. It was also shown that CpxII has no clamping effect in the absence of Ca^{2+} and it has no Ca^{2+}-dependent stimulatory effect as seen with reconstituted

VAMP2/Syt1 SUVs. The assay is unique, since it demonstrates for the first time content mixing of isolated synaptic vesicles *in vitro* and since it uses GUVs a flat target membrane mimic. The observation that synaptic vesicles are constitutively active independent of the Ca^{2+}–concentration is in line with previous publications, which are however based t-SNARE SUV lipid mixing assays. The fusion kinetics of the isolated and purified rat brain synaptic vesicles show high similarity to the fusion kinetics of reconstituted VAMP2 SUVs without Synaptotagmin 1. Nevertheless Western blotting revealed the presence of Synaptotagmin 1 on the isolated vesicles, which indicates that the availability of Synaptotagmin 1 on the isolated synaptic vesicles, which are mostly derived from the reserve pool, is functionally down-regulated. The availability of fusogenic Synaptotagmin 1 on synaptic vesicles could possibly be reduced by protein-protein interactions with the synaptic vesicle glycoproteins SV2A, SV2B and SV2C or with the dephosphorylated CSP. Furthermore phosphorylation of Synaptotagmin 1 by CaMKII or PKC was shown to increase the binding affinity of Synaptotagmin 1 to Stx1A and SNAP-25, thus stimulating neurotransmitter release.