Identification of molecular presynaptic organization by two distinct proximity biotinylation strategies

Introduction

The general function of synapses is neurotransmission. The presynaptic compartment is responsible for the release of neurotransmitters (NT), that are stored in synaptic vesicles, into the synaptic cleft. There are many proteins involved in this process, whereas the most important ones are the proteins which form the so-called SNARE (soluble N-ethyl-maleimide-sensitive-factor attachment protein receptor) complex. This complex binds the vesicle membrane with the plasma membrane and is essential for synaptic vesicle exocytosis [1].
Munc13-proteins are essential for neurotransmission, as they interact with SNARE-proteins and regulate the assembly of the SNARE complex [2,3]. How Munc13-proteins exactly control the assembly of SNARE complexes is not fully understood yet.

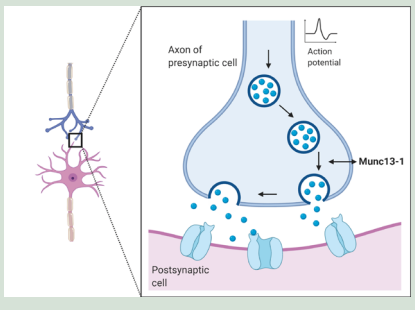


Figure 1. **Transmission at a chemical synapse**

The major aim of the department is to understand how presynaptic proteins work, how they interact with other proteins and how different mutations affect specific functions and cause brain diseases.
To make predictions about the function of a protein, its molecular environment needs to be known. By an atypical use of the SNAP-tag it is possible to identify interacting proteins and one aim of my work is to discover the multi protein complex of Munc13-1 in neuronal cultures.

Methods

Proximity Labeling (PL)

Method that is used to identify the molecular environment of proteins.
It relies on random covalent biotinylation of biomolecules proximal to a protein of interest (POI) via a chemical or an enzymatic tag [4]. Biotinylated proteins can later be isolated using a high-affinity one step pulldown on streptavidin beads.

Figure 2. **Structure of the SNAP-tag** (PDB: 3KZZ [7])

SNAP-tag

The SNAP-tag is a 20 kDa self-labeling protein tag which can be fused to a POI. It is an engineered version of the DNA repair protein O6-alkylguanine-DNA alkyltransferase [5].
Labeling of the tag is achieved by the addition of a chemically synthesized SNAP-tag substrate. This substrate consists of benzylguanine (BnG), which is recognized by the SNAP-tag, and different functional groups [6].

Photoproximity Labeling (PPL)

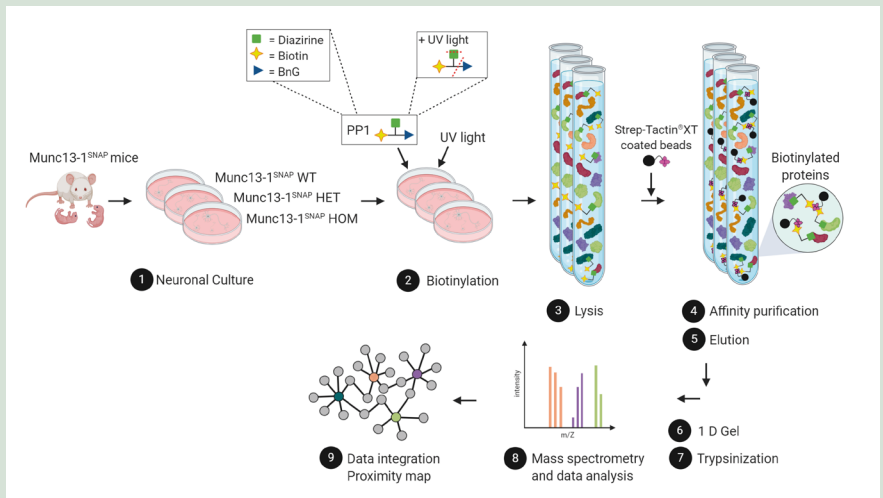


Figure 3. **Experimental procedure of PPL.** 1) Preparation of neuronal cultures of Munc13-1^{SNAP} mice as described below. 2) Addition of the chemically synthesized SNAP-tag substrate (PP1 [8]) to the cultures, incubation and washout of free PP1. Irradiation with 365 nm light triggers cleavage (depicted with red dashes) and diffusion of PP1 away from the POI leading to covalent labeling of proximal proteins. 3) Harvesting and lysis of cells with RIPA buffer. 4) Affinity purification of biotinylated proteins due to the addition of Strep-Tactin®XT coated magnetic beads (IBA Lifesciences,). 5) Elution of the biotinylated proteins under denaturing conditions by boiling in 1x Laemmli. 6) By loading the samples on a gel, 7) performing protein trypsinization and 8) quantitative analysis via mass spectrometry, 9) the proximal "social network" of Munc13-1 can be identified.

Neuron Culture

A neuronal culture is made from brains of P0 (postnatal day 0; just born) Munc13-1^{SNAP} mice. The cortex is cut out, dissected, dissociated and 5 to 6 million neuronal cells are plated on 10 cm culture dishes.
The cultures were kept at 37 °C and 5 % CO_2. After three to four weeks, synapses have developed, and the SNAP-tag substrate can be added.

Results

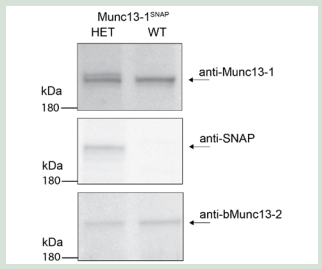
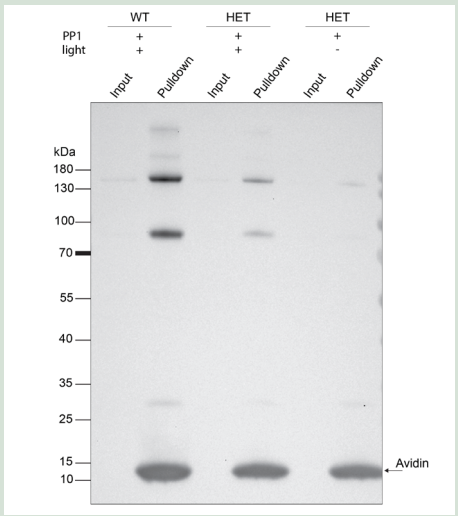


Figure 4. **Validation of the Munc13-1^{SNAP} mouse line**
Neuronal cultures of Munc13-1^{SNAP} heterozygous (HET) and wildtype (WT) mice were harvested with RIPA buffer and analyzed via western blot with different antibodies [rabbit-anti-Munc13-1 (1:1000, Brose Lab, 40), rabbit-anti-SNAP-tag (1:200, NEB), rabbit-anti-bMunc13-2 (1:500, Brose Lab, 50), goat-anti-rabbit-HRP (1:10000, IgG, affinity purified, Jackson Immunoresearch)]. The combination of an additional band with a higher molecular size together with the one band, that is visible after the use of the anti-SNAP antibody, demonstrates that the SNAP-tag is expressed. Besides, the expression of bMunc13-2, which is a family member of Munc13-1, is not affected by the Munc13-1^{SNAP} expression.

Figure 5.
Representative experiment of PPL
Analysis of steps 1 to 6 of the PPL treatment of neuronal Munc13-1^{SNAP} wildtype (WT) and heterozygous (HET) mice cultures via western blot. Input lanes show the total protein content captured by streptavidin-HRP (1:1000, Thermo Fisher) after lysis in RIPA buffer. Pulldown lanes show the captured proteins after enrichment through affinity purification with Strep-Tactin®XT coated magnetic beads (IBA Lifesciences).
No Biotinylation has occurred in WT and HET. The visible bands represent carboxylases, proteins naturally carrying biotin as a prosthetic group. The HET without light serves as a control since there was no irradiation with light, thus no cleavage of PP1 and no proximal labeling with biotin could occur. In addition, the HET lanes don't show a band of the size of Munc13-1, indicating that there is no binding of PP1 to the tag.



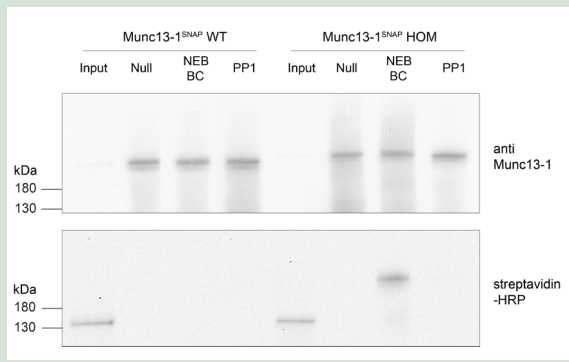


Figure 6.
Binding of PP1 to the SNAP-tag
Immunoprecipitation (IP) starting with soluble proteins from synaptosomes isolated from brains of an adult Munc13-1^{SNAP} WT mouse and an adult Munc13-1^{SNAP} homozygous (HOM) mouse was performed. An anti-Munc13-1 antibody and Protein G Sepharose 4 Fast Flow beads (Sigma-Aldrich) were used to capture Munc13-1 and Munc13-1^{SNAP}. Besides, PP1 and a commercial BnG-biotin compound (NEB) serving as a control were added to see if binding to the SNAP-tag could occur.

Analysis via western blot using a primary anti-Munc13-1 antibody and a secondary goat-anti-rabbit-HRP antibody showed that the IP procedure to capture Munc13-1 worked. By using streptavidin-HRP it was revealed that PP1 is not able to bind to the SNAP-tag, but the control is. This explains why further PPL experiments could not have worked. The visible band in both input lanes represents a carboxylase, that naturally carries biotin as a prosthetic group and is therefore captured by the streptavidin-HRP.
Null, no compound; NEB BC, commercially available SNAP-biotin from NEB.

Summary and Perspective

- PPL offers many advantages for discovering the molecular environment of proteins: applicability to soluble and insoluble proteins; temporal and spatial control mediated by necessary light induction and washout of free PP1; capturing of weak and transient interactions
- Currently, the experimental procedure of PPL in neuronal cultures of Munc13-1^{SNAP} mice doesn't lead to biotinylation
- Optimization and examination of potential errors by e.g. testing whether the compound is designed correctly, redesigning the compound and testing different light sources
- Advantages make it worthwhile to continue and optimize the experiment.

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[1] Rizo J, Rosenmund C (2008) Synaptic vesicle fusion. Nature Structural & Molecular Biology 15:665–674. [2] Lipstein N et al. (2017) Synaptic UNC13A protein variant causes increased neurotransmission and dyskinetic movement disorder. J Clin Invest 127:1005–1018. [3] Ma C, Li W, Xu Y, Rizo J (2011) Munc13 mediates the transition from the closed syntaxin–Munc18 complex to the SNARE complex. Nature Structural & Molecular Biology 18:542–549. [4] Che Y, Khavari PA (2017) Research Techniques Made Simple. Emerging Methods to Elucidate Protein Interactions through Spatial Proximity. The Journal of investigative dermatology 137: e197-e203 [5] Mollwitz B et al. (2012) Directed Evolution of the Suicide Protein O6-Alkylguanine-DNA Alkyltransferase for Increased Reactivity Results in an Alkylated Protein with Exceptional Stability. Biochemistry 51:986–994. [6] Johnsson K (2008) SNAP-tag® Technologies: Novel Tools to Study Protein Function. https://www.neb.com/tools-and-resources/feature-articles/snap-tag-technologies-novel-tools-to-study-protein-function (22.09.2020) [7] Schmitt S, Pojer F, Schiltz M, Johnsson K Crystal structure of SNAP-tag bound to its substrate benzylguanine. [8] McCutcheon DC, Lee G, Carlos A, Montgomery JE, Moellering RE (2020) Photoproximity Profiling of Protein-Protein Interactions in Cells. Journal of the American Chemical Society 142:146–153. Figures 1, 2, 3 are created with BioRender.com.