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Duration of training: 3 Months

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During my stay at Milosevic's lab at European Neuroscience Institute (ENI) in Göttingen, Germany, I focused on phosphatidylinositols (PtdIns; PI), an important lipid that is especially abundant in brain tissue. PtdIns are the precursor of phosphoinositides (PIP) and its phosphorylation leads to several products as phosphatidylinositol-4,5-biphosphate (PtdIns(4,5)P_2 or PIP_2), on which I based my research. PI(4,5)P_2 represents only 1% of the lipids in the plasma membrane. It is a precursor of three second messenger: a) inositol-1,4,5-triphosphate (Ins(1,4,5)P_3); b) diacylglycerol (DAG) and c) PtsIns(3,4,5)P_3. PI(4,5)P_2 has diverse functions, as playing a role in endocytosis, exocytosis, actin-binding proteins, ion-channel activation, etc[1, 2].

PI(4,5)P_2 can regulate fusion via syntaxin-1 and synaptotagmins as a fusion restraint, or as an essential cofactor with a role in recruiting proteins to facilitate SNARE-dependent fusion[3]. Yet, syntaxin-1 clusters are facilitated by electrostatic interactions with PI(4,5)P_2. This high accumulation of PI(4,5)P_2 was required for syntaxin-1 sequestering, as destruction of PI(4,5)P_2 by the phosphatase, synaptojanin-1, reduced syntaxin-1 clustering[6]. Munc-18, a cytosolic SM protein universally involved in all intracellular membrane fusion reactions, binds tightly to syntaxin-1 in the "closed" conformation, preventing the formation of the SNARE complex[5, 6]. Munc-18 can also bind to syntaxin-1 in its "open" conformation via the N-peptide, when syntaxin-1 is part of the SNARE complex[7].

Hence, we proposed to test the hypothesis that absence of Munc18-1 in neurons changes PI(4,5)P_2/syntaxin-1 clusters by disturbing syntaxin-1 levels and localization.

At Milosevic's lab I studied, using 2-color STED (Stimulated Emission Depletion) super resolution microscopy available at the nearby Max Planck Institute for Biophysical Chemistry in the laboratory of Prof S. Hell, PI(4,5)P_2/syntaxin-1 clusters in Munc18-1 *null* and WT (wild-type) neurons. This experiment was performed by lentiviral overexpression of GFP-PH-PLCδ1 construct, that binds PI(4,5)P_2, allowing a direct detection of PI(4,5)P_2 clusters. We were also interested in syntaxin-1 co-localization with PI(4,5)P_2, thus a syntaxin-1 antibody was used to

stained for this endogenous protein. Performing this experiment allowed us to compare between WT and Munc18-1 *null* neurons at DIV3. As an outcome of this experiment, we expected to observe differences between Munc18-1 *null* and WT $PI(4,5)P_2$/syntaxin-1 clusters. Conversely, the outcome showed no differences between WT and Munc18-1 *null* $PI(4,5)P_2$ clusters. A plausible explanation relies on technical issues, the 2-color STED microscope used for this experiment does not have a 488nm channel and our construct is a GFP-fusion protein. Consequently, we used anti-GFP antibody followed by a secondary probe to visualize our PH-PLCδ1 probe, in addition to anti-syntaxin-1 antibody. Thus, the distribution of $PI(4,5)P_2$ and syntaxin-1, detected by our antibodies could be affected by the size of our probes (≈15nm per Antibody plus 4.2nm for GFP).
We were also interested in quantifying the levels of $PI(4,5)P_2$. Therefore, I learnt how to prepare membranes sheets from chromaffin cells and neuronal cultures, and have used purified PH-PLCδ1-GFP to quantify the levels of $PI(4,5)P_2$ via immunocytochemistry.

Now that I am back at my home institution I will apply the knowledge acquired at Milosevic's lab by: using our microscope to perform super resolution by stochastical optical reconstruction microscopy (STORM) and try to clarify whether there is no difference in $PI(4,5)P_2$/syntaxin-1 clusters or if it is an antibody size issue. Also, $PI(4,5)P_2$ levels will be measured between WT and Munc18-1 *null* cells by neuronal membrane sheets method. Moreover, I will teach this method to my colleague in order to measure $PI(4,5)P_2$ levels from chromaffin cells, important for her project.

I recognize that getting the NENS stipend gave me the opportunity to join one of the most renowned scientific communities in Europe, a great achievement for my career. Yet, my staying at Milovesic's lab was really positive, I had a chance to expand my knowledge and learn new methodology that I can now apply at my home institution.

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2) Di Paolo G., *et al.* Phosphoinositides in cell regulation and membrane dynamics. *Nature* **443(12)**, 651-657 (2006).
3) James, D, et al., Phosphatidylinositol 4,5-bisphosphate regulates SNARE-dependent membrane fusion. *J. Cell Biol.* **182 (2),** 355-366 (2008)
4) Van den Bogaart, G, *et al.*, Membrane protein sequestering by ionic protein-lipid interactions. *Nature* **479**, 552-555 (2011).

5) Déak F., *et al.*, Munc18-1 binding to the neuronal SNARE complex controls synaptic vesicle priming. *J. Cell Biol.* **184(5),** 751-764 (2009).

6) Toonen R., *et al.*, Munc18-1 stabilizes syntaxin-1, but is not essential for syntaxin-1 targeting and SNARE complex formation. *J. Neurosci.* **93**, 1393-1400 (2005).

7) Dulubova I, *et al.*, Munc18-1 binds directly to the neuronal SNARE complex. *PNAS* **104(8)**, 2697-2702 (2006)