

Exocytosis and endocytosis: from synaptic vesicles to nanodiscs

16 – 20 January 2018 | The Canary Islands, Spain

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REGISTRATION

Application and Abstract deadline

15 November 2017

Student/postdoc600 EUR

Academic.....700 EUR

Industry.....1000 EUR

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Exocytosis and endocytosis: From synaptic vesicles to nanodiscs

(w18-34)

Tenerife, Spain, 16-20 January 2018

Programme and Abstract Book

Organizer committee

Erdem Keratekin
Ricardo Borges
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James E. Rothman

Site web: <http://meetings.embo.org/event/18-exo-endocytosis>

Venue

Hotel Sol, Costa Atlantis
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Special thanks to

Ms. Marian Martín Murillo
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Meeting venue

Scientific sessions: *Salón Panorama* (First floor)
Poster exhibition: *Salón Atlantis Mar* (First floor)
Lunches: *Salón Atlantis Teide* (First floor)
Banquet: *Salón Costa* (Basement floor)

Programme

Abstracts & Posters number

Address book

Pages

3

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Exocytosis and endocytosis: From synaptic vesicles to nanodiscs

(w18-34)

Tenerife, Spain, 16-20 January 2018

Programme

Day 1 – 16 January 2018, Tuesday

Opening session

16:00 Registration opens

17:00 Welcome Address

Keynote lecture

17:30 On the Molecular Mechanism of Synchronous Neurotransmitter Release **James E. Rothman**

19:00 Welcome reception/cocktail dinner

20:30 **Special Concert** by “Los Sabandeños”.



Day 2 – 17 January 2018, Wednesday

Endocytosis in vivo

Chair: **Sebastian Barg**

9:00 Single synaptic vesicle endocytosis **Ege Kavalali**

9:30 Selected Talk from Abstracts: Clearance of SNARE complexes by the scaffold protein intersectin 1 **Maria Jaepel**

9:50 Functions of the AP-4 and AP-5 adaptor protein complexes **Margaret S. Robinson ("Scottie")**

10:20 Coffee break/posters

10:50 Selected Talk from Abstracts: Functional recruitment of dynamin requires multimeric interactions for efficient clathrin-mediated endocytosis **David Perrais**

11:10 The contributions of the actin machinery to endocytic membrane bending and vesicle formation
Wanda Kukulski

11:40 Selected Talk from Abstracts: Novel Ecto-Tagged Integrins Reveal their Trafficking in Live Cells
Felix Rivera-Molina

12:00 Lunch/meet the speaker

Exocytosis and endocytosis: From synaptic vesicles to nanodiscs

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Tenerife, Spain, 16-20 January 2018

Endocytosis in vitro

Chair: **Sandy Schmid**

14:00 Regulation of clathrin-mediated endocytosis **Sandra Schmid**

14:30 Selected Talk from Abstracts: Mechanisms of force production and force sensing during clathrin-mediated endocytosis in yeast **Julien Berro**

14:50 Endocytosis in a test tube **Patricia Bassereau**

15:20 Coffee break/posters/ Group photo

15:50 Selected Talk from Abstracts: Mechanical properties of lipid bilayer during membrane fission

Pavel Bashkirov

16:10 Lipid regulation of membrane fission **Vadim Frolov**

16:40 Selected Talk from Abstracts: Multicellular endocytosis and phagocytosis

Francoise Brochard-Wyart

17:00 Break/posters

Keynote lecture

17:30 Otoferlin, the calcium sensor orchestrating the vesicle cycle at ribbon synapses

Christine Petit

18:30 Poster session 1

20:00 Dinner in town

Day 3 – 18 January 2018, Thursday

8:30 Excursion to the National Park of Las Cañadas del Teide

12:00 Picnic Lunch at the Excursion

15:00 Return to hotel



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Exocytosis in vivo

Chair: **Luis Gutiérrez**

16:00 Deficits in axonal transport in ALS and Charcot-Marie-Tooth disease models

Giampietro Schiavo

16:30 Selected Talk from Abstracts: VAMP2 controls the exit of Munc18-1 from nanocluster confinement during SNARE complex assembly **Ravi Kiran Kasula**

16:50 The organization of synaptic vesicle pools in hippocampal synapses **Guillermo Alvarez de Toledo**

17:20 Coffee break/posters

17:50 Selected Talk from Abstracts: Birth of a nanodomain: Vesicle docking is initiated by Rab3 positive vesicles identifying RIM sites to tether at the plasma membrane

Nikhil Gandasi

18:10 Measuring the messenger content of single vesicles in live cells in vitro and in vivo and relating this to exocytotic release **Andrew Ewing**

18:40 Selected Talk from Abstracts: Tight distribution of synaptic vesicle release sites generated by Unc13 limits and synchronizes neurotransmission **Alexander Walter**

19:00 **Poster session 2**

20:00 Free time and dinner in town in small groups

Day 4 – 19 January 2018, Friday

Exocytosis in vitro

Chair: **Josh Zimmerberg**

9:00 On the importance of autoinhibitory interactions for the exquisite regulation of neurotransmitter release **Josep Rizo**

9:30 Selected Talk from Abstracts: Entropic forces drive self-organization and membrane fusion by SNARE proteins **Ben O'Shaughnessy**

9:50 Formation and structure of transport vesicles **Felix Wieland**

10:20 Coffee break/posters

10:50 Selected Talk from Abstracts: Do membranes really hate edges? **Joshua Zimmerberg**

11:10 Secretory vesicles, model cells and biosensors **Ann-Sofie Cans**

11:40 Selected Talk from Abstracts: Three-dimensional, high-resolution study of protein diffusion and trafficking in a live cell via interferometric scattering microscopy (iSCAT) **Richard Taylor**

12:00 Lunch/meet the speaker

Exocytosis and endocytosis: From synaptic vesicles to nanodiscs

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Specialized secretory/endocytic systems

Chair: **Tina Pangrsic**

14:00 Calcium-binding proteins (CaBPs) influence inner hair cell (IHC) presynaptic calcium signal and sound encoding **Tina Pangrsic**

14:30 Selected Talk from Abstracts: Coordinated formation and disassembly of a contractile actomyosin network mediates content release from large secretory vesicles **Eyal Schejter**

14:50 Insulin, the fusion pore and diabetes **Patrik Rorsman**

15:20 Coffee break/posters

15:50 Selected Talk from Abstracts: Lipid composition and the ATPase EHD2 determines the surface stability of caveolae **Madlen Hubert**

16:10 MicroRNA exocytosis by vesicle fusion in neuroendocrine cells **Yongsoo Park**

16:40 Selected Talk from Abstracts: A novel quantitative single molecule colocalization assay reveals otoferlin as a multivalent calcium-sensitive scaffold linking SNAREs and calcium channels **Colin Johnson**

17:00 Inside a large dense core vesicle **Ricardo Borges**

17:30 Coffee break

18:00 Poster session 3

19:30 Dinner banquet

Day 5 – 20 January 2018 Saturday

Emerging techniques

Chair: **Wanda Kukulski**

8:30 The mechanism of fusion pore formation **Manfred Lindau**

9:00 Selected Talk from Abstracts: Traffic Light peptides to photocontrol clathrin-mediated endocytosis in yeast **Davia Prischich**

9:20 Nanodiscs-cell fusion: a biochemically defined assay with single pore sensitivity and sub-millisecond time resolution **Erdem Karatekin**

9:50 Coffee break/posters

10:20 Selected Talk from Abstracts: Visualizing membrane structural remodeling of fusion and fission in live cells **Ling-Gang Wu**

10:40 Xenapses: synapses on a chip for studying presynaptic structure and function **Jurgen Klingauf**

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11:00 Round table discussion. Poster prizes, feedback.

Keynote Lecture

11:45 Regulation of SNARE-mediated membrane fusion

Reinhard Jahn

13:00 Meeting adjourned

Poster exhibition

Poster exhibition hall. (*Salón Atlantic Mar*)

Each poster has a number corresponding to the space assigned. Posters will be exhibited during the whole meeting. Please check the number of your poster from the abstract book available at the EMBO Workshop website;

<http://meetings.embo.org/event/18-exo-endocytosis>.

Presenters:

#1 – #20 in poster session 1.

#21 – #40 in poster session 2.

#41 – #63 in poster session 3.

...have to be at their poster during the poster exhibition day assigned.

Poster sessions. (*Salón Panorama*)

Poster presenters are requested to prepare 1-2 slides for a 2 min talk introducing his/her results during the poster sessions 1, 2 or 3. This oral presentation will take place in the main congress hall.

Poster awards. (*Salón Panorama*)

FASEB Journal and Journal of General Physiology will award those posters selected by a scientific committee. The award ceremony will be held during Round table discussion (Day 5).

#1

"Cd74, invariant chain, a modulator of endosomal maturation."

Oddmund Bakke, et al.

Department of Biosciences, University of Oslo, Norway

Invariant chain (CD74, Ii), is a multifunctional molecule expressed in antigen presenting cells – mostly in concert with MHC II but also decoupled, for instance in mature dendritic cells (DCs). In addition CD74, which is mostly rapidly internalized from the plasma membrane is a membrane receptor for MIF, macrophage migration inhibitory factor.

CD74, with its two leucine based endosomal sorting signals that binds AP1 and AP2 adaptors was first found to be an essential partner for the proper trafficking of MHC II and therefore efficient antigen loading in the endosomal pathway and antigen presentation. A portion of CD74, CLIP, occupies the antigen-binding groove of MHCII before a more specific antigen replaces it. The trimeric CD74 delays furthermore endosomal maturation participating in forming the peptide loading compartment, or the immunoendosome. The immunoendosome is an organelle that needs extensive reorganization of the endosomal pathway in antigen presenting cells, properties that we believe are introduced mainly by CD74 and its interactors.

The fusogenic properties of CD74 depend on basically all the domains of the molecule and can be eliminated by a single point mutation of the cytosolic tail. The endosomal fusion property of the molecule is independent on Rab5, PI3 kinase and EEA1 and I will discuss its role in endosomal maturation and how this property can be exploited to study maturation, endosomal fusion and fission and the role of SNARES and SNARE like proteins.

Interestingly, CD74 has been found to interact with several molecules including MHCI and can be used as a vector for simultaneously increasing both MHCI and MHCII mediated immune responses towards specific antigens. this vector is now ready to be tested in clinical therapeutic dendritic cell based cancer immunotherapy. The basis for this vaccine is targeting of the antigen to the immunoendosome and different sorting signal/adaptor binding gives different immune responses.

#2

Fusion pore regulation by cAMP and endocytosis proteins

Alenka Gucek, Nikhil Gandasi, Sebastian Barg

*Dept of Medical Cell Biology
Uppsala University, Sweden*

Exocytosis proceeds through an intermediate state in which a narrow fusion pore connects the secretory vesicle lumen with the extracellular environment. The fusion pore acts as a molecular sieve that allows release of ions and small transmitter molecules, but traps the larger peptide hormones. There is some evidence that fusion pore behavior is affected by second messengers and the GTPase dynamin. We studied fusion pore expansion in insulin secreting beta-cells using high resolution imaging, by exploiting the luminal pH change during pore opening. We report that elevated cytosolic cAMP favors stable fusion pores that allow rapid release of nucleotides, but restrict exit of peptides. The effect is mediated by the cAMP-dependent Rap-GEF Epac2, because cAMP-dependent fusion pore regulation is absent when Epac2 is inactivated pharmacologically or in Epac2-knockout mice. We show further that overexpression of Epac2 and several proteins involved in endocytosis favor cAMP-dependent pore restriction, and are recruited specifically to restricted fusion pores during exocytosis. We conclude that Epac2 controls cAMP-dependent fusion pore restriction and thereby affects differentially the release of hormones and transmitter molecules during exocytosis.

#3

Mechanical properties of lipid bilayer during membrane fission.

Pavel Bashkirov^{1,2}, Ksenia Chekashkina^{1,2}, Anna Shnyrova³, Pedro Arrasate³, Vadim Frolov^{3,4}

¹*Federal Research and Clinical Center of Physical-Chemical Medicine, Moscow, Russia*

²*A.N. Frumkin Institute of Physical Chemistry and Electrochemistry of RAS, Moscow, Russia*

³*Biofisika Institute (CSIC, UPV/EHU) and Department of Biochemistry and Molecular Biology, University of the Basque Country, Leioa, Spain*

⁴*IKERBASQUE, Basque Foundation for Science, Bilbao, Spain*

Leakage-free fusion and fission of cellular membrane require formation of nanoconfined intermembrane bridges of different topology, such as stalks and pores. Lipid monolayers in these structures are extremely bent. The curvature stress has long been considered as the major barrier for lipid rearrangements during both fusion and fission. However, nanomechanics of lipid layers bent to such high curvatures remains poorly characterized. Here we report real time measurement of the mean curvature bending modulus of biomimetic lipid membranes at the curvatures characteristic for spontaneous membrane fission. We used electric field to rapidly and reversibly perturb the shape of highly constricted membrane nanotubes (NT). Analyzing the corresponding changes in the I/V characteristic of the ionic permeability through the NT lumen we extracted the NT luminal radius and the apparent elastic modulus of its membrane. We found that the NT deformations were accurately described within the framework of linear curvature elasticity with the appropriate macroscopic bending modulus. Fission was not associated with any perceptible deviation from the linear elastic behavior. We further analyzed the elasticity of the NTs undergoing spontaneous fission under extreme curvature stress imposed by proteins or osmotic pressure. We found no changes in membrane elasticity due to the stress thus substantiating widely accepted application of linear elasticity to the shared intermediates of fusion and fission reactions. However, we found that increasing curvature stress facilitated formation of pore in the NT membrane and could even cause membrane rupture. We further demonstrated that such undesirable effects could be minimized by applying stress locally and transiently, suggesting the basic functional design principle for membrane fusion/fission catalysts.

#4

Mechanisms of force production and force sensing during clathrin-mediated endocytosis in yeast

Julien Berro, et al.

Yale University

During clathrin-mediated endocytosis (CME) in yeast, the endocytic machinery has to overcome high membrane tension and turgor pressure to shape a ~50-nm diameter vesicle from the flat plasma membrane. Actin dynamics is essential for CME to proceed but it remains unknown a) how the actin network produces the forces necessary to deform the plasma membrane and b) how its dynamic behavior adapts to different membrane tension. Quantitative microscopy methods combined with mathematical modeling have been invaluable tools to decipher key regulations of the endocytic machinery. These methods allowed us to uncover the key role of severing by ADF/cofilin during actin disassembly and to make several predictions regarding the assembly of the actin machinery, its turnover, and possible mechanisms of force production.

Actin polymerization alone is able to produce only a very small fraction of the force necessary to elongate a clathrin-coated pit during CME in yeast. The mechanisms for production of the missing force remains unknown. Using mathematical modeling, we recently uncovered a new mechanism that allows networks of short actin filaments (50-200 nm) to harvest and store elastic energy by dynamic actin filament crosslinking, which we called “Brownian energy ratchet”. We also proposed mechanisms to transform this energy into force and torque, which will account for part of the missing endocytic force.

We have also used quantitative microscopy to determine how the endocytic machinery adapts to a sudden increase in membrane tension and uncover mechanisms cells use to regulate their membrane tension during such an acute shock.

#5

Overlapping and distinct molecular mechanisms of rapid and chronic homeostatic plasticity maintain reliable neurotransmission

Mathias Böhme^{1,2,3}, Anthony McCarthy¹, Andreas Grasskamp^{1,2}, Meida Jusyte^{1,2}, Fabian Goettfert⁴, Stefan Hell⁴, Stephan Sigrist^{2,3}, Alexander Walter¹

¹Leibniz-Forschungsinstitut für Molekulare Pharmakologie; Molecular and Theoretical Neuroscience, Berlin, Germany

²NeuroCure Cluster of Excellence, Charité Universitätsmedizin, Berlin, Germany

³Freie Universität; Institut für Biologie / Genetik, Berlin Germany

⁴Max Planck Institute for Biophysical Chemistry/NanoBiophotonik, Göttingen, Germany

Neural cells must maintain stable synaptic transmission in the face of many plastic challenges, including environmental perturbations during learning or development. Here, presynaptic homeostatic plasticity is a fundamental and adaptive form of plasticity where disturbances of postsynaptic excitability are counterbalanced by an enhancement in presynaptic release. However, the mechanisms by which the presynapse adapts to enhance neurotransmitter release is incompletely understood. For instance, whether and how structural adaptations of the active zone (AZ) cytomatrix tune transmitter release, how these tunings are achieved, and which effector proteins among the release machinery are responsible, is largely unknown.

Here, we show that rapid homeostatic plasticity of *Drosophila* glutamatergic AZs – acutely induced by application of a glutamate receptor blocker – depends on cellular transport and increases the AZ-levels of the scaffolding proteins Bruchpilot (BRP) and Rim-binding protein (RBP) within minutes, augmenting NT release by localizing the release factor Unc13 to the AZ. We identify Unc13 as the necessary downstream target, as interfering with its AZ-localization via deleting the N-terminal BRP/RBP interaction sequence blocks the homeostatic adaptation. In addition, AZ-levels of the SNARE protein Syntaxin-1A (Syx-1A) were also increased upon rapid plasticity, but not that of the chaperone and release factor Unc18.

We investigated whether similar or complementary mechanisms exist to maintain synaptic transmission during life-long diminished postsynaptic glutamate-sensitivity in glutamate receptor mutants. On this longer timescale, homeostatic plasticity also increased BRP, RBP, Syx-1A, and Unc13 AZ-levels, but to a larger extent. In addition, synaptic Unc18 levels increased dramatically (8-fold) and were redistributed to the AZ. Our results reveal a full sequence of presynaptic events altering AZ architectures on different timescales, converging on essential release factors to enhance NT release and enable presynaptic homeostatic plasticity.

#6

Multicellular endocytosis and phagocytosis

Francoise Brochard-Wyart¹, Francoise Winnik², Gregory Beaune²

¹UMR 168, Institut Curie, 26 rue d'Ulm, 75248 Paris, France

²WPI international center for materials nanoarchitectonics Tsukuba Japan

We describe the properties of mixtures of cells and particles. We study both small particles (from nm up to few microns), which are digested by cells by endocytosis and phagocytosis, and larger particles, which do not enter in the cells.

i) Nanostickers (size 20 nm): We show that nanoparticles can be used as a glue “nanostickers”to enable the formation of self-assembled aggregates by promoting cell–cell interactions. We model the cell-cell adhesion induced by the nanostickers using a three states dynamical model where the NPs are free, adsorbed on the membrane or internalized by endocytosis. Nanostickers by increasing the cohesion of tissues and tumors may have important applications for cellular therapy and cancer treatment.

ii) Microparticles (size 1micron) "gluttonous cells": We study the spreading of cellular aggregates deposited on adhesive substrates decorated with microparticles. A cell monolayer expands around the aggregate. The cells at the periphery uptake the microparticles by phagocytosis, clearing the substrate and forming an aureole of cells full of particles. We study the dynamics of spreading, the width of the aureole, and the level of cell internalization as a function of the size, nature and density of the beads. The radius and width of the aureole allow quantification of the MP volume fraction incorporated by the cell.

iii) Macroparticles (size 2-20 microns)” Dancing” : When the particles are too big to be eaten, they are put into motion by the cells. We also form hybrid cells-MPs aggregate by the pendant drop technic. As the concentration of MPs increases, we observe a phase separation predicted by simulations for a mixture of particles with different level of activity.

#7

Photoswitchable dynamin inhibitors to control endocytosis with light

Nuria Camarero^{1,2}, Ana Trapero^{1,3}, Eric Macia⁴, Andrés Martín-Quirós¹, Laura Nevola⁵, Artur Llobet⁶, Amadeu Llebaria³, Jordi Hernando⁷, Ernest Giralt^{5,8}, Pau Gorostiza^{1,2,9}

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⁷*Universitat Autònoma de Barcelona (UAB);*

⁸*Universitat de Barcelona (UB)*

⁹*ICREA*

Genetic approaches to interfere with the action of a protein are slow, irreversible and often lead to compensatory mechanisms, which complicate the outcomes and interpretation of experiments. Acute stimulation with small molecules targets endogenous proteins and is advantageous to control the dynamics and reversibility of responses, but diffusion poses limitations on the temporal and spatial precision that can be achieved. This is important when investigating transient or irreversible events or when disturbing the activity of proteins essential for cell survival. These problems can be solved with compounds whose biological activity can be turned on and off remotely and reversibly, like light-regulated drugs. The uptake of extracellular materials by endocytosis exemplifies both the interest and challenges to manipulate the cellular spatiotemporal dynamics. The progress in understanding its molecular mechanisms has been hampered by the lack of acute manipulation tools. Dynamin mediates the scission of endocytic vesicles and is an appealing target to manipulate endocytosis in spatiotemporally designated patterns with light. Here we describe the development of dynazos, the first light-regulated small-molecule inhibitors of dynamin. A stepwise design of the photochromic and steric properties of the switch considering the properties of the pharmacophore, lead to a series of photoswitchable inhibitors of endocytosis that are water soluble, photostable and cell permeable. We characterized their photochromism with UV-visible and transient absorption spectroscopy, and their biological activity with transferrin uptake assays using confocal microscopy and flow cytometry. Dynazos enable the photoswitchable inhibition of clathrin-mediated endocytosis at micromolar concentration using violet light and rapidly relax in the dark, thus allowing spatiotemporally precise, single-wavelength operation. Photoswitchable dynamin inhibitors can be useful to dissect the role of endocytosis in essential functions such as cell growth, differentiation, motility and invasiveness, modulation of receptor signaling and synaptic transmission.

#8

Optical detection of ultrafast single synaptic vesicle endocytosis at hippocampal synapses

Natali L. Chanaday, Ege T. Kavalali

Department of Neuroscience, the University of Texas Southwestern Medical Center, Dallas, TX 75390-9111, USA

Coupling of synaptic vesicle exocytosis and endocytosis constitutes a core mechanism underlying presynaptic efficacy, recovery and plasticity. In recent years, the differences between endocytic processes occurring at room temperature, an extremely common experimental condition, and more physiological temperatures received considerable attention due to the discovery of ultrafast endocytosis. While ultrafast endocytosis has been supported via fast-freeze electron microscopy and improved capacitance measurements, optical approaches gave variable and contradicting results. In the present work, we aimed to address these issues and analyze single vesicle endocytic events in the timescale of milliseconds, with the goal of elucidating the fastest time course of endocytosis as well as its regulation by Ca^{2+} and temperature. We show that the kinetics of both exocytosis and endocytosis are regulated by Ca^{2+} and temperature, supporting previous reports. While increasing extracellular Ca^{2+} concentration slows down endocytic rate of single synaptic vesicles, the rise in temperature causes an acceleration of retrieval after single synaptic vesicle fusion. Nevertheless, the time course of endocytic events with ultrafast kinetics (~ 150 – 250 ms) were mildly affected by the increase in temperature. In contrast, endocytosis after 40 Hz stimulation (resulting in fusion of multiple vesicles in rapid succession) and the slow single vesicle retrieval events (5 – 12 s) were more susceptible to modulation by Ca^{2+} and temperature compared to the ultrafast events. Future work will be directed to identification of the molecular mechanisms involved in each kinetic component of endocytosis and their coupling to different modes of release.

#9

Membrane deformation by demixing of lipids of different molecular shape

Ksenia Chekashkina^{1,2}, Pavel Bashkirov^{1,2}, Ariana Velasco del Olmo³, Anna Shnyrova³, Piotr Kuzmin², Vadim Frolov^{3,4}

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Cellular membranes contain large amounts of cone-shaped lipids, such as phosphatidylethanolamine (PE), that alone do not form stable lipid bilayer at physiological conditions. Such lipids has long been implicated as regulators of cellular membrane remodeling, specifically, membrane fusion and fission involving highly curved non-bilayer lipid intermediates. The effect of lipid shape(s) has been generally considered in terms of their average concentration or, equivalently, stationary spontaneous curvature of a membrane monolayer. Here we demonstrate that membrane deformations cause pronounced demixing of lipids of different effective molecular architecture abundant in the plasma membrane: cones (PE) and cylinders (phosphatidylcholine, PC). Lipid demixing results in substantial decrease of apparent membrane rigidity. Conversely, chemical bonding between different shapes could result in composite behavior leading to increased rigidity. We further demonstrate that lipid demixing constitutes an independent elastic deformation mode characterized by an elastic modulus defined by the variability of molecular shapes in the membrane. Under physiological conditions the entropic elasticity can contribute as much as a half of the apparent membrane elasticity, making lipid shape a powerful regulator of cellular membrane remodeling.

#10

Local translation at synapse

Dominik Cysewski¹, Magdalena Dziembowska²

¹*Institute of Biochemistry and Biophysics, Polish Academy of Sciences*

²*Center of New Technologies, Warsaw University*

Synapses are structures that permit neurons to communicate. One result of such communication is the remodeling of neuronal network connections, a phenomenon known as synaptic plasticity. The changes to the neuronal network are usually supported by the dynamic structural changes of synapses that require local synthesis of the new synaptic proteins. Some of the proteins transported to the dendrites and axons are supplied by the perikarion/soma; however, in order to enable a quick and directed response to the given stimulus, synthesis within the synapses themselves takes place as well. This phenomenon is known as local translation. It is made possible due to mRNA transported in the RNP granules and ribosomes located within the synapse. In the present study we used synaptoneurosomes as a model of in vitro isolated synapses. Synaptoneurosomes (SN) are a fraction freshly prepared from mouse brain artificially extracted via mechanical separation, and heavily enriched in synaptic connections, containing both pre- and post-synaptic parts of the synapse, along with postsynaptic density. The SNs possess the biochemical potential for protein synthesis in response to the stimulation, e.g. chemical. Isolated synaptoneurosomes were chemically stimulated in order to model the LTP-like (Long Term Potentiation) model of neuronal activity. To study the locally produced proteins three methods of quantitative proteomics were used: iTRAQ, TMT, LFQ. Over 3000 proteins were identified, among which 440 showed signs of growth. The newly synthesized proteins are responsible for synaptic remodeling, secretion functions, cell membrane receptor recruitment. Additionally, several phosphorylations were identified. The identified proteins form a dense network of interactions and functions. That confirms the potential of local translation in the synapse as regards the maintenance or alteration of the synaptic proteome, and, in consequence to synaptic remodeling in response to stimulation.

#11

Epigenetic cross-talk governing neurotransmitter exocytosis: A Regrade plasticity phenomena limiting hypobaric hypoxia induced neurodegeneration

DEBASHREE DAS

Defence Institute of High Altitude Research, C/o 56 APO, Leh-Ladakh, Jammu & Kashmir, Pin- 901205, India

Exposure to global hypoxia and ischemia has been reported to cause neurodegeneration in the hippocampus with CA3 pyramidal neurons. But stagnancy in such progressive neurodegenerative phenomena observed under chronic hypoxic conditions has been an enigma since the inception of such studies. Understanding the underlying mechanisms resulting in such stagnancy could reveal crucial information related to survival strategies adopted by CA3 neurons on prolonged exposure to a global hypoxia. Simulations studies using Sprague-Dawley rats show stagnancy in neurodegeneration in CA3 region beyond 14 days of chronic exposure to hypobaria simulating an altitude of 25,000 ft. Despite increased synaptosomal glutamate and higher expression of NR1 subunit of NMDA receptors, we observed decrease in post-synaptic density and accumulation of synaptic vesicles at the pre-synaptic terminals. Molecular investigations involving western blot and real-time PCR showed duration-dependent decrease in the expression of SNARE protein SNAP-25, resulting in reduced vesicular docking and impairment of exocytosis of glutamate neurotransmitter. Investigation into the role of epigenetic factors in regulating expression of SNAP-25 during chronic hypobaric hypoxia using ChIP assays showed decreased expression of H3K9Ac and H3K14Ac, resulting in SNAP-25 promoter silencing. Administration of sodium butyrate, a non-specific HDAC inhibitor, during 21 days hypoxic exposure prevented SNAP-25 downregulation but increased CA3 neurodegeneration. This epigenetic regulation of SNAP-25 promoter was however found to be independent of increased DNMT3b expression and promoter methylation. Our findings therefore provide a novel insight into epigenetic factor-mediated synaptic phenomena leading to re-calibration of excitatory neurotransmitter exocytosis to prevent excitotoxic neurodegeneration on prolonged exposure to global hypobaric hypoxia. To summarize our findings, the present study reveals a retrograde plasticity phenomenon of activity-dependent modulation of neurotransmitter vesicle exocytosis in chronic hypobaric hypoxia preventing progressive neurodegeneration due to excitotoxicity.

#12

Effect of Membrane Tension on SNARE-Mediated Single Fusion Pores

Natasha Dudzinski^{1,4}, Erdem Karatekin^{2,3,4,5}

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Fast and temporally controlled release is imperative for neurotransmitter release via evoked synaptic vesicle exocytosis. This process is driven by the interaction between vesicle v-SNAREs with target t-SNAREs on the plasma membrane. The initial, nanometer-sized connection between two fusing lipid bilayers is called the fusion pore. This structure only lasts for a short time before it either dilates completely (resulting in full fusion), or reseals again (kiss-and-run fusion). Fusion pore dynamics determine the amount and size of cargo released, as well as vesicle recycling. Thus they constitute a fundamental mechanism that controls the extent of postsynaptic neuronal activity. Due to its small size and transient nature, the fusion pore has been an elusive object to study. We have recently developed an assay using biochemically well-defined components to look at current flow through single SNARE-mediated fusion pores. The components of the assay include HeLa cells with “flipped” t-SNARE proteins expressed on the outside of the cell (t-cells), and nanodiscs with v-SNARE proteins embedded within them (v-discs). When we fill a patch pipette with v-discs and establish a cell-attached patch onto a t-cell, the v- and t-SNAREs interact, leading to the formation of a fusion pore connecting the cytosol to the pipette solution. Under voltage clamp, currents through these fusion pores report single fusion pore dynamics.

Membrane tension has been shown to affect fusion pore dynamics in neuroendocrine cells. In our assay we can quickly and accurately change the pressure in the patch pipette, and thereby modulate membrane tension, using a high-speed pressure clamp. We will show how an increase in membrane tension causes extended pore lifetimes, as well as increased pore conductance and charge influx through the pore.

#13

Birth of a nanodomain: Vesicle docking is initiated by Rab3 positive vesicles identifying RIM sites to tether at the plasma membrane

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Insulin is released by regulated exocytosis, which requires secretory vesicles to be docked at the plasma membrane. The number of release ready vesicles at the plasma membrane is therefore rate limiting for hormone secretion. Stable docking is preceded by a loosely tethered state, and we showed previously that this transition occurs within seconds after arrival of the vesicle by recruitment of syntaxin to the docking site. The molecular nature of the tethered state is not known and it remains elusive whether the vesicles tether to a pre-existing receptor complex in the plasma membrane or attach to random sites. To answer this we quantified GTP-binding Rab proteins and their effectors at the docking site by imaging GFP-tagged proteins using TIRF microscopy. Clusters of the Rab3 interacting protein RIM and Rabphilin existed at docking sites prior to vesicle tethering and docking. A further increase in RIM fluorescence was seen at vesicles during their maturation into the releasable pool, confirming a role of RIM in priming. Vesicles that successfully docked carried Rab3 and Rabphilin whereas those that only temporarily tethered did not. In contrast, Rab27 and its effector Granophilin were present on both types of vesicles. Vesicles which had Rab3 co-localized to munc18 in the cytosol. This suggests munc18 binding to syntaxin to initiate syntaxin clustering and hence stable docking. These results suggest that Rab3/munc18 and Rabphilin act on the incoming granules as signal to initiate the docking process. Since RIM is thus far the only protein found to be enriched at the docking site it may act as a docking receptor for the incoming vesicle.

#14

Chromaffin adrenergic cells without adrenaline

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Adrenal chromaffin cells release catecholamines and other soluble substances to the blood stream as part of the adaptive responses to stressful situations. Chromaffin cells have been classified as ‘adrenergic’ or ‘noradrenergic’ cells depending on the catecholamine content. The proportion between adrenergic and noradrenergic largely changes depending on the animal species. In addition, it is reported that chromaffin granules from both cells exhibit a different pattern in electron microscopy images. Also, adrenaline and noradrenaline can be selectively released depending on the physiological stimuli (cold, exercise, muscarinic or histaminergic activation).

Recently knockout mouse with non-functional PNMT are become available offering the possibility to check how is an adrenergic cell without adrenaline.

As we expected, the lack of PNMT severely took out adrenaline but surprisingly was not fully compensated by noradrenaline, achieving a loss of about half in the total amine content. Unexpectedly, adrenal medulla does not exhibit a different distribution of potassium dichromate staining. Moreover, electron microscopy images show no differences between PNMT-KO and WT indicating that the shape of granule matrix is not caused by the condensation of noradrenaline.

Another was especially relevant observation was the response to hypoglycaemia elicited by insulin, which resulted in a potent adrenaline release in WT mice but a just a little noradrenaline release from PNMT-KO.

Secretory responses, studied by amperometry, showed some interesting differences caused by the absence of adrenaline.

Our results indicate that the handling of noradrenaline by adrenergic cells is deficient or not correctly accomplished. Thus, it strongly clarifies an old question about chromaffin: the presence of adrenaline is not the only feature to call cell ‘adrenergic’.

This work is partially paid by the grant BFU2013-45253-P from the MINECO (Spain) to RB and JDM.

#15

The involvement of vesicular ATP in the storage and exocytosis of catecholamines of bovine chromaffin cells

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Chromaffin secretory granules are organelles similar to the large dense core vesicles (LDCV) present in many secretory cell types including neurons. They accumulate solutes at high concentrations (catecholamines, 0.5–1 M; ATP, 120–300 mM; or Ca²⁺, 40 mM) and can achieve a theoretical osmolarity around 1.5-2 mOsm, a tonicity much greater than cytoplasm. While the transport and function of catecholamines and Ca²⁺ into chromaffin granules have been extensively characterized, there is very little information on the mechanism of ATP transport and function into LDCV.

The colligative properties of ATP and catecholamines demonstrated *in vitro* are thought to be responsible for the extraordinary accumulation of solutes inside of LDCV and also a subtlety factor that reduces the apparent diffusion of epinephrine in aqueous media.

Because functional cells cannot be deprived of ATP, we have knocked-down the expression of the vesicular nucleotide carrier, the VNUT, to show that a reduction in vesicular ATP is accompanied by a drastic fall in the quantal release of catecholamines. This phenomenon is particularly evident in newly synthesized vesicles, which we show are the first to be released.

Surprisingly, we find that inhibiting VNUT expression also reduces the frequency of exocytosis, whereas the overexpression of VNUT drastically increases the quantal size of exocytotic events. To our knowledge, our data provide the first demonstration that ATP is an essential element in the concentration of catecholamines in secretory vesicles. In this way, cells can use ATP to accumulate neurotransmitters and other secreted substances at high concentrations, supporting quantal transmission.

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This work is supported by the grant BFU2013-45253-P from the MINECO to RB and JDM.

#16

Characterization of release modes at individual active zones in *Drosophila* NMJs reveals interdependences

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Neuronal communication relies on chemical signals that are conveyed by neurotransmitters stored in synaptic vesicles (SVs) and liberated by SV-exocytosis at active zones (AZs). SV-exocytosis can either be evoked by action potentials (AP) inducing presynaptic calcium influx or it can occur spontaneously. It is not clear whether spontaneous release serves a specific function, as opposed to being a non-regulated, unavoidable “byproduct” of the AP-inducible release machinery, and if there is a mechanism regulating it. Additionally, only few results exist on whether both release mechanisms are dependent on each other, which could explain factors of synaptic functionality like SV pool composition. The dependence of release modes on presynaptic AZ-structure and on each other can be studied using postsynaptically expressed calcium indicators like GCaMP, which reports Ca²⁺-influx through neurotransmitter receptors. This was investigated at neuromuscular junctions of *Drosophila* larvae, whose synapses display a large heterogeneity in structure and activity, likely due to differences in maturation. We could show that both spontaneous and evoked activity depend on the synaptic abundance of the release-site generating protein Unc13A, and indirectly on the cytomatrix protein BRP (Reddy-Alla et al., Neuron 2017). Recently, it has also been a controversial issue whether synapses show preference for either release mode. We now show that AZs (IHC-stained for BRP) engaging in AP-evoked release are more likely to release SVs spontaneously, and vice-versa. This implies a common pool of SVs operating in both release modes and partly resolves the issue of synaptic release mode preference. Furthermore, we show that unlike expected for truly spontaneous events, AP-independent release events are interdependent: following one event, the likelihood to observe another event shortly after.

#17

Pleiotropic mechanisms driving F-actin-dependent transport of organelles during secretion in neuroendocrine chromaffin cells

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Neuroendocrine chromaffin cells have been used as an excellent model to study the molecular mechanisms linked to the exo-endocytosis cycle of neurotransmitter release. In this model, the F-actin cytoskeleton forms an intricate cortical network that has been found essential not only to promote the active transport of dense vesicles to the plasma membrane but also to directly participate in the molecular mechanism of membrane fusion and fission associated with the secretory cycle¹. Recently, by using high resolution confocal microscopy and expression of EGFP-Lifeact and vesicular markers we have analyzed with unprecedented detail the re-organizations of the F-actin cytoskeleton associated with secretion. These changes affect specific regions of the submembranal space in form of expansions driving the transport of dense core vesicles toward the plasmalemma. Interestingly, during these expansions not only vesicles but also other organelles such as mitochondria approached the secretory machinery sites. Simultaneously, we found F-actin cytoskeletal retractions withdrawing vesicles from the subplasmalemmal space and the formation of new empty internal spaces where organelles could be transported.

In addition to these well-coordinated F-actin-myosin II dependent processes driving the transport of the majority of the vesicles, we also found infrequent fast transport of chromaffin vesicles using F-actin comet tails nucleated from the granular membrane.

Thus, during cell stimulation, F-actin structures adopt a variety of mechanisms to accomplish its complex role in the processes of exo-endocytosis.

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#18

Plasma membrane levels of glycosphingolipids strongly influence cell surface association of caveolae

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Caveolae are small omega-shaped nanodomains of the plasma membrane (PM), which are enriched in cholesterol and sphingolipids. The formation of caveolae is primarily driven by the integral membrane protein caveolin1 (Cav1) and the cavin-coat complex, whilst the ATPase EHD2 controls caveolae dynamics. These structures are extremely abundant in fat cells, where they have been proposed to play a role in lipid metabolism, although the specific contributions of caveolae have yet to be elucidated.

Our work aims to explain how the coat proteins cooperate with lipids to regulate the equilibrium between stable and dynamic caveolae. By using fusogenic liposomes we can directly insert specific fluorescently labeled sphingolipids into the PM and follow their rapid distribution using live-cell TIRF microscopy. A stable mammalian cell line expressing Cav1-mCherry enables us to monitor caveolae and analyze their dynamics before and after lipid addition using single-particle tracking of labeled Cav1. We observed that sphingomyelin stabilizes caveolae at the cell surface, whereas glycosphingolipids such as lactosylceramide and GM1 greatly enhance their mobility. Previous studies have linked caveolae to sorting and trafficking of excess lipids however, there is no knowledge on how lipids influence caveolae and their dynamics. By using 3D confocal imaging we can show that lipids accumulate in caveolae and ultrastructural analysis indicate that the morphology of caveolae is altered. Visualization of caveolae associated endogenous proteins with immunofluorescence revealed a crucial role of EHD2 in coping with excess lipids. The knowledge gained from this work will be applied in studies using adipocytes to help understand the role of caveolae in lipodystrophy and other lipid related diseases.

#19

MACC1, a prognostic biomarker for colorectal cancer is involved in clathrin-mediated endocytosis

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Colorectal cancer (CRC) progression and metastasis formation is directly linked to poor overall patient survival. The recently identified gene MACC1 (Metastasis Associated in Colon Cancer 1) is a prognostic biomarker for metastasis and survival in CRC. MACC1 shows a 40% homology with SH3BP4 also known as transferrin trafficking protein (TTP). SH3BP4 has been characterized as an important protein involved in Clathrin-mediated endocytosis (CME) and in particular its perturbation selectively affects the transferrin receptor (TfR) trafficking from the plasma membrane to endosomes. MACC1 and SH3BP4 show at their N-Terminus some CME cassettes, involved in binding proteins such as a clathrin box, NPF- and DPF-sites, and an SH3 domain for potential binding of clathrin (CLTC), epsin15 homology (EH) domain proteins, adaptor protein 2 (AP2), and dynamin (DNM2), respectively. Here we aim to understand the involvement of the metastasis inducer MACC1 in CME. To dissect the functional relevance of MACC1 in CME we performed a mass spectrometry analysis of interaction partners. By analyzing the interaction partners, we focused on a set of CME related proteins. We validated the interaction of MACC1 and factors of CME (clathrin, DNM2 and AP2) and three receptors TfR, EGFR and MET co-IP. We noticed an increase of colocalization of MACC1 within the cytoplasm with TfR, CLTC, and DNM2 upon TfR stimulation. MACC1 involvement in CME might be directly dependent on both its N-terminus -with NPF and DPF binding motifs, and the clathrin box - and the SH3 domain. MACC1 overexpression downregulates the internalization of TfR, its surface distribution, and defines a faster recycling of TfR to the plasma membrane. Our next step is to determine whether the deletion of potential CME binding sites influences MACC1-dependent changes of endocytosis in CRC cell lines. In summary, our data indicate a role of MACC1 in receptor endocytosis.

#20

Release Site Clearance of SNARE Complexes by the scaffold protein intersectin 1

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Synaptic vesicle cycling in the presynapse is essential for maintaining normal synapse and brain function and relies on tight coupling of exo- and endocytosis. One aspect of this coupling is the removal of postfusion SNARE complexes from release sites to enable subsequent release events and thus to avoid clogging of active zones.

In this study we identified the multidomain scaffold protein intersectin 1 as an important factor in this process. Interaction studies revealed intersectin 1 as an interaction partner of the v-SNARE synaptobrevin 2. This interaction was even more pronounced if synaptobrevin 2 was organized in the SNARE complex. Electrophysiological investigations at the calyx of Held synapse revealed that this interaction is crucial for efficient replenishment of fast-releasing synaptic vesicles.

By preventing the intersectin 1 – SNARE complex interaction by either using intersectin 1 KO neurons or wild-type neurons expressing an intersectin 1 binding deficient mutant of synaptobrevin 2 we were able to show different phenotypes caused by impaired release site clearance. For example by applying pHluorin imaging we detected a surface accumulation of SNARE proteins as well as exocytic depression upon sustained stimulations.

Our results suggest a role for intersectin 1 in removal of SNARE complexes from release sites to provide accessible sites for subsequent exocytosis events and proper refilling of the synaptic vesicle pool.

To confirm our hypothesis we currently establish a proximity ligation assay to directly analyze the amount of postfusion SNARE complexes. Furthermore we use superresolution STED microscopy to look at newly exocytosed synaptobrevin 2 molecules and their localization in relation to the active zone upon loss of the intersectin 1 – SNARE complex interaction.

#21

Imaging synaptic vesicles from live hippocampal neurons by subdiffractional tracking of internalized molecules (sdTIM)

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Our understanding of endocytic pathway dynamics is restricted by the diffraction limit of light microscopy prompting many to turn to super-resolution techniques. However, highly crowded nerve terminals drastically limit the tracking of multiple endocytic vesicles, such as synaptic vesicles (SVs) simultaneously, which restricts the analytical dissection of SV discrete diffusional and transport states. To address this, our lab implemented a novel pulse-chase technique based on the subdiffractional tracking of internalized molecules (sdTIM). The technique allowed us to image anti-green fluorescent protein Atto647N-tagged nanobodies trapped in SVs from live hippocampal nerve terminals expressing vesicle-associated membrane protein 2 (VAMP2)-pHluorin[1]. Our results showed that, once internalized, VAMP2-pHluorin/Atto647N-tagged nanobodies exhibited a markedly lower mobility than on the plasma membrane, an effect that was reversed upon restimulation in presynapses but not in neighbouring axons[1]. Combining sdTIM with Bayesian model selection applied to hidden Markov modelling, allows determining both (i) the number of active transport and diffusive states underlying a particular particle trajectory, and (ii) when transitions between these states occur. Our results showed that SVs oscillate between diffusive states, or a combination of diffusive and transport states with opposite directionality. Importantly, SVs exhibiting diffusive motion were less likely to switch to the transport motion. We have further extended our protocol for dual-colour super-resolution imaging of Atto565-tagged nanobodies and Alexa647-tagged cholera toxin subunit-B internalized in SVs and signalling endosomes, respectively, undergoing long-range axonal retrograde transport with 30-50 nm localization precision[2]. This technique is therefore highly quantitative and should be instrumental in defining the precise role of Tau and Synapsin in governing SVs mobility. Overall, sdTIM technique offers a transferable approach to track multiple subdiffractional endocytic structures in live neurons and other cell types.

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#22

Ral Small GTPase Signalling Control Intestinal Stem Cell Dynamics by Promoting Internalisation of WNT Signalosomes

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Internalisation of frizzled receptors is crucial for proper activation of canonical WNT-signalling, and is of importance, not only to normal development and homeostasis of WNT-dependent organs, but also to cancer where hyper activation of the WNT pathway often is a triggering event that drives tumour progression. Although endocytosis is of importance for WNT-signalling, it is not fully known if activation of oncogenic pathways in cancer cells can influence endocytosis of Frizzled receptors, if this contributes to tumour progression, and if it is could be of interest for new therapeutic strategies against cancer.

Here, we present a new finding that Ral small GTPase signalling, a known effector pathway downstream of oncogenic KRAS, is crucial for Clathrin-mediated endocytosis of Frizzled receptors in intestinal stem cells of mice, and is necessary for proper activation of the WNT pathway. Consequently, we found that deleting floxed RALA and RALB in the intestine of mice, driving CRE expression under the Villin promoter, resulted in profound loss of stem cells and proliferation in the gut. The mechanism was traced to RALA and RALB's role in promoting Clathrin-mediated endocytosis through activation of RALBP1, and using CRISPR-CAS9 to delete RALA, RALB, or RALBP1 in HEK29 cells caused accumulation of Frizzled receptors on the plasma membrane, and impaired internalization of endogenous and SNAP-tagged Frizzled proteins. Finally, tracking expansion of individual stem cell clones, expressing fluorescent tdTomato, we were able to conclude that RALA and RALB deficient stem cells are less competitive compare to wild type stem cells of the gut.

Our findings suggest that regulation of vesicle trafficking of Frizzled receptors through Ral GTPases provides an important link to how oncogenic KRAS can fuel wnt-signalling and provide stem cells in the gut with a competitive advantage compared to non-transformed stem cells, and highlights that vesicle trafficking could be interesting for anti-cancer treatments.

#23

A novel quantitative single molecule colocalization assay reveals otoferlin as a multivalent calcium-sensitive scaffold linking SNAREs and calcium channels

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Sensory hair cells rely on otoferlin as the calcium sensor for exocytosis and encoding of sound preferentially over the neuronal calcium sensor synaptotagmin. Although it is established that synaptotagmin cannot rescue the otoferlin KO phenotype, the large size and low solubility of otoferlin have prohibited direct biochemical comparisons that could establish functional differences between these two proteins. To address this challenge, we have developed a single molecule colocalization binding titration assay (smCoBRA) that can quantitatively characterize full-length otoferlin from mammalian cell lysate. Using smCoBRA, we found that, although both otoferlin and synaptotagmin bind membrane fusion SNARE proteins, only otoferlin interacts with the L-type calcium channel Cav1.3, showing a significant difference between the synaptic proteins. Furthermore, otoferlin was found capable of interacting with multiple SNARE and Cav1.3 proteins simultaneously, forming a heterooligomer complex. We also found that a deafness-causing missense mutation in otoferlin attenuates binding between otoferlin and Cav1.3, suggesting that deficiencies in this interaction may form the basis for otoferlin-related hearing loss. Based on our results, we propose a model in which otoferlin acts as a calcium-sensitive scaffolding protein, localizing SNARE proteins proximal to the calcium channel so as to synchronize calcium influx with membrane fusion. Our findings also provide a molecular-level explanation for the observation that synaptotagmin and otoferlin are not functionally redundant. This study also validates a generally applicable methodology for quantitatively characterizing large, multivalent membrane proteins.

#24

VAMP2 controls the exit of Munc18-1 from nanocluster confinement during SNARE complex assembly

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Munc18-1 is a key regulatory protein essential for docking and priming of secretory vesicles at the plasma membrane through its involvement in SNARE complex assembly. Using super resolution techniques our lab has previously demonstrated that Munc18-1 domain 3a hinge-loop controls (1) syntaxin-1A opening, (2) its ensued engagement into SNARE complex and Munc18-1 release from the confinement of plasma membrane nanoclusters¹. The release of Munc18-1 from syntaxin-1A could define a specific set of “ready for fusion” nanoclusters of open syntaxin-1A as previously envisaged². Alternatively, Munc18-1 could directly trigger SNARE complex formation via opening of syntaxin-1A within the context of vesicular SNARE, VAMP2. To assess the potential role of VAMP2-Munc18-1 interaction in triggering SNARE complex formation we examined the nanoscale organisation and dynamics of Munc18-1WT and two mutants with highly reduced binding to VAMP2 (A297H and T304H). These constructs are tagged with photoconvertible mEos2 and expressed in Munc18-1/2 double knockout (DKO)-PC12 cells. We demonstrated that Munc18-1 secretagogue stimulation increased the mobility of Munc18-1 due to its release from syntaxin-1A. However, expression of the two VAMP2 deficient binding mutants blocked this increase in mobility suggesting that these residues are necessary for triggering the release of Munc18-1 from the nanocluster confinement in an activity-dependent manner. We also showed that both mutants reduced Ba²⁺-induced stimulated secretion. Our results demonstrate that syntaxin-1A opening also requires Munc18-1 interaction with VAMP2 suggesting that such opening only occur within the confinement of primed vesicles upon VAMP2 binding to Munc18-1.

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#25

UNC-16/JIP3 regulates early events in polarized trafficking of synaptic vesicle proteins via LRK-1/LRRK2 and AP complexes

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JIP3/UNC-16/dSYD is a MAPK-scaffolding protein with roles in protein trafficking. We show that it is present on the Golgi and is necessary for the polarized distribution of synaptic vesicle proteins (SVPs) and dendritic proteins in neurons. UNC-16 excludes Golgi enzymes from SVP transport carriers and facilitates inclusion of specific SVPs into the same transport carrier. The SVP trafficking roles of UNC-16 are mediated through LRK-1, whose localization to the Golgi is reduced in *unc-16* animals. UNC-16, through LRK-1, also enables Golgi-localization of the μ -subunit of the AP-1 complex. AP1 regulates the size but not the composition of SVP transport carriers. Additionally, UNC-16 and LRK-1 through the AP-3 complex regulates the composition but not the size of the SVP transport carrier. These early biogenesis steps are essential for dependence on the synaptic vesicle motor, UNC-104 for axonal transport. Our results show that UNC-16 and its downstream effectors, LRK-1 and the AP complexes function at the Golgi and/or post-Golgi compartments to control early steps of SV biogenesis. The UNC-16 dependent steps of exclusion, inclusion and motor recruitment are critical for polarized distribution of neuronal cargo.

#26

Dysregulation of the ER and Golgi associated Rab18 pathway in a human neurological disorder

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Rab18 has been implicated in the regulation of (endoplasmic reticulum) ER and Golgi function, lipid droplets, and the formation of regulated storage granules. We have previously found that loss of function mutations in Rab18 and components of the Rab3GAP complex are associated with Warburg Micro Syndrome (WMS), a severe human neurological disorder caused by defects in the development of the brain. The Rab3GAP complex comprises of two subunits Rab3GAP1 and Rab3GAP2, and has both GTP hydrolysis promoting (GAP) activity towards Rab3 and GDP-GTP exchange (GEF) activity towards Rab18. The Rab3GAP activity is associated with a C-terminal domain of Rab3GAP1, while Rab18 GEF activity requires the Rab3GAP1/2 complex. Because mutations in either Rab3GAP1 or Rab3GAP2 result in loss of Rab18 GEF activity, we have proposed that WMS is primarily a disorder of Rab18 regulation.

We have previously found that the Rab3GAP complex localises to punctate structures associated with the ER and Golgi. Because of the important of site-specific activation of Rab GTPases, we therefore determined the molecular basis of Rab3GAP targeting to ER and Golgi membranes to better understand the function of the Rab18 pathway and the trafficking defects underpinning WMS. Our unpublished data reveal that the Rab3GAP complex binds to membranes through two different signals. Structural and biophysical analysis show that a phenylalanine acidic tract (FFAT) motif in Rab3GAP1 binds to the ER and Golgi membrane protein VAP. A conserved beta-propellor domain in Rab3GAP interacts with PI-4P. We have found these binding properties enable the Rab3GAP complex to tether VAP containing ER-like membranes to PI-4P containing Golgi-like membranes and trigger the vectorial activation of Rab18. We therefore propose that the Rab3GAP complex acts as a minimal ER-Golgi membrane tethering and Rab18 GEF system.

#27

Connecting the dots: how does the ATPase EHD2 regulate caveolae dynamics?

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Caveolae are plasma membrane invaginations enriched in certain cell types such as adipocytes and endothelial cells. Due to their dynamic behaviour, ranging from stably surface connected to short ranged kiss-and-run cycles with the cell surface, caveolae are believed to serve as tension regulators of the plasma membrane. The Eps15-homology domain containing protein 2 (EHD2) has been identified as a major regulator of the caveolae dynamics. EHD2 belongs to a family of dynamin-related ATPases that bind and remodel cellular membrane by mechanical scaffolding. We have recently shown that EHD2 undergoes major conformational changes from a closed auto-inhibited form found in solution to an open conformation bound to lipids. The transition from closed to open state requires apart from lipids the loading of ATP, whereby a pocket in the G-domain is unlocked enabling oligomerisation of the protein. EHD2 oligomerises at the neck of caveolae and stabilises them at the cell surface; however, the exact mechanism is still not known. With a stopped flow FRET assay we are able to measure the immediate impact that different lipid compositions have on the oligomerisation rate of EHD2 and monitor the binding kinetics. In order to visualize caveolae, we established a stable cell line that allows us to induce the expression of mCherry tagged Caveolin1, the major component of caveolae. By combining microinjection of fluorescently labelled purified EHD2 into these cells, we are able to timely study the acute and initial steps of EHD2 localisation to caveolae and how this affects their dynamics. By injecting EHD2, we can stabilise caveolae to the cell surface, however there is a delicate balance as too much EHD2 causes degradation of caveolin1, thus loss of caveolae.

#28

The activity of the polybasic juxtamembrane region of syntaxin 1A in promoting Ca²⁺-triggered release requires CK2 phosphorylation

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We have recently showed in PC12 cells* that the polybasic juxtamembrane region (5RK) of the plasma membrane neuronal SNARE, syntaxin 1A (Syx), is absolutely required for a depolarization-induced Ca²⁺-dependent close-to-open transition (CDO) of Syx, and functions as a fusion clamp, making release dependent on stimulation by Ca²⁺. The mechanism underlying 5RK activity has not been characterized. Here, we show that phosphorylation of Syx by protein kinase CK2 is required for 5RK activity. Following biochemical identification of constitutive S14 phosphorylation of Syx in PC12 cells, dynamic FRET analysis of 35hosphor-null and 35hosphor-mimetic mutations of our Syx-based FRET probe* revealed that S14 phosphorylation imposes the 5RK requirement for CDO. CK2 is the likely *in vivo* Syx protein kinase based on inhibition of phosphorylation by tetrabromo-2-benzotriazole in PC12 cells, which abolished the 5RK requirement, and by the identity of the phosphorylation site. Further, using the light chain of tetanus toxin, we show that S14 phosphorylation is required for CDO to involve the vesicular SNARE, synaptobrevin 2. Prompted by these results, which underscore a potentially significant role of S14 phosphorylation in vesicle exocytosis, we next amperometrically 35hosphor catecholamine release from PC12 cells, revealing that the 35hosphor-null mutant does not support Ca²⁺-triggered release. Collectively, these results identify a functionally important N-terminal CK2 phosphorylation site in Syx, which is required for CDO to occur within a vesicular context and to be regulated by 5RK, making release dependent on stimulation by Ca²⁺. We suggest a model of interrelated roles of CK2 phosphorylation and 5RK in Syx functioning during Ca²⁺-triggered release.

References

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#29

Lipid composition and the ATPase EHD2 determines the surface stability of caveolae

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Caveolae are small omega shaped invagination of the cell surface which have been implicated in muscle, pulmonary, and lipid disorders. Caveolae are typically enriched in lipids such as cholesterol and sphingolipids. The dynamic behaviour of caveolae range from stable surface association, to short-range cycles of fission and fusion with the plasma membrane and endocytosis. The dynamin-related ATPase EHD2 confines caveolae to the cell surface by restricting the scission and subsequent endocytosis of these membrane pits. Here, we establish functionally, and structurally how EHD2 cycles between an active, membrane-bound state and an inactive state in solution. We show that the regulatory N-terminal residues and the EH-domain keep the EHD2 dimer in an autoinhibited conformation in solution. We determine that ATP-binding enables partial insertion of the open conformation of EHD2 into the membrane, which furthermore enables G-domain-mediated oligomerization of EHD2. This stringently regulated mechanistic cycle of EHD2 promotes surface stability of caveolae. Furthermore, we show that the lipid composition of the cell surface influence caveolae dynamics. We incorporate different lipid species in the plasma membrane using fusogenic liposomes and show that both glycosphingolipids and sphingomyelin, which initially are evenly distributed in the bilayer, subsequently accumulate in caveolae. Incorporation of glycosphingolipids, but not other lipid species, in the plasma membrane counteracts the role of EHD2 and decreases the surface stability of caveolae. We show that overexpression or microinjection of EHD2 in cells can rescue the effect induced by increased glycosphingolipids levels, suggesting that caveolae dynamics is determined by the degree of EHD2 oligomerisation and the lipid composition.

#30

Conformation-specific recognition and retrograde sorting of yeast Synaptobrevin by the Snx4-Atg20 SNX-BAR protein

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Synaptobrevin/VAMP2 is an essential R-SNARE protein that has been extensively studied in its role in synaptic vesicle exocytosis. How Synaptobrevin is sorted and trafficked between different organelles of the cell is not well understood. We find that the yeast Synaptobrevin homologue, Snc1, functions in a Golgi-to-endosome trafficking circuit, in addition to its role in exocytic Golgi-to plasma membrane transport. Retrieval of Snc1 from the endosome requires the SNX-BAR protein, Snx4 and Atg20, which dimerize to constitute a BAR domain that is capable of topological remodeling the topology of a membrane bilayer in vitro. A retrograde sorting signal in Snc1 was identified in the “linker” segment of Snc1 that joins the SNARE motif to the transmembrane domain, and biochemical reconstitution studies show that Snx4-Atg20 directly recognizes this signal. Curiously, amino acid substitutions within the transmembrane domain of Snc1 abolish retrograde sorting in vivo and ablate Snc1 recognition by Snx4-Atg20 in vitro. Published structural analyses of Synaptobrevin suggest that the conformation of the retrograde sorting signal is constrained by interactions between conserved basic residues within the linker and the headgroups of anionic membrane lipids. Studies of Snx4-Atg20 lipid binding specificity show that it binds preferentially to membrane containing a high content of anionic lipids, a property that distinguishes it from all other yeast SNX-BAR proteins. Importantly, the region of Snc1 that is recognized by Snx4-Atg20 corresponds to a region of Synaptobrevin that, when incorporated into a cis SNARE complex, contacts other SNAREs, indicating that SNARE complex formation and Snx4-Atg20 binding are mutually exclusive. This work identifies a mechanism for the selection of a SNARE protein into an endosome-derived retrograde transport carrier in which recognition of the SNARE by Snx4-Atg20 depends critically on conformational constraints imposed on the retrograde sorting signal by the transmembrane domain and interactions of linker residues with vicinal lipids.

#31

Role of SNAREs in the mechanism of Invariant chain-mediated endosomal enlargement

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The major histocompatibility complex class II associated invariant chain (Ii) is a type II transmembrane glycoprotein first known for trafficking of the MHC class II to endosomal compartment and preventing peptides from binding the MHCII groove in the ER. Moreover, Ii affects endocytic transport by delaying endosomal maturation and it has fusogenic properties and increases endosomal size. The delayed endosomal maturation and slower degradation could impact antigen processing and antigen loading creating a less proteolytic environment compared to endosomal degradation without Ii. How Ii influence the maturation process and increase the size of endosomes is not clear. We have previously demonstrated that the early endosomal fusion machinery, involving Rab5 and EEA1, is not affected by Ii expression and it is not responsible for Ii-mediated endosomal enlargement. In fact, the enlargement takes place without the PI-3 kinase dependent Rab5/EEA1. On the other hand, SNARE proteins are involved in this process as the general SNARE inhibitor NEM affects the size of Ii-positive enlarged endosomes. SNARE proteins mediate fusion of vesicles by forming a complex of four SNAREs. Sec1/Munc18 (SM) proteins are important regulators of SNAREs. After several two hybrid screens which gave not verifiably candidates we performed a siRNA screen against all SNARE and SM proteins and analyzed the effect of the silencing on endosomal size when Ii was expressed. Interestingly, several candidates showed a decreased endosomal size compared to control cells. Four individual siRNAs are used to exclude off-target effects and silencing is checked by WB. We are at present characterizing candidates by live imaging and biochemical approaches. We aim by this approach to characterize potential SNARE complex involved in Ii-mediated endosomal enlargement and thereby clarify mechanism by which Ii modulates endosomal maturation and possibly creating compartments for antigen loading (also called MIIC), both in model cell lines and in professional antigen presenting cells.

#32

Characterizing synaptic vesicle exocytosis-endocytosis with new pH-sensitive red fluorescent dyes

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Specific labelling of biomolecules with bright, photostable fluorophores is the keystone of cellular imaging. Notably, proteins with pH-sensitive fluorescence are valuable tools for imaging of exocytosis and endocytosis. The pH-sensitive variant of GFP, pHluorin, coupled to the luminal domain of vesicular proteins such as synaptobrevin and synaptotagmin is particularly well suited to these applications. A useful extension of this technology would be the creation of pH sensors based on red fluorescent fluorophores because longer wavelengths are less phototoxic, elicit lower levels of autofluorescence, and allow multicolor imaging experiments. Thus, we generated two bright red pH-sensitive fluorescent dyes, Carbofluorescein (CFI) and Virginia Orange (VO), which exhibit a pH sensitivity similar to that of pHluorin. Using the self-labelling SNAPtag system, both dyes reported activity-dependent exocytosis as efficiently as pHluorin or its red variant pHuji. When expressed with the GFP-based indicator GCaMP6f in the same neuron, Synaptobrevin-SNAPtag-CFI enabled concomitant imaging of synaptic vesicle cycle and presynaptic Ca²⁺ transients at single nerve terminals. Coupling of VO to antibodies directed against endogenous vesicular proteins showed a higher signal-to-background ratio compared to the genetically encoded pH-sensors, making it amenable for detection of single vesicle exocytosis and endocytosis events. Overall, these tools permit a higher flexibility in order to investigate the dynamic properties of the presynapse.

#33

EXO- AND ENDOCYTOSIS AT A RETINAL INHIBITORY SYNAPSE DURING CROSSOVER INHIBITION.

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Visual information is split into ON and OFF bipolar cell (BC) channels, which depolarize in response to dark and bright stimuli. The AII amacrine cell is an inhibitory interneuron that makes electrical synapses with ON BCs. Excitation to the AII triggers the synaptic release of glycine onto the OFF pathway; this is crossover inhibition. We performed whole-cell voltage-clamp recordings, using the “sine + DC” method to measure membrane capacitance with high temporal resolution. This approach allows us directly measure the fusion (exocytosis) and recovery (endocytosis) of synaptic vesicles at lobular varicosities near the AII soma during crossover inhibition. AIIs were held at -80 mV and L-type currents were evoked with a 100 ms, 70 mV depolarizing step, triggering calcium dependent vesicle fusion. Our observed capacitance jumps ranged from 14.81-152.3 fF, corresponding to the fusion of 370-3800 vesicles (n=38). After 4 minutes of treatment of 1 mM cAMP added to our internal recording solution, we observed presynaptic potentiation (n=14). In recordings from older animals (9-13 months) we consistently observed a gradual decay in capacitance, indicative of vesicular membrane retrieval (endocytosis). Tau ranged from 3.90 to 7.11 s (n=9), suggesting that AIIs are suited for fast membrane recovery. We propose that the variability in exocytosis for these interneurons is due to their diverse cell-to-cell lobular morphologies. In order to test this hypothesis, we performed capacitance recordings in conjunction with wide-field optical sectioning of dye filled AII. We compared 3D reconstructions of the lobular varicosities from individual AII to their net levels of exocytosis. Our results show that the magnitude of glycine release via exocytosis positively correlates to the number and overall size of these lobules (n=11). We believe that cells with larger lobules have more synaptic connections with OFF BCs and/or ganglion cells, providing more net inhibitory output.

#34

Fusion pore kinetics are altered in chromaffin cells of the SOD1G93A mouse model of ALS at symptomatic stage but not at early stage in spite of dramatic changes in mitochondria morphology and function

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Amyotrophic lateral sclerosis (ALS) is characterized with a selective loss of motor neurons that cause paralysis and respiratory failure. Hyperexcitability and Ca²⁺-dependent glutamate excitotoxicity has been hypothesized to be involved in ALS pathogenesis. Exploring the chromaffin cell (CC) of SOD1G93A mouse model of familiar ALS, we found that the fusion pore kinetics of exocytosis is slowed but with higher catecholamine quantal size when the disease is already established (Calvo-Gallardo et al., *Am J Physiol Cell Physiol* 2015;308:C1-C19). Here we have investigated whether exocytosis is also altered at presymptomatic stages in this model. Analyzing more than 2000 amperometric spikes of 1 minute acetylcholine stimulus, we fail in observe such significant differences in the fusion pore kinetics with respect with control C57BL/6 mouse CC. We only observe a faster decay time in SOD1G93A CCs (5.228 ± 0.298 ms vs 6.290 ± 0.360 ms in the control), contrary to the slowed secretion previously observed at symptomatic stages. To go further in the study of this interesting alterations in the neurosecretion, we started studying mitochondrial ultrastructure and function in these cells, as a crucial organelle involved in the process by distributing Ca²⁺ microdomains and providing ATP. Transmission electron microscopy indicated that mitochondria from SOD1G93A CCs showed the following alterations with respect to wildtype CCs: i) more number and small sized; ii) increased mitochondrial intermembrane space; iii) swollen and lower number of cristae. These ultrastructural changes were accompanied by lower ATP production and a higher rate of reactive oxygen species generation. This suggests that mitochondria dysfunction in SOD1G93A occurring at early predisease stages precede the exocytotic alterations. In spite of having lower clinical relevance than in later stages, these results could generate some clues about the initiation and progression of the disease.

Funding: SAF 2013-44108-P grant to AGG; FPI BES-2014-069005 grant to I.M-L., MINECO, Spain.

#35

The impact of ligand size on endocytosis

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Receptor mediated endocytosis is the process through which cell plasma membrane internalize biological or synthetic nanoscale objects driven by ligand-receptor complexation. Existing biophysical models of endocytosis have mainly focused on studying the free-energy costs associated with the deformation of the lipid bilayer wrapping the invader. Less attention has been paid to the study of how different types of binders (ligands or receptors) affect adhesion free energy. The lack of direct links between molecular and mesoscopic scales hampers understanding of experimental results both in biology and nanotechnological applications.

In this contribution we extend state-of-the-art multivalent calculations and study how mobility and size of the binding complexes impact the adhesion free energy by means of analytic models and simulations. Using the proposed method, we study the internalization pathway of invaders decorated by fixed and freely diffusing ligands. We find that the attachment transition at which the cell membrane starts to wrap the guest is comparable for both systems. However, invaders decorated by freely diffusing and non-mutually-interacting (ideal) ligands are significantly more difficult to internalize than those where ligands are immobile, and are usually found in partially wrapped states. Nevertheless, complete wrapping becomes favorable also in mobile ligand systems when the steric repulsion between ligands is increased. This result is due to the entropic costs of confining bulky ligands in the contact region driving the system towards complete wrapping.

Our model rationalizes the relationship between uptake mechanism and structural details of the invader, such as ligand size, mobility and ligand/receptor affinity, providing a comprehensive picture of pathogen endocytosis and helping the rational design of efficient drug delivery vectors.

#36

Role of OCRL in neurons

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Lowe syndrome, caused by mutation of the phosphatidylinositol polyphosphate 5-phosphatase OCRL, is characterized by anomalies affecting the central nervous system, the eye, and kidney. There has been tremendous progress in understanding the role of OCRL in the endolysosomal pathway in epithelial cells that may be relevant for understanding the renal symptoms of the disease, but whether these underlie the neuronal pathology or if OCRL might have neuronal-specific functions is largely unexplored.

To identify OCRL interactors that may be relevant for its role in the nervous system we analyzed by mass spectrometry the proteins from mouse brain that co-immunoprecipitated with OCRL. The functional annotation of the OCRL interactors found in the brain returned proteins involved in synaptic vesicle recycling, components of the vATPase proton pump, and monomeric and trimeric GTPases. We validated the interaction of the synaptic vesicle protein VAMP2, the best characterized synaptic vesicle protein, by immunoprecipitation and immunofluorescence. Furthermore, we observed an increase in the area of endosomal structures and an accumulation of filamentous actin on endosomes in cultured neuronal cells depleted of OCRL. This is analogous to what we observed previously in OCRL-depleted proximal tubule cells where we demonstrated a PI(4,5)P₂-dependent accumulation of actin which impaired the recycling of endocytic receptors out of early endosomes (Vicinanza et al, *The EMBO Journal*, 2011). We are currently testing the hypothesis that actin accumulation in neuronal endosomes induced by the loss of function of OCRL may affect synaptic vesicle recycling and thus could represent one of the mechanisms underlying neuronal impairment in Lowe syndrome.

#37

Entropic forces drive self-organization and membrane fusion by SNARE proteins

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SNARE proteins are the core of the cell's fusion machinery and mediate intracellular membrane fusion reactions on which exocytosis and trafficking depend. For example, cognition and coordinated motor activity rely on tightly controlled release of neurotransmitters (NTs) at synapses, accomplished by a synaptic fusion machinery that senses Ca²⁺ when an action potential arrives, releasing the SNAREs to accomplish the final step. Fusion is catalyzed when vesicle-associated v-SNAREs form trans-SNARE complexes ("SNAREpins") with target membrane-associated t-SNAREs, a zipper-like process releasing ~65 kT per SNAREpin. Fusion requires several SNAREpins, but how they cooperate is unknown and the reported number required varies widely.

Computational modeling can help establish mechanisms, but a major obstacle is that the collective behavior of multiple fusion machinery components is beyond current approaches, due to massive time requirements. To capture the long timescales of fusion, we use a highly coarse-grained approach that retains key biophysical SNARE properties such as the zipper energy landscape and the surface charge distribution. In simulations the ~65 kT zipper energy was almost entirely dissipated, with fully assembled SNARE motifs but uncomplexed linker domains. The SNAREpins self-organized into a circular cluster at the fusion site, driven by entropic forces that originate in steric-electrostatic interactions among SNAREpins and membranes. Cooperative entropic forces expanded the cluster and pulled the membranes together at the center point with substantial force. We find that even one SNAREpin suffices for fusion, but the fusion rate increases rapidly with the number of SNAREpins due to increasing entropic forces. We hypothesize that this principle finds physiological use to boost fusion rates to meet the demanding timescales of neurotransmission, exploiting the large number of v-SNAREs available in synaptic vesicles. Once in an unfettered cluster, we estimate ≥ 15 SNAREpins are required for fusion within the ~1 ms timescale of neurotransmitter release.

#38

Phosphoinositides are not required for secretory granule docking

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Phosphoinositides (PIs) play important roles in exocytosis and are thought to regulate secretory granule docking by co-clustering with the SNARE protein syntaxin to form a docking receptor in the plasma membrane. Here we tested this idea by high-resolution TIRF imaging of EGFP-labeled PI markers or syntaxin1 at secretory granule release sites in live insulin-secreting cells. In intact cells, PI markers distributed evenly across the plasma membrane with no preference for granule docking sites. In contrast, syntaxin1 was found clustered in the plasma membrane, mostly beneath docked granules. We also observed rapid accumulation of syntaxin1 at sites where granules arrived to dock. Acute depletion of plasma membrane PI(4,5)P₂ by recruitment of a 5'-phosphatase strongly inhibited Ca²⁺-dependent exocytosis, but had no effect on docked granules or the distribution and clustering of syntaxin. Cell permeabilization by α -toxin or formaldehyde-fixation caused PI marker to slowly cluster, in part near docked granules. In summary, our data indicate that PI(4,5)P₂ accelerates granule priming, but challenge a role of PIs in secretory granule docking or clustering of syntaxin1 at the release site.

#39

Novel low-energy, non-axisymmetric modes of deformation in endocytosis

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Endocytosis is a process in which cells form invaginations by internalizing parts of the plasma membrane for the purpose of bringing cargo into the cell. In particular, the cargo material can be various proteins but also pathogens, thus making endocytosis an important mode of intercellular trafficking. Endocytic events are mediated through a protein coat attaching to the lipid bilayer of the cell. The protein's structure induces curvature into the lipid bilayer, thus forming an invagination and subsequently a bud which carries the material to be transported. When the bud is sufficiently constricted, scission occurs at the bud's neck and a vesicle is formed. Despite the fact that endocytosis has been studied extensively, its mechanics are not yet fully understood. Lipid bilayers are usually modeled as liquid shells which do not have any shear resistance in their in-plane direction. There are few non-axisymmetric, continuum studies on lipid bilayers available. Recent findings for lipid bilayers based on a three-dimensional, C1-continuous Finite Element formulation showed that non-axisymmetric solutions can be energetically preferable over axisymmetric solutions. In this work, we investigated under which conditions, non-axisymmetric solutions are favorable. Namely, we find that axisymmetric shapes can branch out into non-axisymmetric shapes depending on factors such as the surrounding tension, viscous dissipation and the shape of the protein coat. We identify two different branches of solutions in which the mean curvature can take a value close to the prescribed spontaneous curvature. Firstly, this condition is met by forming spherical shapes, i.e. shapes with two equal principal curvatures. Secondly, cylindrical shapes, i.e. shapes where one of the principal curvatures is close to zero, can satisfy the same condition. This result and a set of factors determining the branching of solutions will be discussed in detail.

#40

Functional recruitment of dynamin requires multimeric interactions for efficient clathrin-mediated endocytosis

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During clathrin mediated endocytosis (CME), membrane scission is achieved by the concerted action of dynamin and its interacting partners (Ferguson and De Camilli, 2012). Disrupting the interaction between the proline/arginine-rich domain of dynamin (dynPRD) and the Src-homology domain 3 of amphiphysin (amphSH3) by injecting the dynamin-mimicking peptide D15 inhibits endocytosis in living cells (Shupliakov et al., 1997). However, the low affinity of D15 for amphSH3 raises the question of how a weak dynamin-amphiphysin interaction could result in the precise spatio-temporal recruitment of dynamin. Here, we propose that oligomerisation could increase the avidity of the interactions at play by bringing multiple binding sites in close proximity as proteins concentrate around the neck of nascent clathrin coated vesicles (CCVs). To test this hypothesis, we took a multidisciplinary approach using peptide design and in vitro binding assays, quantitative live cell imaging and dynamin mutation screen. We found, using a surface plasmon resonance assay, that divalent dynPRD-derived peptides bind more strongly to multimers than monomers of amphSH3. Consistently, divalent peptides, when dialysed in living cells with a patch-clamp pipette while monitoring CME with the ppH assay (Merrifield et al. 2005), block endocytosis more effectively than monovalent ones. Moreover, mutating dynamin in the D15 motif alone partially rescues endocytosis in dynamin triple knock-out cells, but additionally mutating a flanking SH3-binding site abolishes it. Finally, the recruitment kinetics of these mutated dynamins to nascent endocytic vesicles is not affected, but the maximal amount of dynamin is reduced, suggesting that the rate of recruitment of this essential protein is not a limiting factor for endocytic vesicle formation. We conclude that dynamin drives vesicle scission via multivalent interactions in vivo.

#41

Ubiquitination amplifies entropic forces that drive SNARE-mediated membrane fusion

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SNARE proteins are the core of the cell's membrane fusion machinery. In evoked release of neurotransmitters at neuronal synapses, vesicle- and target membrane-associated SNAREs form trans-SNARE complexes ("SNAREpins") that catalyze fusion. Post-translational attachment of ubiquitin to synaptic proteins regulates neurotransmitter release and synaptic plasticity. SNARE proteins are among the most highly ubiquitinated in the brain, suggesting a fundamental role in modulating neurotransmitter release.

Our highly coarse-grained (CG), molecularly-detailed simulations predict that SNARE-mediated fusion is driven by entropic forces from steric interactions among SNAREpins (Mostafavi et al., PNAS, 2017). The CG representation of SNAREpins had realistic geometry, zipper energy landscape, and surface charges. SNAREpins self-organize into circular clusters at the fusion site, and entropic forces among SNAREpins expand the cluster and push membranes into a close proximity of nanometers. Even one SNAREpin is adequate for fusion, but fusion rates increase with SNAREpin number as the entropic forces get stronger.

A fundamental prediction is that fusion rates would increase if the SNAREpins were bulkier, since entropic forces would then increase. Thus, we used our model to examine the effect of ubiquitination of SNAREpins, as these would render SNAREpins bulkier. We systematically coarse-grained ubiquitin and attached them to CG SNAREpins, varying the attachment site, and measured fusion rates and contact forces. We found that these increased for a given number of SNAREpins irrespective of the attachment site. The effect is strongest when the ubiquitination site is closest to the C-terminal end, proximal to the point of closest contact between membranes, as expected from the entropic crowding effect. This is consistent with preliminary measurements of excitatory post-synaptic current (EPSC) with ubiquitin attached to different sites along the SNAREpin. Thus, our work explores the exciting possibility that modulation of entropic forces is used to regulate neurotransmitter release, and is an important tool for synaptic plasticity.

#42

Phosphatidic acid is involved in clathrin-mediated endocytosis in tip-growing plant cells

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Polar cell growth is a fundamental process in plant development. The cell polarity is mediated by asymmetric distribution of proteins and lipids to specialized domains of the plasma membrane (PM). These polar domains are formed by properly balanced exocytosis, endocytosis, endomembrane trafficking and lateral mobility within PM. Although phosphatidic acid (PA) is structurally the simplest membrane phospholipid, it has been implicated in the regulation of many cellular events, including cytoskeletal dynamics and membrane trafficking. Plant PA is produced by phospholipase D (PLD) or diacylglycerol kinase and shows rapid turnover, but the details about its spatio-temporal distribution and involvement in plant cell polarity regulation are unclear. Here we demonstrate the live-cell imaging of PA dynamics in tip-growing plant cells using a genetically-encoded biosensor, present the characterization of the PA-forming enzymes in tobacco pollen and show that it is involved in the regulation of clathrin-mediated endocytosis.

In growing pollen tubes, PA shows distinct annulus-like fluorescence pattern in the PM domain behind the extreme tip. Coexpression with markers of other signaling lipids phosphatidylinositol 4,5-bisphosphate and diacylglycerol revealed limited colocalization at the shoulders of the cell apex. Enhanced PA subapical localization marks the zone of clathrin-dependent endocytosis visualised by DRP1C- or CLC-based markers. We found that enhanced activity of PLD δ 3, major PM-localized PLD isoform, leads to massive membrane invaginations, suggesting excessive aberrant endocytosis. Co-expression studies with PA marker and endocytosis markers demonstrated that the formation of membrane invaginations coincides with high PA production and that endocytosis machinery is relocalized and enriched there. We isolated novel PA-binding proteins from plant cells, where subunits of adaptor complexes of clathrin-mediated endocytosis were significantly enriched. By combining microscopic, biochemical and computational data, we suggest that AP2 complex is targeted to PM by direct interaction with PA.

#43

Traffic Light peptides to photocontrol clathrin-mediated endocytosis in yeast

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Clathrin-mediated endocytosis (CME) is well known for being fundamental to a variety of physiological processes such as uptake of nutrients through the plasma membrane, signal transduction or neurotransmission. Nonetheless, the extremely complex network of proteins involved in regulating this transient machinery makes it particularly hard to tackle only by means of genetic modification and immunological depletion. In this sense, pharmacological tools are of extreme interest because they allow the study of biological processes by freezing or enhancing them at the point of interest. However, the freely diffusing nature of these molecules does not allow a fine control of their activity. On the contrary, photopharmacology is a powerful tool to manipulate endogenous processes with high spatio-temporal resolution and in a non-invasive manner.

Traffic Light (TLs) peptides are cell-permeable, photoswitchable inhibitors specifically developed to target the main adaptor complex of the CME machinery. They are based on the structure of β -arrestin C-terminal peptide (BAP-long) bound to the β -appendage of AP2 (β -adaptin), which is known to mediate the binding of clathrin to the membrane or to cargo receptors. These peptides, named TL1 and TL2, have already proved capable of inhibiting CME in a light-regulated manner when tested in mammalian cells [1][2].

Here we show that TL peptides can be used as a tool to further investigate the molecular mechanisms behind CME in an extremely versatile eukaryotic model system, i.e. yeast. *S. cerevisiae* spheroplasts were created to allow peptides internalization as observed for mammalian cells. Subsequently, mutants expressing fluorescently tagged Sla1 and Abp1 were used to observe kinetic delays in the dynamics of vesicle formation, confirming the possibility of photoregulating CME events by means of TL peptides.

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#44

Intra- and inter-molecular regulation of dynamin superfamily proteins (DSPs) in membrane fission

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The dynamin superfamily comprises a growing assortment of multi-domain GTPases that participate in various intracellular membrane remodeling events, including the fission and fusion of transport vesicles as well as of large organelles such as the mitochondria. All higher eukaryotes encode for two different classes of DSPs; an extant, ancient branch of DSPs that includes dynamin-related protein 1 (Drp1), a DSP primarily associated with organellar fission, and an evolutionary modern branch of DSPs composed of isoforms of the family namesake ‘dynamin’ that participate in endocytic vesicle fission. Despite the high conservation of their GTPase (G) domains, Drp1 contains various intrinsically disordered polypeptide (IDP) regions within its 3D structure that are supplanted in dynamin by structured modules including the pleckstrin homology (PH) domain that specifically binds phosphatidylinositol-4,5-bisphosphate (PIP2) at the plasma membrane. By contrast, an IDP region in Drp1 termed the ‘variable domain or VD’ preferentially associates with cone-shaped lipids, including cardiolipin (CL) present exclusively in mitochondrial membranes. Additionally, dynamin contains a proline-rich domain (PRD) at its C-terminus, which is entirely absent in ancient DSPs. Furthermore, dynamin and Drp1 respective function in fission relies on interactions with distinct protein partners that specifically recruit and regulate the two DSPs. In spite of these stark differences, Drp1 and dynamin have recently been implicated in both vesicle and mitochondrial fission processes, and are believed to function in concert to mediate membrane remodeling. How so remains unknown. Using truncation, deletion and chimeric mutants of both dynamin and Drp1, we reveal that membrane and partner-protein interactions distinctly, but non-cooperatively, modulate dynamin and Drp1 helical self-assembly and assembly-stimulated GTPase activity, via disparate mechanisms that converge on the facilitation of helical inter-rung G domain dimerization essential for cooperative GTP hydrolysis. Our results provide a plausible explanation for the functional diversity of these distinct DSPs at the two membrane compartments.

#45

Novel Ecto-Tagged Integrins Reveal their Trafficking in Live Cells

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Integrins are abundant heterodimeric cell-surface adhesion receptors essential in multicellular organisms. Integrin function is dynamically modulated by endo-exocytic trafficking, however major mysteries remain about where, when, and how this occurs in living cells. To address this, here we report the generation of functional recombinant β Beta1 integrins with traceable tags inserted in an extracellular loop. We demonstrate that these ‘ecto-tagged’ integrins are cell-surface expressed, localize to adhesions, exhibit normal integrin activation and restore adhesion in β Beta1 integrin knockout fibroblasts. Importantly, β Beta1 integrins containing an extracellular pH-sensitive pHluorin tag allow direct visualization of integrin exocytosis in live cells and revealed targeted delivery of integrin vesicles to focal adhesions. Further, using β Beta1 integrins containing a HaloTag in combination with membrane-permeant and -impermeant Halo dyes allows imaging of integrin endocytosis and recycling. Thus, ecto-tagged integrins provide novel powerful tools to characterize integrin function and trafficking.

#46

Aging-associated changes in synaptic vesicle recycling

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Brain function depends on the ability of neurons to transfer and process information in response to different stimuli. Neuronal communication depends on chemical neurotransmission which is based on the exocytic fusion of synaptic vesicles (SVs) followed by endocytic membrane retrieval and the reformation of SVs. These processes are the main sources of activity-driven energy demands. Age-related cognitive decline likely reflects the manifestation of dysregulated synaptic function and ineffective neurotransmission. Moreover, recent evidence suggests connections between alterations in the presynaptic machinery for exo/endocytosis and aged associated disorders such as Parkinson's and Alzheimer's disease. To analyze changes in synaptic vesicular recycling process and energy metabolism during aging we have obtained cortical and hippocampal synaptosomes from C57BL/6J mice at different ages. The vesicular recycling analysis was assessed by the incorporation of the fluorescent dye FM4-64, measured in real time by confocal microscopy under basal conditions and after depolarization with 50 mM KCl. At all ages, the dye incorporation increased after KCl depolarization, and this incorporation was totally dependent on external Ca^{2+} , indicating an exocytosis event. However, we found changes in the kinetics of the FM4-64 incorporation between synaptosomes at different ages particularly in the initial velocity of the SVs exocytosis. After depolarization, the young synaptosomes incorporate FM4-64 faster than synaptosomes from old mice. On the other hand, we found a lower mitochondrial activity of the aged synaptosomes (9-10 and 18 months) compared to young (2-3 months). At present these results suggest that aging is associated with functional and structural changes of the exo/endocytosis machinery in pre-synapses. Changes in presynaptic calcium or ATP concentrations, which would affect either synaptic vesicle fusion or docking of SVs, might be responsible for the observed changes in the kinetics of synaptic vesicle recycling.

This work was partially supported by Estímulo en Salud "Miguel Alemán Valdés" and CONACYT 594678.

#47

Coordinated formation and disassembly of a contractile actomyosin network mediates content release from large secretory vesicles

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Coordinated formation and disassembly of contractile actin-based structures has been shown to underlie diverse settings of tissue morphogenesis. Here we propose that such a mechanism mediates the contractile activity necessary for content release from large secretory vesicles. A common feature of these systems is formation of an actin coat around each vesicle following their fusion with the apical cell membrane and recruitment of myosin, which together mediate the forces necessary for vesicle contraction. Using live imaging of cultured *Drosophila* larval salivary glands, an established model for such secretory systems, we have followed the dynamics of actomyosin coat formation and content release from glycoprotein (“glue”)-filled vesicles into the gland lumen. We previously demonstrated critical regulatory roles for the Rho1 GTPase in both actin coat formation (via activation of the Formin-family protein Diaphanous) and Myosin II-based contraction (via Rho kinase). Surprisingly, we have now found that disassembly of the actin coat, which accompanies vesicle content release, is necessary for contraction of the actomyosin network. This process was monitored using secretion-arrested vesicles, and found to be dependent on Rho1 inactivation, mediated by a dedicated RhoGAP and branched-actin polymerization. The sequential temporal recruitment of active Rho and its inhibitors is evident by cycles of active Rho1 and actin coat accumulation and depletion in such vesicles, implying that a feedback-based mechanism regulates actin coat disassembly from the vesicle surface. Contraction-driven content release, the final step of this form of exocytosis, is therefore achieved by coordinating formation and disassembly of the contractile machinery.

#48

Distinct membrane-binding properties of plant exocyst complex EXO70 subunit isoforms imply their different roles in cell polarity regulation

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The exocyst subunit EXO70, which directly binds phosphatidylinositol 4,5-bisphosphate (PIP2) in animal cells, is required for targeting of the complex and is represented by many isoforms in plant cells. The presented project combines live cell imaging, in vitro lipid binding assays and structural homology modeling to unravel specific roles of EXO70 isoforms in vesicular trafficking and their differential lipid binding properties. We use pollen tube, a well established model system for investigating plant cell polarity. Pollen tube bears advantage of spatially separated zones of exocytosis and endocytosis, as well as spatially separated maxima of signalling phospholipids. Various EXO70 isoforms localize to different regions of the pollen tube plasma membrane, apical vesicle-rich inverted cone region, nucleus and cytoplasm. EXO70A1a and EXO70B1 occupy two distinct and mutually exclusive plasma membrane domains. EXO70A1a localizes to the small area previously characterized as the site of exocytosis in the tobacco pollen tube, while EXO70B1 surprisingly colocalizes with the zone of clathrin-mediated endocytosis. In contrast, members of the EXO70C class, specifically expressed in tip-growing cells, exhibited exocytosis-related functional effects in pollen tubes despite the absence of apparent plasma membrane localization. Both EXO70A1a and EXO70B1 colocalize to different degrees with markers for anionic signaling phospholipids PIP2 and phosphatidic acid (PA), an emerging important regulator of plant exocytosis and endocytosis. We also demonstrate that EXO70A1 directly binds both PIP2 and PA in vitro and in vivo and present identified key amino acid residues responsible for these interactions.

#49

Contribution of C2AB-phospholipid interactions in Synaptotagmin-1 driven membrane fusion

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Ca²⁺ sensor proteins tightly control the secretion of various neuroendocrine substances. After the arrival of an action potential in the synaptic terminal, Ca²⁺ influx triggers the fast fusion of neurotransmitter-containing synaptic vesicles with the presynaptic plasma membrane. This process depends on SNAREs, complexin and the calcium sensor synaptotagmin-1 (Syt-1). Syt-1 contains a single transmembrane domain and two cytoplasmic C2 domains (together named C2AB). While the role of Syt-1 in living cells has been described to great detail, it remains a challenge to dissect the contribution of C2AB-induced membrane remodeling to the Ca²⁺-secretion coupling mechanism. Here, we studied unitary fusion events of two optically trapped beads coated with SNARE-free synthetic membranes, where the soluble C2AB domain of Syt-1 strongly affected the probability and strength of membrane-membrane interactions in a strictly Ca²⁺- and protein-dependent manner. Using various fluorescent labels, we characterized the membranous interface for membrane continuity, content mixing and C2AB protein localization. Our approach can be further extended to explore the effect of well-characterized Syt-1 mutations, effects of phospholipid composition and other fusion-related proteins.

* R.S. and M.M. contributed equally to this work* G.J.L.W. and A.J.G. jointly supervised this work

#50

Identification and characterization of a synaptotagmin-like protein involved in rhoptry secretion and invasion of parasites *Toxoplasma* and *Plasmodium*

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Rhoptries are unique club-shaped regulated secretory organelles, specific to invasive stages of apicomplexan parasites, which include the causal agent of malaria (*Plasmodium*) and of toxoplasmosis (*Toxoplasma gondii*). Rhoptries are tethered to the apical end of the parasite, discharged upon contact with the host cell during invasion, and their proteins translocated into the host cell by unknown mechanisms. Rhoptries are therefore key organelles for parasite invasion leading to interference with the host immune response by manipulation of the host cell. While rhoptry proteins have been extensively studied at the molecular level, it is totally unknown how rhoptry organelles discharge their content during invasion.

Here we present the characterization of two related *Toxoplasma* proteins, TgCLAW1 and TgCLAW2, which localize to the apical surface of the rhoptries. While TgCLAW1 is dispensable for the parasite lytic cycle, disruption of TgCLAW2 results in a severe defect in *T. gondii* invasion. In TgCLAW2 depleted parasites, rhoptries are correctly tethered to the apical end, but parasites are unable to secrete their content. CLAW2 is conserved in apicomplexan parasites but has no ortholog outside the phylum. We then generated a *Plasmodium falciparum* conditional CLAW mutant, and showed that CLAW is also a rhoptry secretion factor in *Plasmodium*. TgCLAW1 and 2 and PfCLAW are annotated with no structural domains but a search for predicted 3D domains revealed the presence of a C2 and a PH lipid binding domain that we validated experimentally to bind to phosphoinositides.

In conclusion, we identified the first rhoptry-associated protein involved in the discharge of this organelle in *Toxoplasma* and *Plasmodium*. Based on our observations, we venture to hypothesize that CLAW proteins could function as synaptotagmin-like proteins for docking and priming of the rhoptry organelle with the parasite plasma membrane at the time of invasion.

#51

Role of LAR in the endocytosis of the extracellular matrix proteins nidogens in motor neurons

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Neurons are highly polarized cells characterized by efficient uptake, sorting and transport of cargo over long distances. Tetanus toxin, one the most potent neurotoxins, is known to exploit these pathways to enter neurons and cause spastic paralysis [1]. Uptake of the tetanus toxin is facilitated by its interactions with the extracellular matrix proteins nidogens, both of which are then sorted into signalling endosomes and retrogradely transported towards the neuronal soma [2]. However, the molecular factors that enable the endocytosis of nidogens into neurons are currently unknown.

The receptor protein tyrosine phosphatase (RPTP) family member leukocyte common antigen-related protein (LAR, also known as PTPRF), with roles in synaptic development, axon outgrowth and guidance [3], has previously been demonstrated to interact with the laminin-nidogen complex [4]. A screen aimed at describing the molecular composition of tetanus toxin-carrying signalling endosomes in motor neurons identified LAR as a component of these endosomes [5]. In this context, I am interested in the question: is LAR a causative factor in the endocytosis and sorting of nidogens at the neuronal synapse? Using murine primary motor neurons, I am exploring the interaction between LAR and nidogens and the effects of disrupting this binding on the uptake of the complex.

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#52

Three-dimensional, high-resolution study of protein diffusion and trafficking in a live cell via interferometric scattering microscopy (iSCAT)

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Transmembrane proteins undergo a wide variety of movement in live cells, including diffusion in the plasma membrane and transport into the cell via endocytosis concluding with trafficking along filaments. While a great deal is known about the biochemistry of these processes, their in-situ and real-time visualization with nanoscopic resolution remains an experimental challenge.

Conventional microscopies to monitor diffusion of individual proteins within live cells rely on fluorescent labelling. The limited finite emission rate and rapid photobleaching of fluorophores curtail the resolution in space and time by which one can determine the position of the protein. Interferometric scattering microscopy (iSCAT) [1,2], wherein scattered light from a gold nanoparticle labelled protein is imaged interferometrically, can achieve a spatial resolution of several nanometers in all three dimensions even at microsecond exposure times. Here we label the epidermal growth factor receptor (EGFR) protein in the live HeLa cell with a gold nanoparticle probe and observe the various facets of the protein life cycle.

We present high-resolution data on diffusion in the plasma membrane, revealing key insights into confinement within nano-domains. We further show localisation of the receptor into clathrin-coated pits and uptake via endocytosis as well as trafficking of the endosomal vesicle along actin and microtubule filaments. Our nanometrically precise 3D information reveals exciting new aspects of all these processes which were previously not accessible to optical microscopy [3].

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#53

An Active Tethering Mechanism Controls the Fate of Vesicles

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Vesicle tethers are thought to underpin the efficiency of intracellular fusion that is required for eukaryotic life. However, the interplay between tethering and fusion has remained enigmatic. Here, we report that tethering regulates the mode of vesicle fusion by optogenetically controlling a natural tether, the exocyst complex, or an artificial tether. We found that vesicles mainly undergo kiss-and-run instead of full fusion in the absence of functional exocyst. Full fusion could be rescued by optogenetically restoring exocyst function, in a manner depending on the light dose and frequency of activation. In contrast, a passive artificial tether produced mostly kissing events, suggesting that kiss-and-run is the default mode of fusion, not a defective process. Optogenetic control of tethering further showed that how vesicles fuse has physiological relevance since only full fusion could trigger lamellipodial expansion. These findings suggest that active coupling between tethering and fusion is critical for robust membrane merger.

#54

Ca²⁺-dependent Focal Exocytosis of Golgi-derived Vesicles Helps Phagocytic Uptake in Macrophages.

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National Institute of Immunology

The role of Golgi apparatus during phagocytic uptake by macrophages has been ruled out in the past. Notably, all such reports were limited to Fc receptor-mediated phagocytosis. Here, we unravel a highly devolved mechanism for recruitment of Golgi-derived secretory vesicles during phagosome biogenesis, which was important for uptake of most cargos, except the IgG-coated ones. We report recruitment of mannosidase-II positive Golgi-derived vesicles during uptake of diverse targets, including latex beads, *Escherichia coli*, *Salmonella typhimurium*, and *Mycobacterium tuberculosis* in human and mouse macrophages. The recruitment of mannosidase-II vesicles was an early event mediated by focal exocytosis and coincided with the recruitment of transferrin receptor, VAMP3, and dynamin-2. Brefeldin A treatment inhibited mannosidase-II recruitment and phagocytic uptake of serum-coated or -uncoated latex beads and *E. coli*. However, consistent with previous studies, brefeldin A treatment did not affect uptake of IgG-coated latex beads. Mechanistically, recruitment of mannosidase-II vesicles during phagocytic uptake required Ca²⁺ from both extra- and intracellular sources apart from PI3K, microtubules, and dynamin-2. Extracellular Ca²⁺ via voltage gated Ca²⁺ channels established a Ca²⁺-dependent local phosphatidylinositol 1,4,5-trisphosphate gradient, which guides the focal movement of Golgi-derived vesicles to the site of uptake. We confirmed Golgi-derived vesicles recruited during phagocytosis were secretory vesicles as their recruitment was sensitive to depletion of VAMP2 or NCS1, whereas recruitment of the recycling endosome marker VAMP3 was unaffected. Depletion of both VAMP2 and NCS1 individually resulted in the reduced uptake by macrophages. Together, the study provides a previously unprecedented role of Golgi derived secretory vesicles in phagocytic uptake, the key innate defense function.

#55

A novel endosomal sorting protein in synapses: Sorting nexin-4

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Neurotransmitters are released through the exocytosis of synaptic vesicles, which are locally recycled after their fusion with the plasma membrane to maintain neurotransmission. The endosomal system is suggested to be involved in synaptic vesicle recycling, although its exact role is still topic of debate. Sorting nexin-4 (SNX4) is an evolutionary conserved protein that mediates recycling from the endosomes back to the plasma membrane both in yeast and mammal cells. Therefore, we hypothesized that SNX4 mediates a similar trafficking route from endosome to plasma membrane in synaptic terminals to recycle crucial proteins for synaptic vesicle release. To address this hypothesis, we first aimed to characterize the localization of SNX4 in neurons. We have developed a novel antibody against mouse SNX4 which was validated using a battery of independent shRNAs against endogenous SNX4 and, exogenous SNX4 overexpression. The distribution of SNX4 was addressed using Western Blot, immunohistochemistry, immunocytochemistry and immuno-electron microscopy. Our results showed that SNX4 is ubiquitously expressed in the brain. In primary neuronal cultures, SNX4 partially co-localized with both early and recycling endosomes, which is in accordance with the previously established role of SNX4. Interestingly, the highest co-localization was found with synaptic markers. This synaptic localization might be predominantly presynaptic, since we have also identified SNX4 in the presynaptic terminals. The novel identification of SNX4 as a presynaptic protein suggests that the SNX4-dependent endosome sorting could play a role in the local recycling of synaptic proteins. In addition, the characterization of SNX4 in neurons might help to understand the link between SNX4 and Alzheimer's disease, since SNX4 levels are 70% decreased in Alzheimer's disease brains in the highest Braak stages.

#56

Differential regulation of dynamin isoform by lipid mechanics

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Classical dynamins are large GTPases mediating membrane fission during endocytosis. They act as mechano-chemical enzymes converting the energy obtained from GTP hydrolysis into constriction and scission of endocytic vesicle's neck. The minimal dynamin unit capable of producing mechanical work is a short helical polymer. The helix is widely believed to impose its geometry, changing along the GTP hydrolysis cycle, onto lipid bilayer. However, we demonstrate here that the intrinsic ability of dynamins to create membrane curvature strongly depends on membrane rigidity. We tested two major isoforms of classical dynamins, neuron-specific Dyn1 and ubiquitously expressed Dyn2, on membrane templates of different rigidity. We found that the ability of both isoforms to tubulate planar membranes, as well as stationary membrane curvature imposed by the dynamin helices, depends on the apparent membrane rigidity. The rigidity also modulated mechano-chemical activity of both dynamins. Crucially, Dyn2 and, to lesser extent, Dyn1 could both discriminate moderate differences of the rigidity between biomimetic membrane compositions, implying lipid mechanics as a major regulator of dynamin fission machinery.

#57

Sorting Nexin 3 mutation impairs development and neuronal function in *Caenorhabditis elegans*

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The Sorting Nexins family of proteins (SNXs) plays pleiotropic functions in protein trafficking and intracellular signaling, and has been associated with several disorders, namely Alzheimer's disease and Down's syndrome. Despite the growing association of SNXs with neurodegeneration, not much is known about their function in the nervous system. The aim of this work was to use the nematode *Caenorhabditis elegans* that encodes in its genome eight SNXs orthologs, to dissect the role of distinct SNXs, particularly in the nervous system. By screening the *C. elegans* SNXs deletion mutants for morphological, developmental and behavioral alterations, we show here that *snx-3* gene mutation leads to an array of developmental defects, such as delayed hatching, decreased brood size and life span, and reduced body length. Additionally, Δ *snx-3* worms present increased susceptibility to osmotic, thermo and oxidative stress and distinct behavioral deficits, namely, a chemotaxis defect which is independent of the described *snx-3* role in Wnt secretion. Δ *snx-3* animals also display abnormal GABAergic neuronal architecture and wiring, and altered AIY interneuron structure. Pan-neuronal expression of *C. elegans snx-3* cDNA in the Δ *snx-3* mutant is able to rescue its locomotion defects, as well as its chemotaxis toward isoamyl alcohol. Altogether, the present work provides the first in vivo evidence of the SNX-3 role in the nervous system.

#58

Tight distribution of synaptic vesicle release sites generated by Unc13 limits and synchronizes neurotransmission

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Information processing in our brains depends on the exact timing of synaptic transmission which requires precisely controlled Ca²⁺-activated exocytosis of synaptic vesicles (SVs) from unique release sites within active zones (AZs). While current evidence for release sites comes primarily from statistical analyses, molecules generating and positioning them remained elusive. Here, we identified a function of the (M)Unc13 family member Unc13A in both. By studying the local distribution and stability of the essential release factors Unc18, syntaxin-1 and Unc13A with super-resolution microscopy and intravital imaging of *Drosophila* neuromuscular junction synapses, we found that Unc13A was the only protein positioned with nanometer precision within defined sub-AZ domains where it was stable for hours. In contrast to this, syntaxin-1 and Unc18 were broadly distributed and showed high motility (few minutes). The N-terminal portion of Unc13A functioned in stable sub-AZ positioning, but could not generate release sites. Instead its competition with endogenous Unc13A blocked release sites. In contrast, the C-terminal half of Unc13A showed promiscuous, unstable localization, leading to the generation of excessive release sites atypically close and far from Ca²⁺-channels. Unspecific release site positioning resulted in reduced synaptic transmission, excessive facilitation and loss of temporal precision. Thus, Unc13A functions to generate stable release sites at well-defined distances to Ca²⁺ channels which is required to both promote efficient transmission and to prevent temporally imprecise exocytosis from ectopic AZ locations.

#59

Protein interaction networks in synaptic vesicles

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Synaptic vesicles are small storage organelles for neurotransmitters. They are densely packed with proteins and pass through a trafficking cycle in the nerve terminal. This includes neurotransmitter import, docking, priming and fusion with the presynaptic membrane, neurotransmitter release into the synaptic cleft as well as vesicles recycling. These processes are governed by non-covalent protein interactions that assemble on demand and dissociate when the task is completed. Although the major protein components of synaptic vesicles are identified we have only little knowledge about their interactions. Available models assume random distribution of the proteins in the membrane, however, there is strong evidence that they form functionally active assemblies. We set out to unravel these protein assemblies by employing various cross-linking strategies followed by mass spectrometric analysis.

For this, synaptic vesicles were purified from rat brain and major proteins were identified by LC-MS/MS and database searching. Proteins within intact vesicles were then cross-linked using chemical cross-linkers with different spacer length and targeting different functional groups of various amino acids (lysine specific and/or specific for acidic residues). Applied in this way, chemical cross-linking revealed first interaction networks in synaptic vesicles. These networks reveal many protein interactions with Synaptobrevin which plays a central role in complex formation. Local networks, for instance between Synaptophysin, Synapsin and subunit a of the V-type ATPase, were also observed. Surprisingly, only few interactions were observed with Synaptotagmin, one of the major vesicle components. These results pave the way to unravel the heterogeneous protein interaction networks in synaptic vesicles which are key to our understanding of signal transduction in the neuronal synapse.

#60

Visualizing membrane structural remodeling of fusion and fission in live cells

Ling-Gang Wu, et al.

NINDS, NIH

Fusion and fission mediate many biological processes, such as exocytosis, endocytosis, intracellular trafficking, and viral entry. Despite intense studies, membrane structural remodeling mediating fusion and fission had not been real-time observed, and is thus not well understood. With super-resolution STED microscopy, We observed the hemi-fused Ω -shaped structure in live cells, the neuroendocrine chromaffin cells and pancreatic β -cells. This structure was generated from fusion pore opening or closure (fission) at the plasma membrane. Unexpectedly, its transition to full fusion or fission was determined by competition between fusion and calcium/dynamin-dependent fission mechanisms, and was surprisingly slow (seconds to tens of seconds) in a significant fraction of the events. These results provide key missing evidence proving the hemi-fusion and hemi-fission hypothesis in live cells, and reveal the hemi-fused intermediate as a key structure controlling fusion/fission, as fusion and fission mechanisms compete to determine its transition to fusion or fission. As fusion passes through hemi-fusion, vesicle fusion at the plasma membrane generates an Ω -shape membrane profile, which may enlarge or shrink while maintaining vesicular membrane proteins. Closure of fusion-generated Ω -profiles, which produces various sizes of vesicles, is a major mechanism mediating rapid and slow endocytosis. Strong calcium influx triggers dynamin-mediated closure, whereas weak calcium influx facilitates the merging of Ω -profiles with the plasma membrane via shrinking rather than full-collapse as generally believed. Ω -profile shrinking and merging are mediated by strong membrane tension provided by dynamic assembly of filamentous actin, involving ATP hydrolysis, N-WASP and formin. We are currently attempting to resolve the fusion pore and its dynamics. In summary, we have visualized a series of membrane structural changes that mediate vesicle fusion, merging with the plasma membrane, fission, their crucial function in exo- and endocytosis, and a part of their underlying molecular mechanisms.

#61

A polybasic patch in Synaptotagmin-1's first C2 domain regulates evoked release and fusion pores

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Synaptotagmin-1 (Syt1) is the major calcium sensor for fast, synchronous neurotransmitter release. Syt1 is anchored to the synaptic vesicle through a single-pass transmembrane domain, possesses a short luminal stretch, and is composed mostly of two tandem cytosolic C2 domains (C2A and C2B) that bind calcium, SNAREs, and acidic phospholipids. C2B is thought to be functionally more important than C2A, since disruption of calcium-binding via mutagenesis has more dramatic effects for C2B than for C2A. In addition, C2B interacts with SNAREs, complexin, and acidic phospholipids, whereas such interactions have not been found or explored in detail for C2A. Yet the C2A domain is required for exocytosis, as its removal or replacement by C2B results in severe defects in neurotransmitter release. We sought to understand the role of C2A using a combination of a single-pore assay that utilizes fusion between nanodiscs and “flipped” t-SNARE cells, a high-resolution optical tweezers assay that probes binding of single proteins to lipid bilayers, and electrophysiological assessment of release from cultured cortical neurons. Using the single-pore nanodisc-cell fusion assay, we found that wild-type Syt1 C2AB domains help dilate SNARE-induced fusion pores in a calcium and PI(4,5)P₂-dependent manner. Either domain alone is ineffective in this activity. Interestingly, neutralizing a polybasic patch on C2A abolished C2AB's pore-dilation function. Using neuronal cultures from Syt1-KO mice, evoked release could be rescued with expression of wild-type Syt1, but not with Syt1 harboring the C2A polybasic patch mutation. Using the optical tweezers assay, we found that the C2A polybasic patch mutation lowered the binding energy between C2AB and PI(4,5)P₂ containing membranes by ~4 kT per molecule compared to wild-type. Thus, PI(4,5)P₂ binding by Syt1 C2A's polybasic patch is essential for both evoked release and dilation of fusion pores.

#62

Are cultured chromaffin cells the proper neuroendocrine model to study exocytosis?

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Isolated bovine chromaffin cells have been used extensively as a neuroendocrine model to study regulated exocytosis. In order to extend such experimental findings to the physiological situation, it is necessary to study mayor cellular structures affecting secretion in cultured cells and their counterparts present in the adrenomedullary tissue. Focusing on F-actin cytoskeleton, the recognized organizer for exocytotic machinery elements under plasma membrane line, we have found significant differences on network configuration between isolated and native chromaffin cells from adrenomedullary tissue. Contrasting with the cortical ring organization for cultured cells, fibers show a wide cytosolic distribution in native cells. These differences, not only affect cytoskeleton but also vesicles and mitochondria with a cytoplasmic position defined by these fibers. Amperometric assays combined with data offered by mathematical models including different organelles distribution show us the organelles role by compensatory effect on secretion rates regulation. Consequently, our results imply that we have to consider F-actin structural changes to interpret functional data obtained in cultured neuroendocrine cells and discovered differences for F-actin fibbers organization could cast down isolated chromaffin cells like the best model for neurosecretion studies.

#63

Do membranes really hate edges?

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Our work focused on proteins acting as catalysts lowering energy barriers to membrane remodeling. By combining quantitative light microscopy with electrophysiology, and reconstitution of fusion and fission in lipid bilayer membranes, we constructed hypotheses with predicted fusion intermediates whose dimensions were deduced by continuum theory and fits to experiments. We aimed to understand how proteins catalyze the new configurations of lipids that ultimately mediate these processes. By successfully using a new technology, the Volta Phase Plate in cryo-electron microscopy, we managed to visualize the predicted hemifusion diaphragm mediated by the hemagglutinin (HA) of influenza virus (IFV), and the measurements of its dimensions fit the predictions of continuum theory. But another result was unexpected: HA catalyzed the actual breakage of membranes, leading to free membrane edges – often in great profusion. To understand how this was unexpected, we must consider the physical forces that act on lipids in solution. Membranes hate edges. The lipid bilayer is self-assembling because its free energy of cohesion (that comes in part from enthalpic attractive forces between hydrocarbon chains and in part from the entropic hydrophobic effect that minimizes interfacial area) automatically ensures stability of the lipid bilayer. Formally, the edge of an otherwise lamellar membrane has a large linear tension, i.e. should be a high energy region that the membrane seeks to minimize. Nevertheless, we observe that ‘free edges’ actually outnumber hemifusion diaphragms for certain lipid compositions of target membranes. Such edges only occur in close vicinity of activated HA molecules, indicating that they are triggered to form by the same event that triggers full fusion: namely, the amphipathic helix of HA being ejected from HA and binding to the target bilayer. We propose that the HA fusion-peptide somehow stabilizes the observed membrane edges, i.e. drastically lowers bilayer line-tension.

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