3534-Pos
SNARE Complex Assembly in Retinal Bipolar Neuron Exocytosis
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Three kinetic components of exocytosis have been described in retinal bipolar neurons. They are thought to reflect the fusion of a docked pool of ribbon-tethered synaptic vesicles termed the rapid pool, the releasable pool of ribbon-tethered vesicles, and a cytoplasmic reserve pool. In neurons, assembly of SNARE proteins facilitates exocytosis. We asked whether these pools could be distinguished on the basis of SNARE complex formation. Syntaxin3B is a t-SNARE in ribbon synapses. We generated a fluorescent peptide based on the syntaxin3B SNARE binding motif from goldfish. The peptide was dialyzed into isolated synaptic terminals of goldfish retinal bipolar cells via a whole-terminal recording electrode. A scrambled peptide served as control. Exocytosis was monitored with membrane capacitance measurements. Beginning one minute after break-in, a 1s stimulation, sufficient to deplete the releasable pool, was given every 60 seconds. The first exocytotic response was not significantly altered by the syntaxin3B peptide. However, by the fourth pulse, the exocytotic response in terminals dialyzed with the syntaxin3B peptide was reduced by 89% relative to the first, whereas that with the control peptide was reduced by only 45% ($p < 0.04$). This effect was not due to a reduction in calcium influx. Next, we implemented a pulse train protocol that captures the three components of release. Control terminals showed both depletion and replenishment of the pools. Terminals dialyzed with the syntaxin3B peptide showed: 1) immediate loss of the exocytotic component attributed to the reserve pool 2) decreased refilling of the rapid and releasable pools. The results demonstrate that reserve pool vesicles are unlike those in the rapid and releasable pools. It is likely that pool refilling is inhibited by the syntaxin3B peptide because reserve vesicles establish new SNARE complexes when they join a fusion-competent vesicle pool.

3535-Pos
Role of the Synaptobrevin C-terminus in Fusion Pore Formation
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Neurotransmitter release is mediated by the SNARE proteins synaptobrevin II (sybII) also called VAMP2, syntaxin and SNAP-25 generating a force transfer to the membranes and inducing fusion pore formation. However, the molecular mechanism by which this force leads to fusion pore formation remains elusive. To determine a possible role of the sybII TM domain in fusion pore formation, sybII constructs in which one or two polar or non-polar residues were added at the C-terminus of the protein were expressed in double knock-out (DKO) embryonic mouse chromaffin cells deficient in sybII and cellubrevin1. Exocytosis was stimulated by flash photolysis of caged-calcium (NP-EGTA), and the capability of the mutated constructs to support exocytosis was monitored by whole-cell patch clamp capacitance measurements while the associated transmitter release was monitored by carbon fiber amperometry. We found that the ability of sybII to support exocytosis is inhibited by addition of one or two residues to the sybII C-terminus depending on their energy of transfer from water to the membrane interface2, following a Boltzmann distribution. These results suggest that C-terminal zipping of the SNARE complex pulls the C-terminus of sybII deeper into the vesicle membrane, with this movement disrupting the vesicular membrane continuity and leading to fusion pore formation. In contrast to current models, fusion thus begins with molecular rearrangements at the intravesicular membrane leaflet and not between the apposed endoplasmic leaflets.
1 M. Borisovska, Y. Zhao, Y. Tsytsyura et al., Embo J 24 (12), 2114 (2005).
2 W. C. Wimley and S. H. White, Nat Struct Biol 3 (10), 842 (1996).
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3536-Pos
Effects of Rab3 and Rab27 on Exocytotic Activity and Docking in Mouse Chromaffin Cells
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The small GTPases Rab3A and Rab27A are endogenously expressed and specifically localized to dense-core vesicles in PC12 cells and are implicated in the late steps of dense-core vesicle exocytosis. What remains unknown is the individual and collective impact of these proteins on the size of the releasable pools and the rate at which vesicles are primed into these pools. Chromaffin cells isolated from Rab3A-/-, Rab27-/-, and double knock-out mice were used to examine the density of docked vesicles, granule mobility at the membrane, and exocytotic activity. Docked granules were visualized using electron micrographs and quantified based on their placement with respect to the plasma membrane. Mobility of granules was examined using total internal reflection fluorescence (TIRF) microscopy with virus-infected chromaffin cells expressing NPY-cherry. Changes in membrane capacitance were recorded from chromaffin cells in adrenal slices using patch clamp electrophysiology, providing data reflecting fusion kinetics and the refilling of releasable pools. The present study has identified the contributions of Rab3 and Rab27 to release readiness of dense-core vesicles in primary mouse chromaffin cells.

3537-Pos
Link between Structure and Cooperativity in the C2a Domains of Synaptotagmin
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Synaptotagmin has recently been shown to be a major Ca+2 sensor in exocytosis; however, the role of Ca+2 binding in the process is poorly described beyond the location of the binding sites. To address this, we have studied the most highly conserved sequences in the C2 family by mutagenesis, X-ray crystallography and biophysical characterization. We have shown that a single, conservative point mutation in this highly region of the C2A domain of Synaptotagmin 1 is capable of altering the cooperativity between the C2 domain and Ca+2. We conclude that this locus is a unique characteristic of the C2 domain and is involved in discriminating between the activities of C2A vs. C2B in the synaptotagmin molecule. We show that static loop motion in the Ca+2 binding pocket can be an important regulatory feature of the synaptotagmin molecule.

3538-Pos
Quantitative Analysis of Domain Formation after Snare Mediated Fusion of Synaptic Vesicles
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In synapses, synaptic vesicles are recycled shortly after fusion with the plasma membrane by endocytosis. If membrane components diffused freely away from the fusion site before this endocytosis, a complete reassembly of these synaptic vesicle membrane components (proteins and lipids) would be necessary for synaptic vesicle recycling. Therefore, this recycling would be highly facilitated in the case that complete mixing of synaptic vesicles with the plasma membrane did not occur, but rather if the synaptic vesicle components remained clustered in a patch on the plasma membrane. To test this hypothesis, we applied Förster resonance energy transfer (FRET) to study the fusion of purified synaptic vesicles with artificial membranes containing the SNAREs syntaxin 1 and SNAP-25 and a lipid composition that mimicked the plasma membrane. We compared the extent of mixing for fluorescently labeled phospholipids (with a varying degree of acyl-chain saturation) upon fusion. We found that phospholipids with saturated acyl-chains mixed with vesicular membrane to a higher extent. Full mixing was not achieved for phospholipids with unsaturated acyl-chains. This indicates that stable membrane domains of different compositions might be present after fusion.

3539-Pos
Primed Vesicles of the Readily Releasable Pool Appear to be in a Hemifused State
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The fusion of secretory vesicles with the plasma membrane occurs in three biochemically distinct steps: initial tethering and docking of the vesicle with the membrane, priming of the fusion machinery, and merging of the lipid bilayers. All three processes revolve around the regulated assembly of low-energy complexes formed by the coil-coiling of three members of the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) protein family. According to the hypothesis of distal to proximal (N- to C-terminal) ''zipping'' of SNAREs during exocytosis, priming of vesicles was suggested to correspond to only partial N-terminal SNARE complex assembly. Here we describe evidence that SNAREs might prime vesicles by hemifusing them with the plasma membrane.

In mouse chromaffin cells expressing green synaptopHluorin in the granules, cell membranes were stained from the outside with red lipophilic dyes that rapidly redistribute by flip-flop between both leaflets. Fusion of secretory granules was monitored by evanescent wave microscopy. Exocytosis was triggered by superfusion with high K+ solution, and double images were taken at 491 and 560 nm excitation, respectively. Fluorescence signals of the membrane probes recorded in the red channel were spatially and temporally aligned with respect to fusion events in the green channel to yield average movies with high signal-to-noise ratio. We found the membrane fluorescent signals to be slightly increased in diffraction-limited spots at locations of docked granules for up to a second prior to fusion. The fluorescent signals, however, rapidly decreased to background levels upon fusion of the granules at the respective sites, with the fluorescence dissipating from the center to the periphery.
Our results are best explained by mixing of lipids prior to fusion in a hemifused state.

3540-Pos
Towards Real Time Analysis in Photoactivation Localization Microscopy
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We investigated a new fast and precise algorithm for analysis of Photoactivation Localization Microscopy (PALM) images, based on real-time template matching used e.g. in face recognition or astrophysics. For generation of the template we measured the Point Spread Function (PSF) with high precision for the experimental setup in use and modelled it by a 2D Gaussian. This Gaussian is now used as template, and the problem of image registration is solved by a fast Sum Square Difference (SSD) Algorithm [1].
To gain subpixel resolution we used a newer class of algorithms [2]. By resampling the template and using an error correction method a maximum error of 10% of the pixel period is expected. The performance is tested in comparison to established algorithms in PALM with regard to computation time and accuracy.
By combining PALM with Total Internal Reflection Microscopy (TIRF) we were able to localize single molecules involved in secretion in fixed and live neurosecretory cells. The fusion of secretory vesicles with the membrane is mediated by the assembly of low-energy complexes formed by the coil-coiling of three members of the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) protein family: SNAP-25, syntaxin, and synaptobrevin 2 (syb2). However it is controversially debated how many SNARE complexes are minimally needed for SV priming and fusion. To this end we expressed syb2 fused to Dronpa or Dendra2 in PC12 cells. With the help of new algorithms real-time localization and counting of syb2 with sampling rates up to 10Hz was possible.
Thus, template matching is a promising strategy towards real-time analysis in FPALM, whenever direct evaluation of recorded images is needed for proper object selection like in live cell imaging.
[1] Barnea u. Silverman, 1972.
[2] Frischholz u. Spinnler, 1993.

3541-Pos
Syntaxin1a Dispersion and Assessment of *cis*-Snare-Complex Formation-Disassembly during Synaptic Transmission in Hippocampal Neurons
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Soluble-N-ethylmaleimide-sensitive-factor-attachment-protein-receptor(SNARE)-complex formation and dissociation are biochemical prerequisites of synaptic transmission that occur during calcium-triggered synaptic vesicle (SV) fusion and subsequent recycling, which prepares cellular machinery for the following responses to action potentials (AP). The exact sequence of molecular events remains unknown, and it's a technological challenge to demonstrate SNARE-protein interactions *in-situ*, during AP-induced synaptic transmission. In this study, C-termini-labeled fluorescent constructs of Syntaxin1a-Cerulean and VAMP2-Citrine were transiently expressed either alone or together in cultured hippocampal neurons. A Syn1a-Cer-probe revealed a more uniform distribution of fluorescence in the plasma-membrane, with slightly higher intensity in synaptic boutons, while VAMP2-Cit-fluorescence was predominantly in boutons, suggesting that trafficking of heterologously-expressed constructs is similar to endogenous SNAREs. Efficiently transmitting boutons were identified by FM4-64-destaining during electrical-stimulation. These boutons revealed both faster transient increase of VAMP2-Cit-fluorescence (synaptopHluorine-effect), and a slower decrease of Syn1a-Cer-fluorescence. The latter effect started with a 3-6 s delay after beginning 30 Hz-stimulation, and developed as stimulation continued for 10-30 s. The largest reduction for averaged data was seen 5-10 s after stimulation completed, and recovery occurred in the following 20-40s. The decrease of Cer-fluorescence that occurred in regions of intensive FM-destaining was often accompanied by a transient increase of fluorescence in adjacent areas. In transmitting boutons co-expressing both Syn1a-Cer and VAMP2-Cit probes, the reduction was slightly stronger (up to 3-4%, although not significant yet, $P<0.15$) compared to the Syn1a-Cer alone (1-2%), even though the latter boutons showed on average larger FM-destaining. The decrease remains if measured in larger areas that presumably include dispersed probe. We hypothesize that a fraction of the Cer-fluorescence reduction can be attributed to FRET between Syn1a-Cer probe as a fluorescence-donor and VAMP2-Cit as an acceptor, which come in close proximity upon cis-SNARE-complex formation after SV-fusion.

3542-Pos
Synaptotagmin-1 and Complexin-2 Work Cooperatively to Promote Exocytosis from Adrenal Chromaffin Cells
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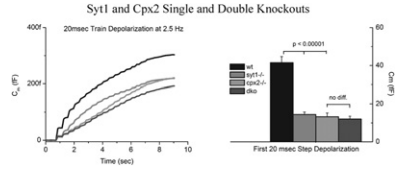
Neurons create secretory vesicles and sites for exocytosis that together promote rapid formation of exocytotic pores in response to fluctuations in $[Ca^{2+}]_i$. The SNARE complex is perfectly positioned to influence this process, and it provides points of entry for accessory SNARE proteins Synaptotagmin1 and Complexins. Recent biochemical studies have proposed that a Syt1-Cpx interaction facilitates membrane fusion, yet earlier studies proposed a competitive relationship. No physiological study has directly addressed this, so here we measured release from chromaffin cells derived from Syt1 and Cpx2 single and double knockout animals. Trains of step depolarizations varying in length were given to probe the number of vesicles in the rapid and sustained release phases. The change in δC_m after 20 and 100msec steps was reduced by ~70% with loss of a single gene, but deletion of Syt1 on the cpx2-/- background (dko) was without consequence (see Figure). Syt1-/- cells showed the greatest recovery in release with more time and Ca^{2+}. Finally, amperometric spike properties were altered. In total, Syt1 and Cpx cooperate in a positive fashion to increase the probability of release and the size of single events.



3543-Pos
Complexin and Synaptotagmin in Synaptic Function and Plasticity
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Neurotransmitter-release is a specialized form of vesicle fusion that shares a common SNARE-mediated fusion mechanism with other vesicle trafficking pathways within cells. With the emergence of multicellular organisms and just before of the formation of the primitive nervous system, two principal families of SNARE complex-binding proteins emerged, synaptotagmins (Syt) and complexins (Cpx). Current data suggest that SNARE binding by the two proteins allows Syt-1 to promote fusion in a calcium dependent manner, while Cpx prevents premature fusion in the absence of calcium influx. However, their precise roles in regulated secretion and effects on short-term synaptic plasticity (STP) is poorly understood. Here, we present a thorough analysis of synaptic transmission at NMJs of mutant and overexpression animals using recordings of evoked and spontaneous EPSCs using voltage-clamp at the *Drosophila* NMJ. Kinetic analysis of eEPSCs reveals that synchronous and asynchronous release critically depends on the level of Cpx and Syt expression. Moreover, sEPSCs analysis indicates that spontaneous release is tightly regulated by Syt and Cpx. Additionally, Syt and Cpx protein levels alter quantal content and STP, suggesting that these proteins play a role in the availability of vesicles for fusion. We propose that Syt minimizes the energy of the transition-state for SNARE-complex fusion, while Cpx decreases this free energy, creating an energetic barrier at a late step of vesicle fusion clamping vesicles in the immediately releasable pool. Finally, we tested the functional conservation of Cpx comparing the two *Drosophila* splice variants and the mammalian isoforms confirming their roles as a vesicle fusion clamp, but also revealing functional diversity in evoked responses.