Morphofunctional changes at the active zone during synaptic vesicle exocytosis

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Abstract

The fusion of synaptic vesicles (SVs) with the plasma membrane (PM) proceeds through intermediate steps that remain poorly resolved. Additionally, the effect of persistent high or low exocytosis activity on intermediate steps remains unknown. Through time-resolved cryo-electron tomography, we ordered events into a sequence. Following stimulation, additional SVs are rapidly primed by forming tethers with the PM. Simultaneously, fusion initiation occurs by membrane curvature ('buckling') of the SV and PM. It is followed by the formation of a fusion pore, and the collapse of SV membrane. At this time, membrane-proximal, but not membrane-distal, vesicles lose their interconnections, allowing them to move towards the PM. A SNARE mutation that arrests spontaneous release caused vesicles to reside further from the membrane while forming more tethers, whereas a mutation stimulating spontaneous fusion caused a complete loss of membrane-proximal triple-tethered SVs, and a loss of intervesicle connectors. Overall, tether formation and connector dissolution is triggered by stimulation and adjusted to the spontaneous fusion rate. These moprhological observations likely correspond to the transition of SVs from one functional pool to another.

- *Need to reformulate the part about 4E mutant (p value is not sufficient)*
- *For 4K mutant: check if the loss of tethered SVs restricted to those with more than only one, or to those with more than two tethers?*
- *Possibly mention here introduce the formation of distal connectors by stimulation*

Introduction

In the central nervous system, neurons communicate through the release of neurotransmitters at synapses. This process relies on synaptic vesicle (SV) exocytosis, i.e. the fusion of SVs with the plasma membrane (PM). This in turn is eminently important for normal brain function such as movement coordination or memory formation. SV exocytosis involves a sequence of steps [1,2]. The vesicle is first docked to the active zone (AZ) PM. Subsequently the exocytosis machinery goes through a maturation process, termed priming, after which the SV is ready to fuse. These SVs form the readily releasable pool (RRP) of SVs. Finally, a calcium influx triggers fusion of the SV with the PM. Docked SVs are defined as the SVs in very close proximity or direct contact with the PM as observed by electron microscopy (EM), whereas priming refers to SV ability to undergo exocytosis immediately upon stimulation. Whether every docked SV is also primed has been debated [1,3]. A recent high-pressure freezing/freeze-substitution EM study of genetically modified synapses has indicated that vesicles that are in direct contact with the PM, i.e. docked, are primed and belong to the RRP and that this situation occurs downstream of vesicle tethering [4]. From a molecular perspective, priming involves several proteins, including the SNARE complex (SNAP-25, syntaxin-1, and synaptobrevin-2), Munc13, Munc18, synaptotagmin-1, and complexin [2,5]. All three SNAREs form a highly stable tight four-helix bundle, known as trans-SNARE complex. The surfaces of the SV and the PM, respectively, are negatively charged and therefore tend to repulse each other. The formation of the trans-SNARE complex counteracts this repulsion and brings the SV and the PM in high proximity [6]. Evidence has suggested that the SNARE complex is only partially zipped in primed SVs [7]. Furthermore, various studies have suggested that the formation of at least three SNARE complexes provides the necessary energy for a SV to become fusion-competent [8,9,10]. Yet in the absence of cytoplasmic Ca^{2+}, minimal spontaneous exocytosis takes place. When the presynaptic terminal gets depolarized by an action potential, Ca^{2+} flows in the cytoplasm and binds to synaptotagmin-1, which is localized at the SV surface. Upon Ca^{2+} binding, synaptotagmin-1 was proposed to insert between the head groups of the PM anionic phospholipids and trigger membrane curvature and destabilization, leading first to hemifusion and subsequently to fusion[11]. Interestingly, the trans-SNARE bundle surface is negatively charged, which contributes to the electrostatic barrier that minimizes spontaneous fusion and allows synaptotagmin-1 to act as an electrostatic switch that triggers exocytosis [12]. Introducing

negatively charged side chains by site-directed mutagenesis reduces the rate of spontaneous and evoked exocytosis whereas introducing more positive side chains enhances the rate of spontaneous exocytosis and depletes the RRP.

Cryo-electron tomography (cryo-ET), which preserves samples to atomic resolution, revealed that under resting conditions, no SV is in direct contact with the PM and the majority of AZ-proximal SVs are connected to the PM by a variable number of short tethers [13,14]. The observed gap between the SV and the PM is consistent with the model of an electrostatic barrier formed by the negative charges of the SV, the PM, and the trans-SNARE bundle [12]. In synaptosomes treated with hypertonic sucrose solution, which depletes the RRP, the majority of tethered vesicles had 1 or 2 tethers [13,15,16]. This observation suggested that the RRP consists of SV that are linked to the PM by 3 or more tethers. The RRP, as identified by morphological criteria, only represents a minority of AZ-proximal vesicles. This is in agreement with previous reports. In one of them the term pre-primed pool was used for the few vesicles (~1 vesicle at hippocampal synapses) that are rapidly released and another publication showed that the immediately releasable pool is made up of only 10-20% of the vesicles located on the AZ (equal to ~1 vesicle on hippocampal synapses) [17,18]. The ensemble of proximal vesicles that are not in the RRP have been termed non-RRP and presumably belong to the recycling pool that releases more slowly [13,19]. Farther away from the AZ, partially intermixed with the recycling pool, is the reserve pool containing vesicles that only release upon high frequency stimulation. Vesicles in the reserve pool are tightly clustered and well inter-connected by structures that were termed connectors [13,19]. It should be noted that the molecular nature of connectors is not known and is possibly heterogenous. Synapsin has been proposed as a molecular constituent but since the deletion of all forms of synapsin does not lead to the complete absence of connectors, it is clear that not all connectors contain synapsin [20,21]. The second row of SVs near the active zone, immediately after the proximal vesicles, is called the intermediate region. Resting state intermediate SVs are less densely packed and also less connected than proximal SVs [14]. This suggests that, after exocytosis of RRP SVs, intermediate SVs could be rapidly recruited in the RRP by diffusion [22]. Synaptic activity enhances the mobility of a fraction of SVs, whereas it induces synapsin dissociation from SVs in a synapsin phosphorylation-dependent manner [23,24]. The same mobility enhancement can be achieved through inhibition of synapsin dephosphorylation, which leads to synapsin dissociation from SVs, or by knocking out all three synapsin forms [25,26,27]. Interestingly, ribbon synapses do not express synapsin and show higher SV mobility than conventional synapses [28]. It is therefore conceivable that inter-SV connectors restrain SV diffusion and that synaptic activity influences the level of inter-SV connectivity and thereby their mobility.

To investigate this hypothesis and to better understand the impact of depolarization and synaptic activity on SV tethering, we designed two sets of cyro-ET experiments. On the one hand, we compared the morphology of wild-type rat synaptosome in resting state and a few milliseconds after depolarization. On the other hand, we imaged autapses in mice neuronal culture expressing either wild-type SNAP-25, a more positively charged SNAP-25 mutant, or a more negatively charged mutant of SNAP-25. The more positive SNAP-25 mutant, which is consitutively active and whose RRP is permantly depleted showed no triple-tethered SV, which confirmed the morphological definition of the RRP. Our experiments revealed that immediately after depolarization additional SVs are recruited to the RRP. Shortly after exocytosis the level of inter-SV connectivity was decreased among SVs situated in a 25 to 75-nm distance range from the AZ PM. Altogether, our results indicate that connectors regulate SV mobility and their recruitment at the AZ PM.

Say something about the size of the RRP Do we want to talk about kiss-and-run? I have the feeling that we better not to keep the message nice and clear

Results

Discussion

Materials and methods

Synaptosome

Synaptosome preparation

Synaptosomes were prepared as previously described [29], with some modifications. Adult male or female Wistar rats at an age of 6-8 weeks were slightly stunned by CO2 and quickly decapitated with a guillotine. Animals were obtained from the central animal facilities of the department of clinical research of the University of Bern. The procedures used were in accordance with the Swiss Veterinary Law guidelines. The cerebral cortex and the hippocampi were removed in sucrose buffer (SEH: 0.32 M sucrose, 1 mM EDTA, 10 mM HEPES; HEPES, #H4034, Sigma-Aldrich Corporate Offices. St. Louis, MO, USA) on ice. Homogenization of the tissue was done in SEH with a Potter-Elvehjem grinder (#358011, Wheaton. Millville, New Jersey, USA), four strokes at the bottom and 6 from top to bottom were applied to the tissue at a speed of 800 turns/min as described in [29]. The whole process from decapitation to homogenization was done within 2-3 min, to obtain functional synaptosomes. Homogenized tissue was then centrifuged at 1000 g for 10 min at 4°C to remove meninges and blood vessels. The resulting supernatant containing synaptosomes, but also gliosomes and mitochondria was then added to a discontinuous, isoosmotic Percoll (#P1644, Sigma) gradient with 5%, 10% and 23% in 0.32 M sucrose, 1 mM EDTA in centrifuge tubes (#344060, Beckman Coulter). The samples were spun in an ultracentrifuge (rotor: SW 40 Ti; Beckman Coulter. Nyon, Switzerland) at 16400 rpm for 12 min at 4°C. The layer with the highest amount of functional synaptosomes was between 10-23 % [29]. The layer was carefully taken out and diluted 1:10 in HBM (140 mM NaCl, 5 mM KCl, 5 mM NaHCO3, 1.2 mM Na2HPO4, 1 mM MgCl2, 10 mM Glucose, 20 mM HEPES). The obtained solution was further spun with an ultracentrifuge (rotor 45 Ti; Beckman Coulter) at 11200 rpm for 20 min at 4°C. The pellet was carefully and quickly aspirated with a Pasteur pipette to avoid mixture with the solution and then diluted in HBM.

Plunge freezing and spray-mixing (synaptosomes)

The following steps from incubation to plunge freezing were all done at room temperature (RT), equivalent to 23-25°C. The synaptosomal solution was incubated with calcein blue AM (#C1429, Molecular Probes-Thermo Fisher Scientific. Waltham, MA, USA) 30 min prior to plunge freezing to visualize the cytosol of functional – esterase containing – cellular compartments such as synaptosomes. Additionally, 1.3 mM CaCl2 and 10 nm gold fiducials were added (gold fiducials, #s10110/8. AURION Immuno Gold Reagents & Accessories. Wageningen, The Netherlands). CaCl2 is necessary to trigger exocytosis and gold fiducials are important to align the acquired tilt series for tomogram reconstruction. The sprayed solution contained 1 mM CaCl2 and 52 mM KCl in HBM to depolarize synaptosomes and trigger exocytosis. It also contained fluorescein (#46955, Sigma) to trace the spray droplets on the EM grid in cryo-FM. The synaptosomal solution was applied to a 200-mesh lacey finder carbon film grid (#AGS166-H2. Agar Scientific. Elektron Technology UK Ltd. Stansted, UK). Excess liquid on the grid was removed by blotting with a filter paper and the grid was immediately plunge frozen in liquid ethane with a homebuilt plunge freezer and was sprayed on the fly. The plunge freezer and the spraying device (atomizer) were computer controlled with a LabView script (National Instruments Corporation. Mopac Expwy Austin, TX, USA). The spraying device was set similarly to the device in [**doi:10.1016/0304-3991?**]. Nitrogen gas pressure necessary to drive spraying was set to 2.5 bar. The grid was set to pass in front of the spray nozzle at a distance of 3-4 mm. The plunge freezer was accelerated to 0.75 m/s and the minimum spray delay was ~7 ms (Table S3). The atomizer sprays scattered droplets of various size on the EM grid. During the time lapse between spraying and

freezing the content of the droplets spreads by diffusion. KCl diffuses approximately 4x faster than fluorescein. Cryo-ET imaging was done within the diffusion distance of KCl but outside of the visible spray droplet because the center of the spray droplet would usually be too thick for imaging. This reduces the effective stimulation duration to anything between 0 ms and less than the given spray-freeze delay. Moreover, through diffusion, KCl concentration rapidly rises and then decreases. Hence synaptosomes are not permanently depolarized.

Cryo-fluorescence microscopy (synaptosomes)

After plunge freezing, the sample was imaged at the fluorescent microscope under cryo conditions, with a Zeiss Axio Scope.A1, equipped with an AxioCam MRm camera (Carl Zeiss AG. Germany), and a fluorescence lamp (HXP 120 C). The correlative microscopy stage (#CMS196. Linkam Scientific Instruments. UK) was cooled down to -190°C by liquid nitrogen and the frozen EM grid was placed into the chamber of the cryostage on a bridge that was not submerged in liquid nitrogen and was close to the objective, where the temperature was around -150°C. The filter set used for imaging fluorescein was #38 (#000000-1031-346, Zeiss) (BP 470/40, FT 495, BP 525/50; corresponds to GFP) and the one for calcein blue AM was #49 (#488049-9901-000, Zeiss) (G 365, FT 395, BP 445/50; corresponds to DAPI). The objective used was either a 10x (#420941-9911, NA = 0.25 Ph1, Zeiss) or a 50x (#422472-9900, NA = 0.55 Dic, Zeiss), the acquisition software used was AxioVision (AxioVs40x64 V 4.8.3.0, Zeiss) and the processing software was ZEN lite (Zeiss).

Cryo-electron microscopy (synaptosomes)

Following cryo-FM, the grid was mounted in a cryo-holder (Gatan, Pleasonton, CA, USA) and transferred to a Tecnai F20 (FEI, Eindhoven, The Netherlands) which was set to low dose conditions, operated at 200 kV, and equipped with a field emission gun. Images were recorded with a 2k x 2k CCD camera (Gatan) mounted after a GIF Tridiem post-column filter (Gatan) operated in zero-loss mode. The sample was kept at about -180°C. Tilt series were acquired using SerialEM [30] for automated acquisition recorded typically from -50° to 50° with a 2° angular increment and an unbinned pixel size of 0.75 or 1.2 nm. Due to sample thickness (400-700 nm), tomograms were usually not recorded with higher tilt angles. Defocus was set between -8 to -12 µm and the total electron dose used was about 80-100 e-/Å2. Some tomograms were acquired at the Titan Krios, equipped with a K2 direct electron detector (Gatan) without energy filter. The K2 camera was operated in superresolution counting mode and between 8-40 frames per tilt angle were taken. Tilt series were acquired using the Latitude software (Gatan) for automated acquisition recorded typically from -60° to 60° with a 2° angular increment and an unbinned pixel size of 0.6 nm. Defocus was set between -8 to -12 µm and the total electron dose used was about 80-100 e-/Å2. Prior to image processing the frames at each tilt angle, frames were aligned and averaged in 2dx MC_Automator [31] with motioncor [32]. 3D reconstruction was done in IMOD [33]. The alignments were done using the automated fiducial tracking function and the 3D reconstructions were done using the weighted back projection followed by a nonlinear anisotropic diffusion (NAD) filtering. Following tomogram reconstruction only synaptosomes that fulfilled the following criteria were used: 1) even and non-broken PM, 2) synaptic cleft still attached to the presynapse, 3) spherical vesicles, and 4) a mitochondrion in the presynapse necessary to cover the energy demands of the synapse. These criteria indicate that the synaptosome is functional [**doi:10.1016/0006-8993?**].

Manual and automatic segmentation procedures

Manual segmentation of SVs, mitochondria, and the active zone PM was done in IMOD (Figure S4A&B). The boundary marked the region to be analyzed by Pyto [34]. The analysis by Pyto was essentially the same as described previously [13] [34]. In short, the segmented area is divided in 1 voxel thick layers parallel to the active zone for distance calculations. A hierarchical connectivity segmentation detects

densities interconnecting vesicles (so called connectors) and densities connecting vesicles to the active zone PM (so called tethers) (Figure S4C). Distance calculations are done with the center of the vesicle. Mainly default settings were used. The segmentation procedure is conservative and tends to miss some tethers and connectors because of noise. Consequently, the numbers of tethers and connectors should not be considered as absolute values but rather to compare experimental groups. All tomograms analyzed by Pyto were obtained on the same microscope with the same tilt range. The margin of error for false negatives and positives was found to be less than 10% by comparison with ground truth [34]. As it was done before, an upper limit was set between 2100 and 3200 nm3 on segment volume. The tomograms that were used for this analysis were binned by a factor of 2 to 3, resulting in voxel sizes between 2.1 and 2.4 nm. Tether and connector length were calculated using the midpoint method [34]. From the stimulated synaptosomes only those that showed visible signs of exocytosis where used for analysis in Pyto.

Data analysis

If not stated otherwise data in the text are described as mean ± standard error to the mean (SEM). Wherever possible, data were presented as box plots with the following settings: orange bar, median; box extremities, lower and upper quartiles; whiskers extend up to 1.5 x interquartile range; dots, outliers. We used the same statistical tests as in [13] [35]. For normal distributed data, the Student's t-test was used. For data deviating from the normal distribution, the Kruskal-Wallis (K-W) test was used. In addition, for data that required to be split into discrete bins (e.g. fraction of connected vesicles by distance to active zone), the $\chi 2$ test was used. To calculate the correlation coefficient for paired samples (such as vesicle distance to active zone and number of tethers), Spearman's rank correlation was used (ρ-test). The confidence values were calculated using two-tailed tests and were indicated in the graphs by *, P<0.05;* ***, P<0.01;*** , P<0.001. We did not apply statistical methods to predetermine sample size but similar sample sizes as previously reported have been used (7,8). It was not necessary to apply randomization.

Neuron culture on EM grids

Animals

SNAP-25 KO C57/Bl6-mice: Heterozygous animals were routinely backcrossed to Bl6 to generate new heterozygotes. The strain was kept in the heterozygous condition and timed heterozygous crosses and caesarean section were used to recover knockout embryos at embryonic day 18 (E18). Pregnant females were killed by cervical dislocation; embryos of either sex were collected and killed by decapitation. Permission to keep and breed SNAP-25 and syt-1 mice was obtained from the Danish Animal Experiments Inspectorate and followed institutional guidelines as overseen by the Institutional Animal Care and Use Committee (IACUC). CD1 outbred mice stock: these were used to create astrocytic cultures. Newborns (P0-P2) of either sex were used. Pups were killed by decapitation.

Preparation of astrocytic and neuronal culture

The procedure has been published before [36]. The following changes were made. Glial cells were ready to be used after 10 days, once they were triturated and counted with a Buerker chamber, 100,000 cells/ml were plated onto untreated 12 well plates containing 10% DMEM. After 2 days, neurons were plated. 20 µl solution containing 250,000 cells/ml were plated onto the flame sterilized gold R2/2 or R2/1 EM grids as previously described in (Shahmoradian et al., 2014). Following a 30 min incubation at 37°C, the grid was transferred into the 12 well plate containing the astrocytes and medium was replaced with NB medium (Neurobasal with 2% ml B-27, 1 M HEPES, 0.26% Glutamax, 14.3 mMb-mercaptoethanol, 20000 IU Penicillin, 20 mg Streptomycin) for the E18 pups or NB-A

medium (Neurobasal-A with 2% B27, 1% Glutamax 1, 20000 IU Penicillin, 20 mg Streptomycin) for the P0-P1.

Constructs and viruses

SNAP-25B was N-terminally fused to GFP and cloned into a pLenti construct with a CMV promoter [37]. Mutations were made using the QuikChange II XL kit (Agilent). The mutations were verified by sequencing and have been published before [12]. The preparation of lentiviral particles followed standard protocols.

Cryo-electron microscopy

Grids were plunge frozen with a Vitrobot (TFS) with a blot time of 3 s and a blot force of -10, wait time and drain time were not used, humidity was set to 100% at 4°C. 4 µl undiluted 10 nm BSA gold tracer (Aurion) was added directly onto the grid prior to plunge freezing. Tomograms were acquired at the Titan Krios, equipped with a Falcon 3 direct electron detector (Thermo Fisher Scientific (TFS)) without energy filter. The Falcon camera was operated in linear mode. Tilt series were acquired using the TEM Tomography software (TFS) for automated acquisition recorded typically from -60° to 60° with a 2° angular increment and an unbinned pixel size of 0.37 nm. Defocus was set between -6 to -10 µm and the total electron dose used was about 80-100 e-/Å2. 3D reconstruction was done in IMOD [33]. The alignments were done using the automated fiducial tracking function and the 3D reconstructions were done using the weighted back projection followed by a nonlinear anisotropic diffusion (NAD) filtering. Following tomogram reconstruction all synapses with a clear synaptic cleft and attached postsynapse were used for semi-automatic segmentation and analysis of tethers and connectors.

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