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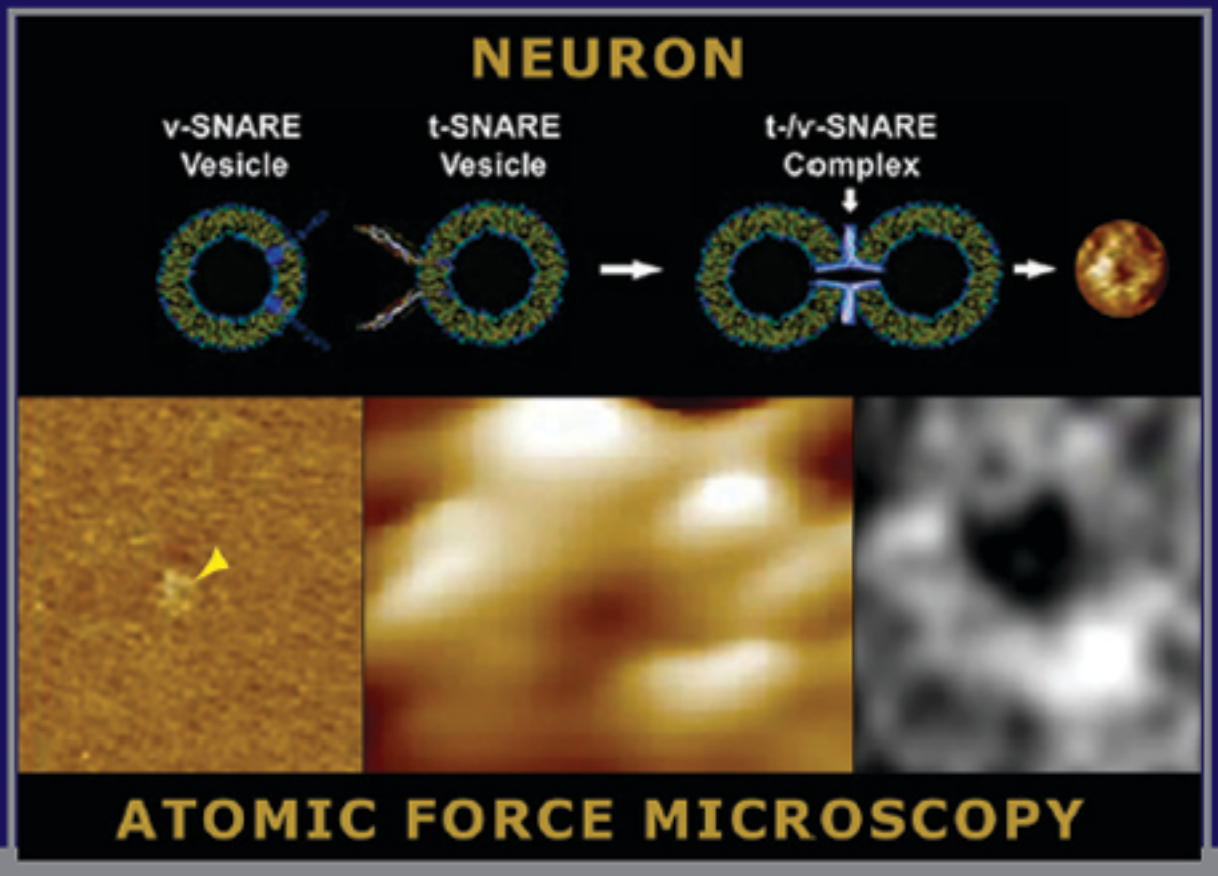

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Journal of **CELLULAR** and **MOLECULAR MEDICINE**

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Cover: Interaction between 50 nm in diameter t- and v-SNARE reconstituted liposomes, result in the formation of 6 nm in diameter t-/v-SNARE ring complexes. AFM micrograph of the complex (yellow arrowhead) at low magnification (left lower panel), and at high resolution (lower center panel), and an EM image (lower right panel). **See: B. Jena *et al.***, *J Cell Mol Med.* vol. 13 issue 10 pages [4169–4173].

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Structure of membrane-associated neuronal SNARE complex: implication in neurotransmitter release

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Abstract

To enable fusion between biological membranes, t-SNAREs and v-SNARE present in opposing bilayers, interact and assemble in a circular configuration forming ring-complexes, which establish continuity between the opposing membranes, in presence of calcium ions. The size of a t-/v-SNARE ring complex is dictated by the curvature of the opposing membrane. Hence smaller vesicles form small SNARE-ring complexes, as opposed to large vesicles. Neuronal communication depends on the fusion of 40–50 nm in diameter membrane-bound synaptic vesicles containing neurotransmitters at the nerve terminal. At the presynaptic membrane, 12–17 nm in diameter cup-shaped neuronal porosomes are present where synaptic vesicles transiently dock and fuse. Studies demonstrate the presence of SNAREs at the porosome base. Atomic force microscopy (AFM), electron microscopy (EM), and electron density measurement studies demonstrate that at the porosome base, where synaptic vesicles dock and transiently fuse, proteins, possibly comprised of t-SNAREs, are found assembled in a ring conformation. To further determine the structure and arrangement of the neuronal t-/v-SNARE complex, 50 nm t-and v-SNARE proteoliposomes were mixed, allowing t-SNARE-vesicles to interact with v-SNARE vesicles, followed by detergent solubilization and imaging of the resultant t-/v-SNARE complexes formed using both AFM and EM. Our results demonstrate formation of 6–7 nm membrane-directed self-assembled t-/v-SNARE ring complexes, similar to, but twice as large as the ring structures present at the base of neuronal porosomes. The smaller SNARE ring at the porosome base may reflect the 3–4 nm base diameter, where 40–50 nm in diameter v-SNARE-associated synaptic vesicle transiently dock and fuse to release neurotransmitters.

Keywords: SNARE complex • membrane fusion • neurotransmission

Introduction

A general understanding of membrane fusion in cells has been made possible following discovery of an *N*-ethylmaleimide-sensitive factor (NSF) [1] and SNARE proteins [2–4], and the mechanism of their participation [5–11]. Target membrane proteins at the cell plasma membrane SNAP-25 and syntaxin termed t-SNAREs, and secretory vesicle-associated membrane protein VAMP or v-SNARE, are part of the conserved protein complex involved in fusion of opposing cellular membranes. VAMP and syntaxin are both integral membrane proteins, whereas the soluble SNAP-25 protein associates with syntaxin. Therefore, the key to our understanding of SNARE-induced membrane fusion requires determination of the atomic arrangement and interaction between membrane-associated v- and t-SNAREs. Ideally, the atomic coordinates of membrane-associated SNARE complex using x-ray crystallography would help elucidate the chemistry of SNARE-induced membrane fusion in cells. So far such structural details at the atomic level of membrane-associated t-/v-SNARE complex have not been realized, primarily due to solubility problems of membrane-associated SNAREs and due to the fact that v-SNARE and t-SNAREs need to reside in opposing membranes when they meet to be able to assemble in a physiologically relevant conformation [6, 8, 9]. The remaining option has been the use of nuclear magnetic resonance spectroscopy (NMR), which too has been unsuccessful due to the size of the t-/v-SNARE complex being larger than current NMR capabilities. Regardless of these setbacks, atomic force microscopy (AFM) force spectroscopy has provided, at nm

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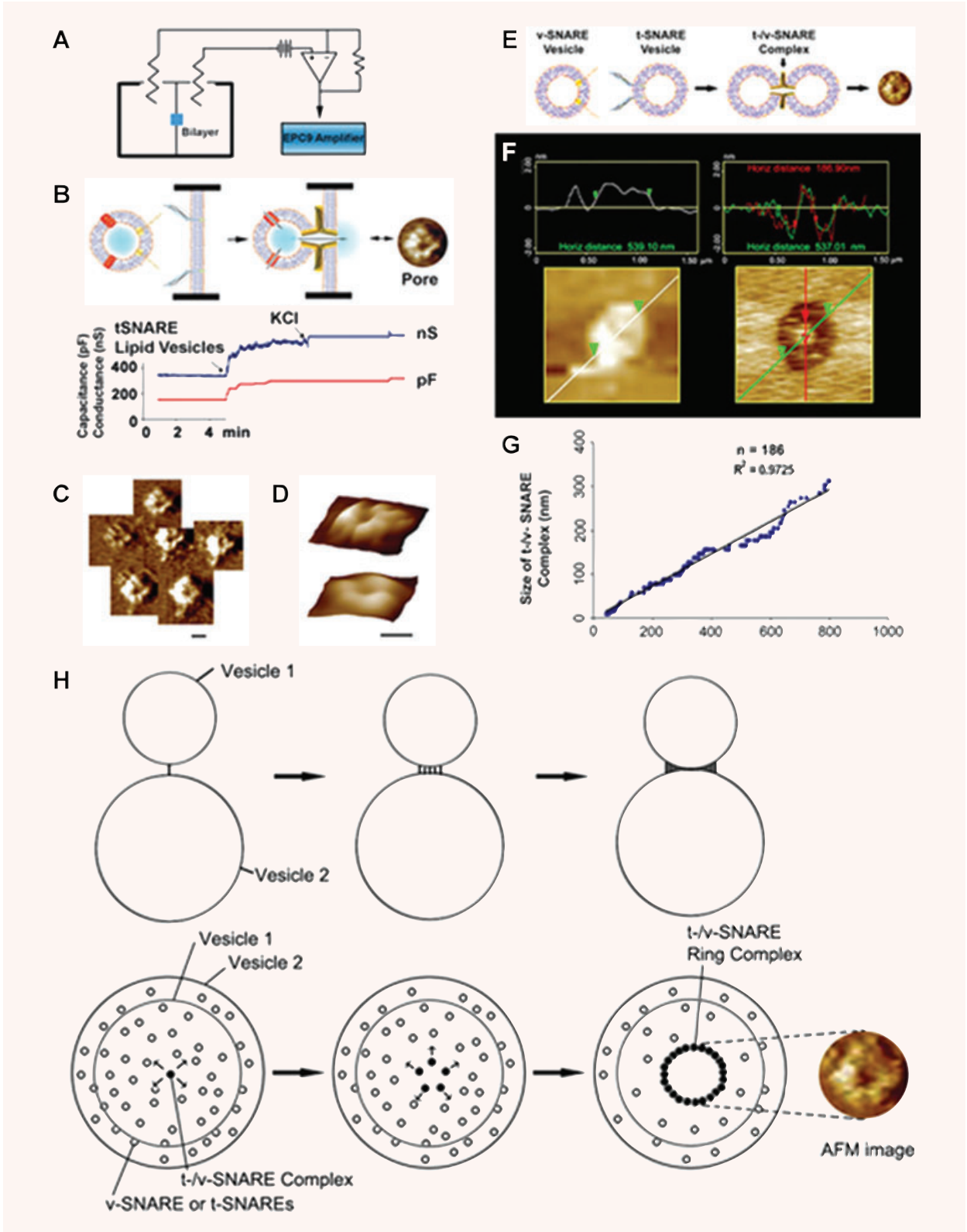

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Fig. 1 Opposing bilayers containing t- and v-SNAREs, respectively, interact in a circular array to form conducting channels in presence of calcium. (**A**) Schematic diagram of the bilayer-electrophysiology setup (EPC9). (**B**) Lipid vesicle containing nystatin channels (*red*) and membrane bilayer with SNAREs demonstrate significant changes in capacitance and conductance. When t-SNARE vesicles were added to a v-SNARE membrane support, the SNAREs in opposing bilayers arranged in a ring pattern, forming pores (as seen in the AFM micrograph on the extreme right) and there were seen stepwise increases in capacitance and conductance (–60 mV holding potential). Docking and fusion of the vesicle at the bilayer membrane open vesicle-associated nystatin channels and SNARE-induced pore formation, allowing conductance of ions from *cis* to the *trans* side of the bilayer membrane. Then further addition of KCl to induce gradient-driven fusion resulted in little or no further increase in conductance and capacitance, demonstrating that docked vesicles have already fused [6]. (**C**) t-/v-SNARE ring complex at low and high resolution (**D**) is shown. Bar = 100 nm. (**E–G**) The size of the t-/v-SNARE complex is directly proportional to the size of the SNARE-reconstituted vesicles [8]. (**E**) Schematic diagram depicting the interaction of t-SNARE-reconstituted and v-SNARE-reconstituted liposomes [8]. (**F**) AFM images of vesicle before and after their removal using the AFM cantilever tip, exposing the t-/v-SNARE-ring complex at the centre. (**G**) Note the high correlation coefficient between vesicle diameter and size of the SNARE complex [8]. (**H**) Schematic diagram depicting the possible molecular mechanism of SNARE ring complex formation, when t-SNARE-vesicles and V-SNARE-vesicles meet. The process may occur due to a progressive recruitment of t-/v-SNARE pairs as the opposing vesicles are pulled toward each other, until a complete ring is established, preventing any further recruitment of t-/v-SNARE pairs to the complex. The top panel is a side view of two vesicles (one t-SNARE-reconstituted, and the other v-SNARE reconstituted) interacting to form a single t-/v-SNARE complex, leading progressively (from left to right) to the formation of the ring complex. The lower panel is a top view of the two interacting vesicles [16].

resolution, an understanding of the structure, assembly, and disassembly of membrane-associated t-/v-SNARE complex, in physiological buffered solution [6, 8, 9, 11]. The structure and arrangement of SNARE-associated with lipid bilayer were first determined using AFM (Fig. 1) [6]. A bilayer electrophysiological setup allowed measurements of membrane conductance and capacitance during fusion of v-SNARE-reconstituted liposomes with t-SNARE-reconstituted membrane, and vice-versa. Results from these studies demonstrated that t-SNAREs and v-SNARE when present in opposing membranes interact and assemble in a circular array (ring complexes), and form conducting channels in presence of calcium [6]. The interaction between t-SNAREs and v-SNARE proteins to form such conducting channel is strictly dependent on the association of SNAREs in opposing bilayers. In the absence of membrane, either v-SNARE or t-SNAREs, fail to appropriately interact and form the ring complex, and to establish continuity between the opposing bilayers [6]. The size of the t-/v-SNARE complex is dictated by the curvature of the opposing membranes hence smaller vesicles form smaller SNARE-ring complexes (Fig. 1) [8].

Neuronal communication depends on the fusion of 40–50 nm in diameter membrane-bound synaptic vesicles containing neurotransmitters at the presynaptic membrane. In earlier studies [12, 13], 12–17 nm in diameter cup-shaped neuronal porosomes at the presynaptic membrane have been demonstrated. Neuronal porosomes are permanent structures at the presynaptic membrane, where synaptic vesicles transiently dock and fuse to release neurotransmitters. The morphology, isolation, composition, and functional reconstitution of porosomes present at the nerve terminal have previously been described [12, 13]. Earlier studies demonstrate the presence of t-SNAREs at the base of porosomes [14]. AFM, electron microscopy (EM), and electron density measurement studies [15] followed by contour mapping, and 3D topography, provide for the first time, the arrangement of proteins at nm resolution within the neuronal porosome complex. Results from this study demonstrate that proteins at the central plug of the porosome, interact with proteins at the periphery of the complex, conforming to its eight-fold symmetry. Furthermore, at the centre of the porosome complex representing the porosome base, where synaptic vesicles dock and transiently fuse, proteins, possibly comprised of t-SNAREs, were found assembled in a ring conformation. To further determine the structure and arrangement of the neuronal t-/v-SNARE complex, separate sets of 48–52 nm PC:PS vesicles were reconstituted with t-SNAREs and v-SNARE, respectively, mixed to allow t-SNARE-vesicles to interact with v-SNARE vesicles, followed by detergent solubilization and imaging of the resultant complexes formed using AFM and EM. Our results demonstrate formation of the smallest membrane-directed self-assembly of t-/v-SNARE ring complexes observed so far. Besides AFM imaging, for the first time, the t-/v-SNARE ring complex was imaged by high-resolution EM.

Materials and methods

Protein purification

N-terminal 6xHis-tag constructs for SNAP25, C-terminal 6xHis-tag constructs for Syntaxin 1A and VAMP2 were generated. All three proteins were expressed with 6xHis at full length in *E. coli (BL21DE3)* and isolated by Ni-NTA (nickel-nitrilotriacetic acid) affinity chromatography (Qiagen, Valencia, CA, USA). Protein concentration was determined by BCA assay.

Preparation of proteoliposomes

All lipids were obtained from Avanti Polar Lipids (Alabaster, AL, USA). A 5-mM lipid stock solution was prepared by mixing lipid solution in chloroform-DOPC (1,2-dioleoyl phosphatidylcholine): DOPS (1,2-dioleoyl

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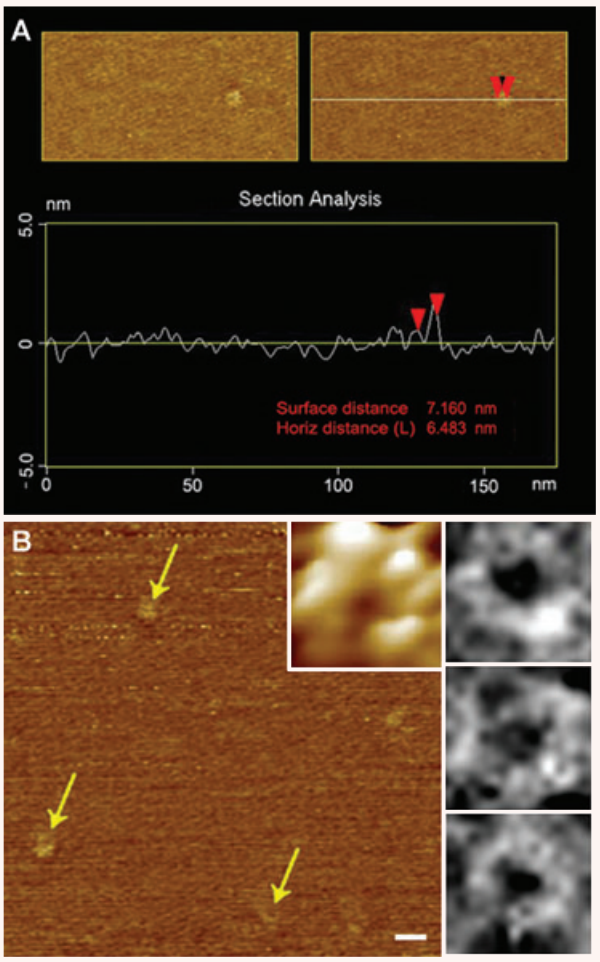

Fig. 2 Meeting of 48–52 nm in diameter proteoliposomes, one reconstituted with t-SNAREs and the other v-SNARE, results in the formation of 6–7 nm membrane-directed self-assembled t-/v-SNARE ring complexes. (**A**) Note the 6.483 nm t-/v-SNARE ring complex demonstrated in the AFM section analysis. (**B**) The left panel is a low magnification AFM micrograph of 6–7 nm t-/v-SNARE ring complexes (yellow arrows; Bar = 10 nm) and a high-resolution AFM image within inset box of side measuring 6 nm. On the right, three different high-resolution EM images of 5–6 nm t-/v-SNARE ring complexes are shown within inset boxes each side measuring 6 nm.

phosphatidylserine) in 70:30 mol/mol ratios in glass test tubes. The lipid mixture was dried under gentle stream of nitrogen and resuspended in decane. Lipids were suspended in 5 mM sodium phosphate buffer, pH 7.5, by vortexing for 5 min at room temperature. Unilamellar vesicles were formed by sonication for 2 min, followed by a 50-nm pore size extruder. Typically, vesicles ranging in size from 48–52 nm in diameter were obtained as assessed by AFM and photon correlation spectroscopy. Two sets of proteoliposomes were prepared by gently mixing either t-SNARE complex (Syntaxin-1/SNAP-25; final concentration 25 μM) or VAMP2-His_6 (final concentration 25 μM) with liposomes [10, 12], followed by three freeze/thaw cycles to enhance protein reconstitution at the vesicles membrane.

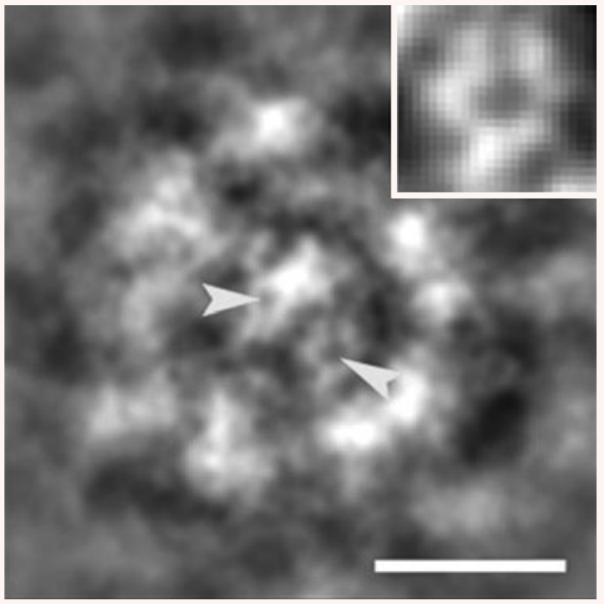

Fig. 3 Negatively stained electron micrograph of isolated neuronal porosome protein complexes. Note the 12–17-nm complexes exhibiting a circular profile and having a central plug. Approximately 8 to 10 interconnected protein densities are observed at the rim of the structure, which are connected to a central element via spoke-like structures. At the centre of the structure, which corresponds to the porosome base, there are proteins, most likely SNAREs arranged in rings (yellow arrowhead). Bar = 5 nm. [15].

Atomic force microscopy

AFM was performed on solubilized membrane-directed t-/v-SNARE complexes placed on mica surface in buffer. The complexes were imaged using the Nanoscope IIIa AFM from Digital Instruments (Santa Barbara, CA, USA). Images were obtained in the 'tapping' mode in fluid, using silicon nitride tips with a spring constant of 0.38 $N.m^{-1}$, and an imaging force of $<$200 pN. Images were obtained at line frequencies of 2 Hz, with 512 lines per image, and constant image gains. Topographical dimensions of the lipid vesicles were analyzed using the software nanoscope IIIa4.43r8, supplied by Digital Instruments.

Transmission electron microscopy

TEM was performed by a minor modification of a published procedure [15]. The solubilized membrane-directed t-/v-SNARE complexes were adhered to carbon-coated 400-mesh copper grids (Cu-400CN, Pacific Grid-Tech, San Francisco, CA, USA) previously rendered hydrophilic by glow discharge for 10 s. The grids were washed with drops of deionized water and then exposed to drops of 2% (w/v) uranyl nitrate as previously reported [15]. Images at 80,000× magnification were recorded at low defocus on 4K × 4K Gatan UltraScan CCD under low electron dose conditions using a Tacnai 20 electron microscope (Philips Electron Optics/FEI, Eindhoven, The Netherlands) operating at 200 kV.

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Results and discussion

Two sets of 48–52 nm PC:PS vesicles, reconstituted with t-SNAREs and v-SNARE respectively, result in the formation of 6–7 nm in diameter t-/v-SNARE ring complexes when mixed to allow interaction of t-SNARE in one vesicle with v-SNARE in the opposing vesicle. This membrane-directed self-assembly of t-/v-SNARE ring complex is observed from high-resolution imaging studies using both AFM and EM (Fig. 2). In their hydrated state, as seen in AFM micrographs, the central pore within a 6-nm SNARE ring complex measures 2 nm in diameter. In contrast, dehydrated SNARE ring complex of a similar size in EM micrographs appears as 3–4 nm channels, suggesting them to be highly hydrated channels in their native state. Additionally, as observed in earlier studies [6, 8, 9], the SNARE pairs forming the ring complex leave no room for additional SNARE complexes or for molecules to pass through, giving the channel the appearance of being almost non leaky. Approximately, 8–9 protein densities are found to form a 6–7 nm in diameter SNARE ring complex, each density possibly representing a SNARE pair. So what is the molecular principle dictating SNARE ring complex formation when t-SNARE-vesicles and V-SNARE-vesicles meet? Unfortunately, there is no direct method to image the various steps involved in the process, leading to the formation of the SNARE ring complex. However, the process may involve a progressive recruitment of t-/v-SNARE pairs as the opposing vesicles are pulled toward each other, until a complete ring is assembled (Fig. 1), preventing any further recruitment of t-/v-SNARE pairs.

The ring complex at the base of neuronal porosome (Fig. 3) appears to possess a diameter almost half that of the t-/v-SNARE ring complexes formed when such 50–60 nm t-SNARE-vesicles and v-SNARE-vesicles meet. This can be explained, since the 12–17 nm in diameter cup-shaped neuronal porosome complex has a mere 3–4 nm in diameter base where 40–50 nm in diameter synaptic vesicle transiently docks and fuses to release neurotransmitters. This allows room for only one or two SNARE ring complexes at the neuronal porosome base, each ring composed of merely three SNARE pairs (Fig. 3 inset), and a central channel measuring just 1–1.5 nm in diameter. Unlike exocrine or neuroendocrine secretions, the 1–1.5 nm in diameter central channel would be adequate for neurotransmitter release as a result of the build-up of intravesicular pressure following synaptic vesicle swelling during neurotransmission [17, 18].

Acknowledgements

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