# SNAREs in Opposing Bilayers Interact in a Circular Array to Form Conducting Pores

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ABSTRACT The process of fusion at the nerve terminal is mediated via a specialized set of proteins in the synaptic vesicles and the presynaptic membrane. Three soluble *N*-ethylmaleimide-sensitive factor (NSF)-attachment protein receptors (SNAREs) have been implicated in membrane fusion. The structure and arrangement of these SNAREs associated with lipid bilayers were examined using atomic force microscopy. A bilayer electrophysiological setup allowed for measurements of membrane conductance and capacitance. Here we demonstrate that the interaction of these proteins to form a fusion pore is dependent on the presence of t-SNAREs and v-SNARE in opposing bilayers. Addition of purified recombinant v-SNARE to a t-SNARE-reconstituted lipid membrane increased only the size of the globular t-SNARE oligomer without influencing the electrical properties of the membrane. However when t-SNARE vesicles were added to a v-SNARE membrane, SNAREs assembles in a ring pattern and a stepwise increase in capacitance, and increase in conductance were observed. Thus, t- and v-SNAREs are required to reside in opposing bilayers to allow appropriate t-/v-SNARE interactions leading to membrane fusion.

## INTRODUCTION

Neurotransmission involves the sequential interaction of proteins in opposing bilayers (Söllner et al., 1993a,b; Rothman, 1994; Jeong et al., 1998). The classical concept of fusion is a three-step process of cell excitation, docking, and fusion, in which docking may occur before cell excitation. Fusion has been implicated to occur via soluble *N*-ethylmaleimide-sensitive factor (NSF)-attachment protein receptors (SNAREs) (Weber et al., 1998). SNAREs are classified as v-SNARE and t-SNAREs, depending on their primary location either in vesicles (v-) or in target (t-) membranes (Rothman, 1994). Studies demonstrate that t- and v-SNAREs reconstituted into lipid vesicle membranes can fuse with one another, suggesting SNAREs to be the minimal membrane fusion machinery (Weber et al., 1998). The structure of the SNARE complex formed by interacting native (Jeong et al. 1998) and recombinant (Hanson et al., 1997; Sutton et al., 1998) t- and v-SNAREs, has been examined using electron microscopy (Hanson et al., 1997; Jeong et al., 1998) and x-ray crystallography (Sutton et al., 1998). However, the morphology and arrangement of SNAREs in lipid bilayers and their interaction and arrangement when associated within opposing bilayers, remains to be established.

Here we examine the structure and arrangement of purified recombinant t- and v-SNAREs in artificial lipid bilayers, using atomic force microscopy (AFM). To further evaluate the functional properties of SNARE proteins in bilayers, conductance and capacitance of membranes in the presence and absence of SNARE proteins was examined (Cohen and Niles, 1993; Kelly and Woodbury, 1996; Woodbury, 1999). If pore structures were to form by direct addition of SNAREs to a single membrane, an increase in conductance would be observed due to the movement of ions through the pore. To determine the interaction between t- and v-SNAREs present in opposing bilayers, we used v-SNARE reconstituted artificial lipid vesicles and challenged them with t-SNARE reconstituted lipid membranes. The structure and arrangement of the SNARE complex formed as a result, and any changes in capacitance or conductance were recorded using AFM (Schneider et al., 1997; Cho et al., 2002a,b,c) and a bilayer apparatus (Woodbury and Miller, 1990).

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## MATERIALS AND METHODS

### Preparation of lipid bilayer

Lipid bilayers were prepared using brain phosphatidylethanolamine (PE) and phosphatidylcholine (PC), and dioleoylphosphatidylcholine (DOPC), and dioleoylphosphatidylserine (DOPS), obtained from Avanti Lipids (Alabaster, AL). A suspension of PE:PC in a ratio of 7:3, and at a concentration of 10 mg/ml was prepared. Lipid suspension, 100 $\mu$l, was dried under nitrogen gas and resuspended in 50 $\mu$l decane. To prepare membranes reconstituted with vesicle-associated membrane protein (VAMP), 625 ng/ml VAMP-2 protein stock was added to the lipid suspension and brushed onto a 200-$\mu$m hole in the bilayer preparation cup until a stable bilayer with a capacitance between 100 and 250 pF was formed.

### Preparation of lipid membrane on mica

To prepare lipid membrane on mica for AFM studies, freshly cleaved mica disks were placed in a fluid chamber. Bilayer bath solution, 180 $\mu$l, containing 140 mM NaCl, 10 mM HEPES, and 1 mM $CaCl_2$, was placed at the center of the cleaved mica disk. PC:PS vesicles, 10 $\mu$l, were added

to the above bath solution. The mixture was then allowed to incubate for 60 min at room temperature, before washing (×10), using 100 μl bath solution/wash. The lipid membrane on mica was then imaged before and after the addition of SNARE proteins or v-SNARE reconstituted vesicles. Ten microliters of t-SNAREs (10 μg/ml stock) and or v-SNAREs (5 μg/ml stock), was added to the lipid membrane. Similarly, 10 μl v-SNARE reconstituted vesicles was added to either the lipid membrane alone or lipid membrane containing t-SNAREs.

### Atomic force microscopy

Atomic force microscopy was performed on mica and on lipid membrane. Lipid membrane alone or in the presence of SNAREs or v-SNARE reconstituted vesicles on mica were imaged using the Nanoscope IIIa AFM (Digital Instruments, Santa Barbara, CA). Images were obtained both in the "contact" and "tapping" mode in fluid. However, all images presented in this manuscript were obtained in the "tapping" mode in fluid, using silicon nitride tips with a spring constant of 0.38 $N \cdot m^{-1}$, 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 SNARE complexes and lipid vesicles were analyzed using the software nanoscopeIIIa4.43r8 supplied by Digital Instruments.

### Electrophysiological bilayer setup

Electrical measurements of the artificial lipid membrane were performed using a bilayer setup (Cohen and Niles, 1993; Kelly and Woodbury, 1996; Woodbury, 1999). Current verses time traces were recorded using pulse software, an EPC9 amplifier and probe from HEKA (Lambrecht, Germany). Briefly, membranes were formed while holding at 0 mV. Once a bilayer was formed and demonstrated to be in the capacitance limits for a stable bilayer membrane according to the hole diameter, the voltage was switched to −60 mV. A baseline current was established before the addition of proteins or vesicles.

### Artificial vesicle preparation

Purified recombinant SNAREs were reconstituted into lipid vesicles using mild sonication. Three hundred microliters of PC:PS, 100 μl ergosterol, and 15 μl nystatin (Sigma Chemical Company, St. Louis, MO.) were dried under nitrogen gas. The lipids were resuspended in 543 μl of 140 mM NaCl, 10 mM HEPES, and 1 mM $CaCl_2$. The suspension was vortexed for 5 min, sonicated for 30 s and aliquoted into 100-μl samples (AVs). Twenty five μl of syntaxin 1A-1 and SNAP-25 (t-SNAREs) at a concentration of 25 μg/ml was added to 100 μl of AVs. The t-SNARE vesicles were frozen and thawed three times and sonicated for 5 s before use. Bilayer bath solutions contained 140 mM NaCl and 10 mM HEPES. KCl at a concentration of 300 mM was used as a control for testing vesicle fusion.

## RESULTS AND DISCUSSION

Unlike in a whole cell with multiple proteins and protein complexes, this pure phospholipid membrane, alone or in association with SNAREs, is much simpler to study. All AFM images of such lipid membrane and membrane-associated SNARE arrangements were obtained using different AFM imaging forces and cantilevers, confirming similar size, structure, and arrangement of SNAREs. Purified recombinant t- and v-SNARE proteins, when applied to a lipid membrane, form globular complexes (Fig. 1, *A–D*) ranging in size from 30 to 100 nm in diameter and 3 to 15 nm in height when examined using AFM. Section analysis of the t-SNARE complexes (Fig. 1 *D*) in a lipid membrane, before (Fig. 1 *B*) and after (Fig. 1 *C*) addition of v-SNARE, demonstrate changes in both shape and size of the complexes. A 5% increase in diameter and 40% increase in height were seen after addition of v-SNARE to the t-SNARE complexes in the lipid membrane. Studies of conductance changes in the bilayer following reconstitution of SNAREs into phospholipid membranes supported the AFM observations. Addition of t-SNAREs to v-SNARE reconstituted lipid membranes did not alter membrane current (Fig. 1 *E*). Likewise, when t-SNAREs were added to the lipid membrane before addition of v-SNARE, (t-SNAREs were brushed onto the lipid bilayer in the chamber followed by addition of v-SNARE), no change in the baseline current of the bilayer membrane was observed (Fig. 1 *F*).

In contrast to the SNARE complex formed when t-/v-SNAREs were added to the same bilayer, t-SNAREs and v-SNARE in opposing bilayers interact and arrange in circular arrays, forming pore-like structures (Fig. 2, *A–D*). These pores are conducting, because some vesicles have discharged their contents and appear flattened (Fig. 2 *B*), measuring 10–15 nm in height as compared to the 40–60 nm size of intact vesicles (Fig. 2 *A*). Because the t-/v-SNARE complex lies between the opposing bilayers, the discharged vesicles clearly reveal t-/v-SNAREs forming a rosette pattern with a dimple or pore-like depression at the center (Fig. 2, *B*, *C*, and *D*). On the contrary, unfused v-SNARE vesicles associated with the t-SNARE reconstituted lipid membrane reveal only the vesicle profile (Fig. 2 *A*). These studies demonstrate that the t-/v-SNARE arrangement is in a circular array, with a pore-like structure formed at the center of the complex.

To determine whether the pore-like structures were capable of fusing the opposing bilayers, changes in current across the bilayer were examined. T-SNARE vesicles containing the antifungal agent nystatin, and the cholesterol homolog ergosterol, where added to the *cis* side of the chamber containing v-SNARE in the bilayer membrane. Nystatin, in the presence of ergosterol, forms a cation-conducting channel in lipid membranes (Woodbury and Miller, 1990; Cohen and Niles, 1993; Kelly and Woodbury, 1996; Woodbury, 1999). When vesicles containing nystatin and ergosterol incorporate into an ergosterol-free membrane, a current spike can be observed because the nystatin channel collapses as ergosterol diffuses into the lipid membrane (Cohen and Niles, 1993; Kelly and Woodbury, 1996; Woodbury, 1999). As a positive control, a KCl gradient was established to test the ability of vesicles to fuse at the lipid membrane (410 mM *cis*: 150 mM *trans*). The KCl gradient provided a driving force for vesicle incorporation that was independent of the presence of SNARE proteins (Kelly and Woodbury, 1996). When t-SNARE vesicles were exposed to v-SNARE reconstituted bilayers, vesicles fused (Fig. 2 *E*). Fusions of t-SNARE-containing vesicles with the membrane were observed as current spikes as described.

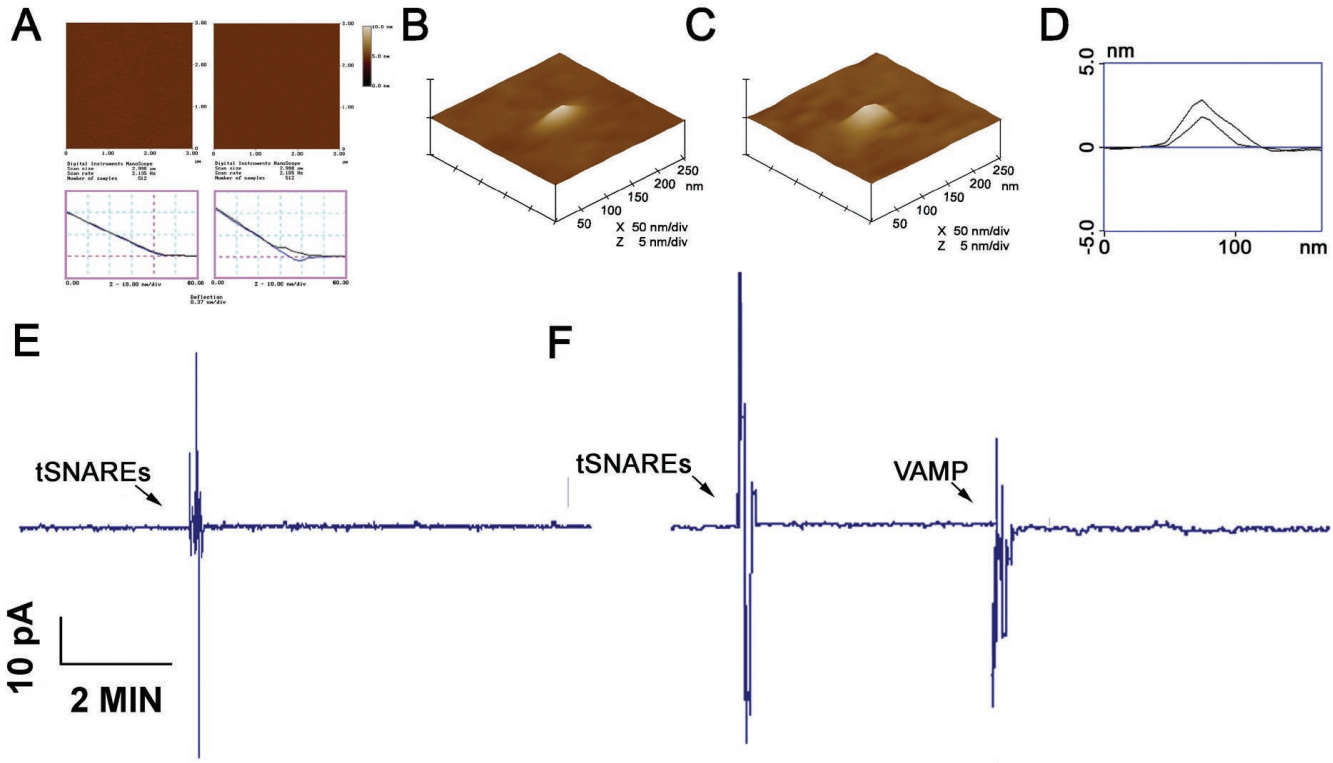


FIGURE 1 AFM micrographs and force plots of mica and lipid surface and of SNAREs on lipid membrane. (*A*) AFM performed on freshly cleaved mica (*left*) and on lipid membrane formed on the same mica surface (*right*), demonstrating differences in the force-versus-distant curves. Note the curvilinear shape exhibited in the force-versus-distant curves of the lipid surface in contrast to mica. Three dimensional AFM micrographs of neuronal t-SNAREs deposited on the lipid membrane (*B*), and after the addition of v-SNARE (*C*). Section analysis of the SNARE complex in (*B*) and (*C*) is depicted in (*D*). Note that the smaller curve belonging to the t-SNARE complex in (*B*) is markedly enlarged after addition of v-SNARE. Artificial bilayer lipid membranes are nonconducting either in the presence or absence of SNAREs (*E*, *F*). Current verses time traces of bilayer membranes containing proteins involved in docking and fusion of synaptic vesicles while the membranes are held at −60 mV (current/reference voltage). (*E*) When t-SNAREs are added to the planar lipid bilayer containing the synaptic vesicle protein, VAMP-2, no occurrence of current spike for fusion event at the bilayer membrane is observed ($n$ = 7). (*F*) Similarly, no current spike is observed when t-SNAREs (syntaxin 1A-1 and SNAP25) are added to the *cis* side of a bilayer chamber, following with VAMP-2. Increasing the concentration of t-SNAREs and VAMP-2 protein.

To verify whether the pore-like structures were continuous across the membrane, capacitance and conductance measurements of the membrane were performed (Fig. 3 *A*). Phospholipid vesicles that come in contact with the bilayer membrane do not readily fuse with the membrane. When phospholipid vesicles were added to the *cis* side of the bilayer chamber containing v-SNARE in the membrane, a small increase in capacitance was observed with little or no further increase. Simultaneously, an increase in conductance was also observed with little or no further increase over a 5-min period. The increase and no further change in conductance or capacitance is consistent with vesicles making contact with the membrane but not fusing (Fig. 3 *B*). These vesicles were fusogenic because of a salt (KCl) gradient across the bilayer membrane, inducing fusion of vesicles with the lipid membrane. When t-SNARE vesicles containing nystatin and ergosterol were added to the *cis* side of the bilayer chamber, an initial increase in capacitance and conductance occurred followed by a stepwise increase in both membrane capacitance and conductance (Fig. 3 *C*), along with several fusion events observed as current spikes in separate recordings. (Fig. 2 *E*). The stepwise increase in capacitance suggests that the t-SNARE vesicles dock and are continuous with the bilayer membrane. The simultaneous increase in membrane conductance is most likely a reflection of the increase in membrane charge associated with the docked vesicles and only secondarily due to open vesicle-associated nystatin channels that are conducting through SNARE-induced pore formation, allowing conductance of ions from *cis* to the *trans* side of the bilayer membrane. SNARE-induced fusion occurred at an average rate of four t-SNARE vesicle incorporations every 5 min into the v-SNARE reconstituted bilayer without osmotic pressure, compared to six vesicles using a KCl gradient ($n$ = 7).

## CONCLUSION

Studies on the fusion of viral coat proteins with the cell plasma membrane have suggested a “hairpin” conformation

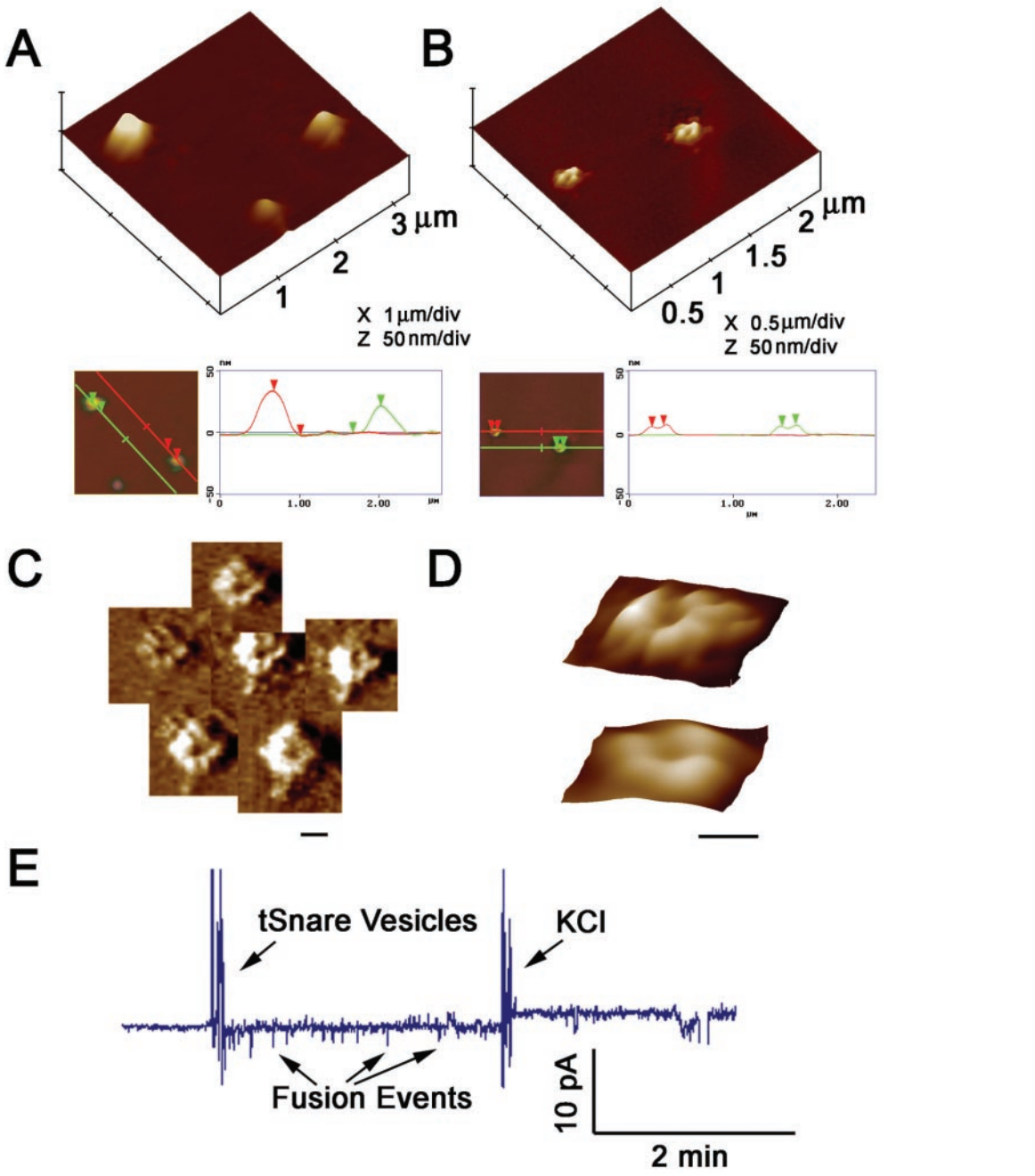


FIGURE 2 Pore-like structures are formed when t-SNAREs and v-SNARE in opposing bilayers interact. (*A*) Unfused v-SNARE vesicles on t-SNARE reconstituted lipid membrane. (*B*) Dislodgement or fusion of v-SNARE-reconstituted vesicles with a t-SNARE-reconstituted lipid membrane, exhibit formation of pore-like structures due to the interaction of v- and t-SNAREs in a circular array. The size of the pores range between 50 and 150 nm (*B–D*). Several 3D AFM amplitude images of SNAREs arranged in a circular array (*C*) and some at higher resolution (*D*), illustrating a pore-like structure at the center is depicted. Scale bar is 100 nm. Recombinant t-SNAREs and v-SNARE in opposing bilayers drive membrane fusion. (*E*) When t-SNARE vesicles were exposed to v-SNARE reconstituted bilayers, vesicles fused. Vesicles containing nystatin/ergosterol and t-SNAREs were added to the *cis* side of the bilayer chamber. Fusion of t-SNARE containing vesicles with the membrane observed as current spikes that collapse as the nystatin spreads into the bilayer membrane. To determine membrane stability, the transmembrane gradient of KCl was increased, allowing gradient driven fusion of nystatin-associated vesicles.

of viral and membrane proteins, leading to the formation of a fusion pore (Chan and Kim, 1998). Analogous to viral fusion, it has been previously hypothesized (Jeong et al., 1998) that molecular assembly of SNAREs in a certain conformation in opposing bilayers, leads to t-/v-SNARE coiling and supercoiling and membrane fusion. In the present study, we demonstrate that, when opposing bilayers meet, SNAREs arrange in a ring pattern resulting in the

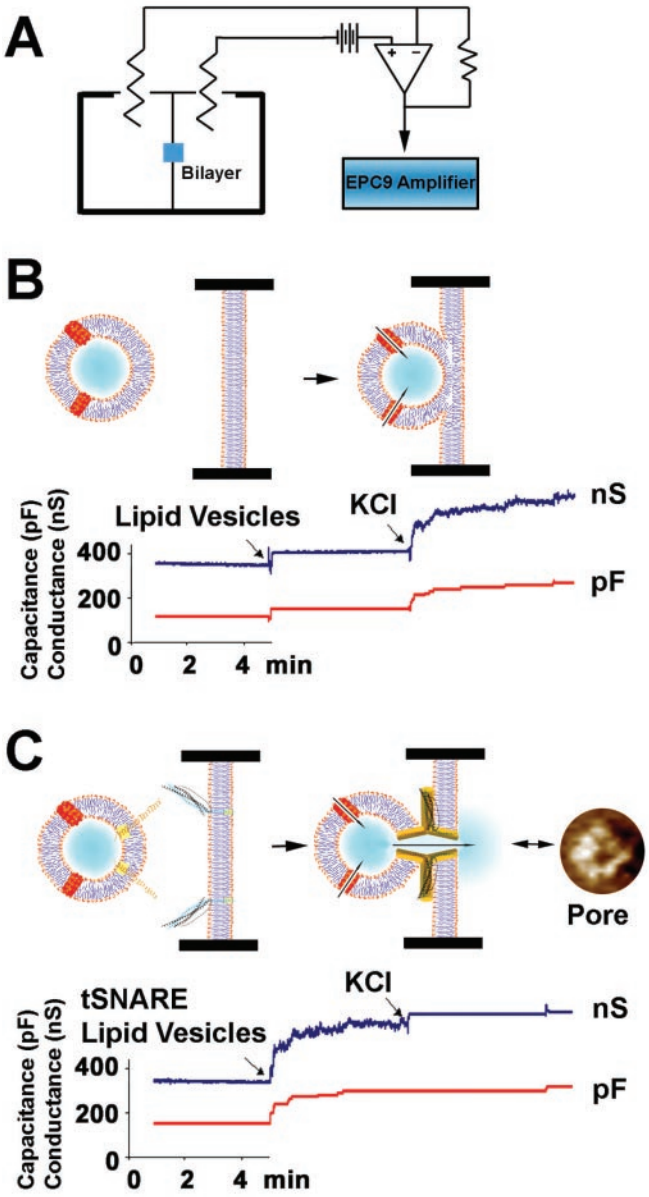


FIGURE 3 Opposing bilayers containing t- and v-SNAREs respectively, interact in a circular array to form conducting pores. (*A*) Schematic diagram of the bilayer-electrophysiology setup. (*B*) Lipid vesicle containing nystatin channels (*red*) and both vesicles and membrane bilayer without SNAREs, demonstrate no significant changes in capacitance and conductance. Initial increase in conductance and capacitance may be due to vesicle–membrane attachment. To demonstrate membrane stability (both bilayer membrane and vesicles), the transmembrane gradient of KCl was increased to allow gradient-driven fusion and a concomitance increase of conductance and capacitance. (*C*) 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, opens 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.

formation of a conducting pore. The next step is to understand the regulation and disassembly of such pores. SNAREs are known to form very stable complexes. Even at 90°C (Fasshaurer et al., 1997) or with sodium dodecyl sulfate treatment (Hayashi et al., 1995), t-/v-SNARE complexes are stable. The soluble *N*-ethylmaleimide-sensitive factor NSF, an ATPase involved in disassembly of SNARE complexes (Söllner et al., 1993a), will be a candidate protein to be used in subsequent studies.

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