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 loose 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 loss of membrane-proximal SVs with more than two tethers, and loss of intervesicle connectors. Overall, tether formation and connector dissolution is triggered by stimulation and adjusted to the spontaneous fusion rate.

- [ ] Need to reformulate the part about 4E mutant
- [ ] For 4K mutant: is the loss of tethered SVs restricted to those with more than only one, or to those with more than two tethers?
- [ ] Possibly 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 conteracts 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 [**doi:0.1038/sj.emboj.7601003?**]. 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 [7,8,9]. 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[10]. 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 [11]. Making the bundle more negative by site-directed mutagenesis reduces the

rate of spontaneous and evoked exocytosis whereas making it more positive 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 [12,13]. 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 [11]. In synaptosomes treated with hypertonic sucrose solution, which depletes the RRP, the majority of tethered vesicles had 1 or 2 tethers [12,14,**doi:10.1016/s0896-6273?**]. 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) [15,16].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 [12,17]. 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 [12,17].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 [18,19]. 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 [13]. This suggests that, after exocytosis of RRP SVs, intermediate SVs could be rapidly recruited in the RRP by diffusion [20]. Synaptic activity enhances the mobility of a fraction of SVs, whereas it induces synapsin dissociation from SVs in a synapsin phosphorylation-dependent manner [21,22]. 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 [23,24,25]. Interestingly, ribbon synapses do not express synapsin and show higher SV mobility than conventional synapses [26]. 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

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2. Ordered list item
    a. Sub-item
    b. Sub-item
        i. Sub-sub-item
3. Ordered list item
    a. Sub-item

- List item
- List item
- List item

subscript: H_2O is a liquid

superscript: 2^{10} is 1024.

unicode superscripts^{0123456789}

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Putting each sentence on its own line has numerous benefits with regard to editing and version control.

Line break without starting a new paragraph by putting
two spaces at end of line.

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Document section headings:

Heading 1

Heading 2

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A heading centered on its own printed page

Horizontal rule:

`Heading 1`'s are recommended to be reserved for the title of the manuscript.

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Figure 1

Figure 2

Figure 3

Figure 4

Table 1

Equation 1

Equation 2

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> Quoted text

> Quoted block of text
>
> Two roads diverged in a wood, and I—
> I took the one less traveled by,
> And that has made all the difference.

Code `in the middle` of normal text, aka `inline code`.

Code block with Python syntax highlighting:

```
from manubot.cite.doi import expand_short_doi

def test_expand_short_doi():
    doi = expand_short_doi("10/c3bp")
    # a string too long to fit within page:
    assert doi == "10.25313/2524-2695-2018-3-vliyanie-enhansera-copia-i-
        insulyatora-gypsy-na-sintez-ernk-modifikatsii-hromatina-i-
        svyazyvanie-insulyatornyh-belkov-vtransfetsirovannyh-geneticheskih-
        konstruktsiyah"
```

Code block with no syntax highlighting:

```
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```

Figures

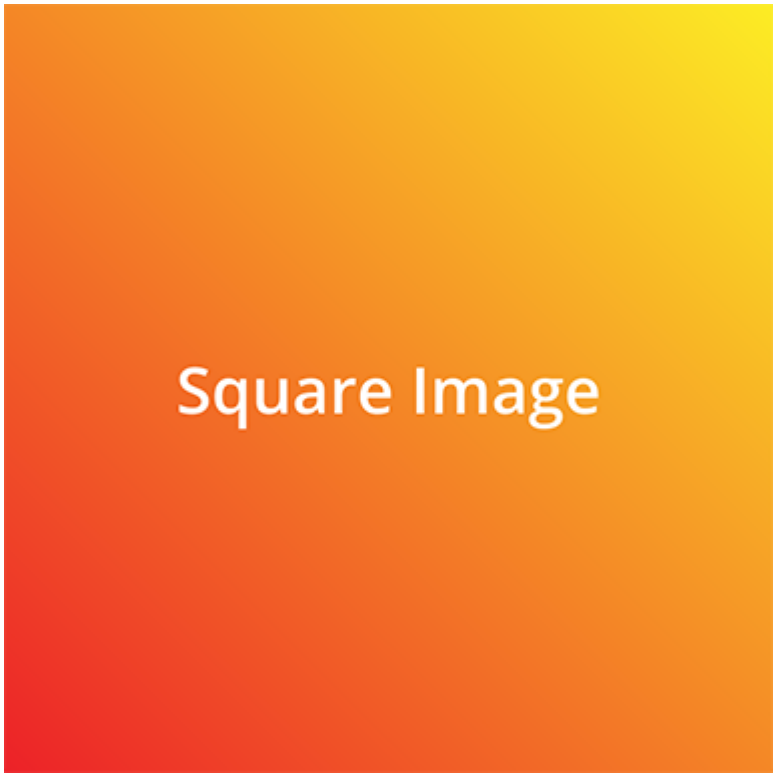

Figure 1: A square image at actual size and with a bottom caption. Loaded from the latest version of image on GitHub.

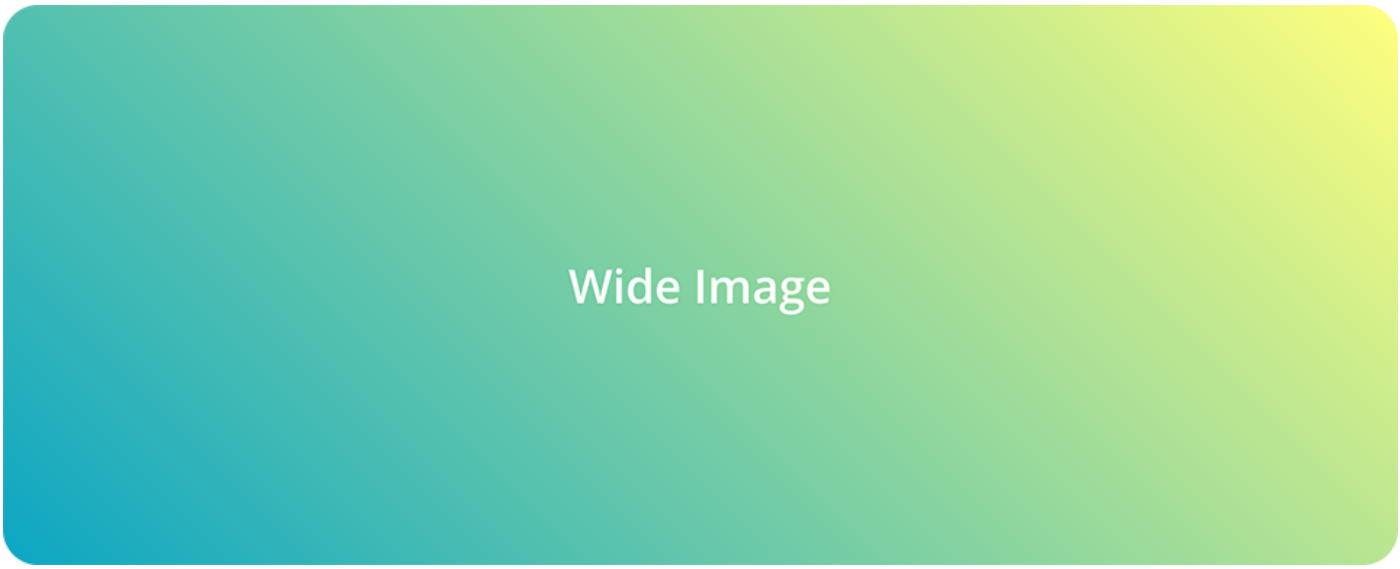

Figure 2: An image too wide to fit within page at full size. Loaded from a specific (hashed) version of the image on GitHub.

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Tables

Table 1: A table with a top caption and specified relative column widths.

Bowling Scores	**Jane**	**John**	**Alice**	**Bob**
Game 1	150	187	210	105
Game 2	98	202	197	102
Game 3	123	180	238	134

Table 2: A table too wide to fit within page.

	Digits 1-33	**Digits 34-66**	**Digits 67-99**	**Ref.**
pi	3.14159265358979323 846264338327950	28841971693993751 0582097494459230	78164062862089986 2803482534211706	`piday.org`
e	2.71828182845904523 536028747135266	24977572470936999 5957496696762772	40766303535475945 7138217852516642	`nasa.gov`

Table 3: A table with merged cells using the `attributes` plugin.

	Colors	
Size	**Text Color**	**Background Color**
big	blue	orange
small	black	white

Equations

A LaTeX equation:

$$\int_0^\infty e^{-x^2} dx = \frac{\sqrt{\pi}}{2} \tag{1}$$

An equation too long to fit within page:

$$x = a + b + c + d + e + f + g + h + i + j + k + l + m + n + o + p + q + r + s + t + u + v + w + x + y + z + 1 + 2 + 3 + 4 + 5 + 6 + 7 + 8 + 9 \tag{2}$$

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