Lecture

# The coordination of synaptic vesicle and neurotransmitter cycles

*S. Voglmaier, M. Santos, H. Li, Z. Hua, Robert H. Edwards*
Departments of Neurology and Physiology, UCSF School of Medicine, 600 16th St., GH-N272B,
San Francisco, CA 94158-2517

**Abstract.** Neurotransmitter release involves the regulated exocytosis of specialized secretory vesicles filled with neurotransmitter. At the nerve terminal, synaptic vesicle membrane recycles locally, through sequential endocytosis and exocytosis. Continued release depends on the ability to fill recycled vesicles with transmitter. Although generally considered independent processes, membrane recycling and the recycling of transmitter now appear to be related through multiple molecular mechanisms. (www.actabiomedica.it)

**Key words:** Vesicular glutamate transporter; endocytosis; endophilin; adaptor protein 3 (AP3); brefeldin A (BFA)

## Introduction

Synaptic vesicles recycle through multiple mechanisms. Kiss-and-run involves the transient opening of a fusion pore, without full fusion to the plasma membrane, and hence bypasses a specific requirement for vesicle recycling. However, most transmitter release is generally considered to involve full fusion, followed by clathrin-dependent endocytosis. This mechanism relies on the recognition of membrane protein cargo by specific adaptors such as the clathrin adaptor protein AP2, followed by recruitment of clathrin and a web of interacting proteins that include amphiphysin, endophilin, dynamin and synaptojanin, each of which performs one or several reactions required for endocytosis (1). It is also generally assumed that all synaptic vesicles contain the same set of membrane proteins, with readily releasable, recycling and reserve pools distinguished through an accident of their history, rather than differences in their molecular composition. We have now observed a direct interaction of the vesicular glutamate transporter VGLUT1 with endophilin (2), indicating the potential for more specific regulation of the mode of endocytosis for this vesicle protein.

## Materials and Methods

To image the recycling of VGLUT1 with high temporal resolution, we inserted the ecliptic pHluorin, a form of GFP shifted in its pH sensitivity so that it is quenched at low pH (3), into the large lumenal loop between transmembrane domains 1 and 2. This fusion protein localizes to nerve terminals, and undergoes activity-dependent increases in fluorescence consistent with exocytosis, followed by quenching that reflects endocytosis (2).

## Outcomes

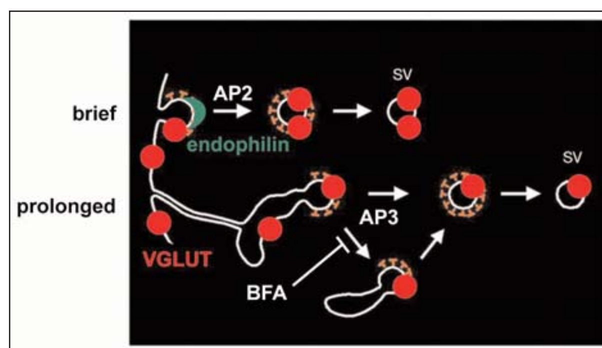
We first used biochemical methods to demonstrate that the interaction with endophilin involves

the second polyproline motif at the cytoplasmic C-terminus of VGLUT1, and the SH3 domain of endophilin. However, we could not demonstrate any effect of the interaction on endocytosis of VGLUT1 in transfected HEK cells by standard cell biological methods. We therefore used the VGLUT1-pHluorin to assess the role of the interaction in transfected hippocampal neurons. Stimulation at moderate frequency for short periods also demonstrates no role for the interaction even in neurons. However, more prolonged stimulation shows that a VGLUT1 mutant incapable of the interaction recycles more slowly during the stimulus, with no difference from wild type in the kinetics of endocytosis after stimulation. In addition, we reconstituted the association of VGLUT1 with endophilin using a heterologous PDZ interaction (4), and showed that endophilin was sufficient as well as necessary for high rates of recycling during prolonged stimulation. Since VGLUT2 and 3 lack polyproline motifs, they presumably recycle more slowly than VGLUT1. We also identified another, dileucine-like sequence at the C-terminus of VGLUT1 that contributes even more strongly to endocytosis and appears in all VGLUT isoforms. The effect of the endophilin interaction depends on this dileucine-like motif, suggesting that they operate in the same pathway, with endophilin acting to accelerate endocytosis mediated by an adaptor protein, presumably AP2 (5). To assess a role for the alternative adaptor AP3 (6), we used the drug brefeldin A (BFA), and found that this could actually rescue the defect in recycling produced by deletion of the polyproline motif in VGLUT1. Further, we observed that BFA reduces the extent of compensatory endocytosis after the stimulus, suggesting a role for the AP3 pathway specifically after prolonged stimulation, presumably as a back-up mechanism to clear excess membrane and protein from the cell surface.

## Conclusion

The results show that VGLUT1 contains signals that target the protein to distinct endocytic pathways, and as such, sets a precedent for other synaptic vesicle proteins, suggesting that synaptic vesicles may vary in their molecular composition. In addition, the results suggest that the AP3 pathway for synaptic vesicle retrieval becomes important specifically after prolonged stimulation, presumably when the classical AP2 pathway becomes saturated. In the case of VGLUT1, inteaction with endophilin helps to restrict the protein to the fast, AP2 pathway but in the absence of this interaction, the protein can use the slower AP3 pathway. Inhibition of the AP3 pathway with BFA rediverts VGLUT1 back to the faster pathway during stimulation, but blocks the compensatory endocytosis that occurs after the stimulus. The results also raise the possibility that AP2 and AP3 pathways generate synaptic vesicles with distinct properties.

The interaction of with endophilin (green) recruits VGLUT1 (red) to the fast, AP2- and clathrin-dependent recycling pathway. In the absence of this interaction, VGLUT1 can also be recycled through a slower, BFA-sensitive AP3-dependent pathway, but only with prolonged, high frequency stimulation. After prolonged stimulation, the AP3 pathway is required to mediate compensatory endocytosis.




**AP2 and AP3 Pathways Compete for the Endocytosis of VGLUT1**

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Correspondence: R. Edwards
Departments of Neurology and Physiology
University of California, San Francisco, CA, USA
E-mail: Robert.Edwards@ucsf.edu