Sumiko Mochida *Editor*

Presynaptic Terminals

Springer

Presynaptic Terminals

Sumiko Mochida
Editor

Presynaptic Terminals

Springer

Editor
Sumiko Mochida
Department of Physiology
Tokyo Medical University
Tokyo, Japan

ISBN 978-4-431-55165-2 ISBN 978-4-431-55166-9 (eBook)
DOI 10.1007/978-4-431-55166-9

Library of Congress Control Number: 2014959280

Springer Tokyo Heidelberg New York Dordrecht London

Printed on acid-free paper

Springer Japan KK is part of Springer Science+Business Media (www.springer.com)

Preface

This book unites leading international experts to discuss recent advances in functional studies on key proteins and protein complexes in stepwise synaptic vesicle phases with the final step of neurotransmitter release (synaptic vesicle exocytosis) in response to a single neuronal signal, action potential, at presynaptic terminals. A number of key proteins and protein complexes also consist of each stepwise synaptic vesicle phase with the first step of synaptic vesicle endocytosis to maintain stable neurotransmitter release in response to unceasing neuronal signals arriving at the presynaptic terminals.

Prior to the synaptic vesicle fusion, synaptic vesicles are docked at the active zone and primed to be ready to fuse. This step is thought to be regulated by a complex of active zone proteins. The complex is important for tethering Ca^{2+} channels to the active zone and loading synaptic vesicles there. For the synaptic vesicle exocytosis in response to Ca^{2+} influx accompanying an action potential, synaptic vesicles fuse with the active zone plasma membrane using a fusion machinery composed of vesicle- and target-SNARE proteins, synaptobrevin-2, syntaxin-1, and SNAP-25. The vesicle fusion machinery is finely regulated by interaction with cytosolic proteins such as complexin, tomosyn, Munc 18, and others. The Ca^{2+} sensor is synaptotagmin expressed as a synaptic vesicle protein having a low-affinity binding property with Ca^{2+}. Synaptotagmin interacts from time to time with Ca^{2+} channels, SNARE proteins, lipid membranes, and others directly or indirectly, and controls synaptic vesicle fusion. Ca^{2+} influx is tuned temporally and spatially by Ca^{2+} binding proteins with higher binding affinity than that of synaptotagmin, such as calmodulin and others, which sense residual Ca^{2+} at the active zone, reflecting a history of presynaptic firing.

Endocytosis is a complicated synaptic vesicle step. There are at least three morphologically distinguished ways of endocytosis: clathrin-coated, kiss-and-run, and bulk endocytosis. These distinct pathways are triggered by different neural firing activity. Dynamin isoforms are selectively activated in relation to synaptic activity for fission of the vesicle membrane neck. The clathrin coat consists of many proteins including adaptor proteins in the inner layer. After fission,

the clathrin-coated vesicles rapidly shed the coat proteins. Endocytosis is important for retrieval of membrane added by synaptic vesicles fusion at the active zone. Thus, it is a rate-limiting step for the next round of exocytosis and is necessary for local recycling of synaptic vesicles to maintain stable neurotransmitter release in a limited space.

The endocyted synaptic vesicles are filled with neurotransmitter by transporters, such as VGLUTs (glutamate), VGATs (GABA), VMAT (monoamine), and VACT (acetylcholine), using H^+ gradient by the H^+ pump V-ATPase. The refilled synaptic vesicles are stored in vesicle pools: a recycling pool and a large reserve (resting) pool. Synaptic vesicles in the reserve (resting) pool are immobilized in a meshwork of cytoskeleton (actin)-binding protein synapsin and remain unreleased even under severe physiological stress, while synaptic vesicles in the recycling pool are stored for incoming physiological neural signals and follow the stepwise synaptic vesicle phases at the presynaptic terminal. The contributions to this book will cover these various facets.

Presynaptic Terminals in a Historical Context

Neurotransmitter Release Is Triggered by Calcium Influx

del Castillo and Katz had found that increasing the extracellular Ca^{2+} concentration enhanced transmitter release, whereas lowering the concentration reduced and ultimately blocked synaptic transmission (del Castillo and Katz 1954b). Because transmitter release is an intracellular process, these findings implied that Ca^{2+} must enter the neuron to influence transmitter release. The extracellular Ca^{2+} concentration, approximately 2 mM in vertebrates, is normally four orders of magnitude greater than the intracellular concentration, approximately 10^{-7} M at rest. However, because these Ca^{2+} channels are sparsely distributed along the axon they cannot, by themselves, provide enough current to produce a regenerative action potential.

Katz and Miledi found that the Ca^{2+} channels were much more abundant at the presynaptic terminal (Katz and Miledi 1967). There, in the presence of tetraethylammonium and tetrodotoxin, a depolarizing current pulse was sometimes able to trigger a regenerative depolarization that required extracellular calcium, a calcium spike. Katz and Miledi therefore proposed that Ca^{2+} serves dual functions. It is a carrier of depolarizing charge like Na^+, and it is a special chemical signal—a second messenger—conveying information about changes in membrane potential to the intracellular machinery responsible for transmitter release. Calcium ions are able to serve as an efficient chemical signal because of their low resting concentration, approximately 10^5-fold lower than the resting concentration of Na^+. As a result, the small amounts of ions that enter or leave a cell during an action potential can lead to large percentage changes in intracellular Ca^{2+} that can trigger various biochemical

reactions. Proof of the importance of Ca^{2+} channels in neurotransmitter release has come from more recent experiments showing that specific toxins that block Ca^{2+} channels also block neurotransmitter release.

The properties of the voltage-gated Ca^{2+} channels at the squid presynaptic terminal were measured by Llinás and his colleagues (Llinás et al. 1981). Using a voltage clamp, Llinás depolarized the terminal while blocking the voltage-gated Na^{+} channels with tetrodotoxin and the K^{+} channels with tetraethylammonium. He found that graded depolarizations activated a graded inward Ca^{2+} current, which in turn resulted in graded release of transmitter. In this book, Karina Leal and I discuss the properties of the voltage-gated Ca^{2+} channels and importance of Ca^{2+} channels activity in neurotransmitter release in Chap. 9.

Transmitter Is Released in Packets with a Fixed Amount of Transmitter

How does the influx of Ca^{2+} trigger release? Katz and his colleagues provided the key insight into this question by showing that neurotransmitter is released in discrete amounts they called *quanta* (del Castillo and Katz 1954a; Fatt and Katz 1952). Each quantum of neurotransmitter produces a postsynaptic potential of fixed size, called the *quantal synaptic potential*. The total postsynaptic potential is made up of a large number of quantal potentials. Excitatory postsynaptic potentials (EPSPs) seem smoothly graded in amplitude because each quantal (or unit) potential is small relative to the total potential.

Katz and Fatt obtained the first clue as to the quantal nature of synaptic transmission in 1951 when they observed spontaneous postsynaptic potentials of approximately 0.5 mV in the nerve–muscle synapse of the frog (Fatt and Katz 1952). Like end-plate potentials (EPPs) evoked by nerve stimulation, these small depolarizing responses were largest at the site of nerve–muscle contact and decayed electronically with distance. The unit EPP in response to a presynaptic action potential is identical in amplitude (approximately 0.5 mV) and shape to the spontaneous miniature postsynaptic potentials (miniature EPPs). Importantly, the amplitude of each EPP is an integral multiple of the unit potential. Increasing the external Ca^{2+} concentration does not change the amplitude of the unit EPP. However, the proportion of failures decreases and the incidence of higher-amplitude responses composed of multiple quantal units increases. These observations show that an increase in external Ca^{2+} concentration does not enhance the *size* of a quantum of neurotransmitter (that is, the number of acetylcholine molecules in each quantum at motor nerve terminals) but rather acts to increase the average number of quanta that are released in response to a presynaptic action potential. The greater the Ca^{2+} influx into the terminal, the larger the number of transmitter quanta released.

Thus, del Castillo and Katz concluded that neurotransmitter is released in packets with a fixed amount of transmitter, a quantum: The amplitude of the EPP varies in a stepwise manner at low levels of acetylcholine release, the amplitude of each step increase is an integral multiple of the unit potential, and the unit potential has the same mean amplitude as that of the spontaneous miniature EPPs.

Quantal Neurotransmitter Release: Vesicular or Not Vesicular?

The quantal unit release of neurotransmitter in packets was then proposed as vesicular release by del Castillo and Katz (1957). The proposal was called the "vesicular hypothesis". Against the "vesicular release", Tauc (1926–1999) and his colleagues proposed a challenging hypothesis "non-vesicular release" (Tauc 1979).

A review paper titled "Quantal Neurotransmitter Release: Vesicular or Not Vesicular?" was written by Tauc in 1997 (Tauc 1997) after establishment of the "vesicular theory". In the introduction he wrote as follows:

> The reader is probably surprised by the incongruity of the question proposed in the title. Where is the problem? Everybody knows, and any textbook will certify that neurotransmitters are released through exocytosis of synaptic vesicles. So be it and there is no need to go further. Yet, a number of researchers question the vesicular release, which is often presented as a fact, whereas it cannot claim more than a status of the hypothesis. The purpose of this paper is to cast doubt on the vesicular hypothesis and to invite the reader to give a second thought to the neurotransmitter release mechanism.

I would recommend the reader of this book to read through his paper.

According to Tauc's paper, in the original formulation of the "vesicular hypothesis" presented by Katz at a meeting in Gif-sur-Yvette in 1955 (del Castillo and Katz 1957), it was assumed that the recently discovered synaptic vesicles contain a transmitter substance, and that when, in their random movement, they touch the presynaptic membrane at critical contact zones, they discharge their content by exocytosis. Against the "vesicular hypothesis", Tauc proposed his unique "non-vesicular theory" in 1979 from observations of acetylcholine release from *Aplysia* neurons (see reviews by Tauc 1979, 1982). He demonstrated that the quantum release of acetylcholine is performed by a complex macromolecular membrane structure, or "vesigate" composed of several functional subunits. It is suggested that the vesigate possesses a number of receptors for cytoplasmic acetylcholine equivalent to the number of acetylcholine molecules in a quantum. The vesigate receptors are thought to be in dynamic equilibrium with cytoplasmic acetylcholine; in order to be releasable, all or most of the receptors have to be occupied by acetylcholine. Synchronized activation of a given number of subunits forms a full quantum. The translocation mechanism is unknown, but it is triggered by Ca^{2+} and may involve a membrane protein identified as "mediatophore" (Dunant and Israël 1995) and some others of the so-called synaptic proteins. It was suggested that the vesigates were located at morphologically identified presynaptic

active zones. Tauc suggested multiple functions of synaptic vesicles; they probably serve as reserves for acetylcholine, but their immediate function may be buffering of Ca^{2+} that enters the terminal during stimulation.

Since Tauc's proposal, 35 years have passed and most of the readers of this book may not know the "non-vesicular theory". The "mediatophore", a "vesigate" component, is the V0c subunit of the V-ATPase (Birman et al. 1990), expressed as a synaptic vesicle H^+ transporter in any presynaptic terminals. Israël and collaborators purified a 150–200 kDa protein complex from a *Torpedo marmorata* electric organ (Birman et al. 1986; Israël et al. 1986). This complex was found to be composed of a single very hydrophobic 17 kDa polypeptide, which upon reconstitution in proteoliposomes showed calcium-dependent pore properties. The 17 kDa protein, initially designated "mediatophore", finally turned out to be the V0c subunit of the V-ATPase (Birman et al. 1990). In this book, Oussama El Far and Michael Seagar demonstrate their recent findings on the V-ATPase in Chap. 7 "The Synaptic Vesicle V-ATPase: A Regulatory Link Between Loading and Fusion?". They discuss the role of the V-ATPase in the presynaptic terminal: in addition to the primary role of vesicle loading, the V0 sector in regulating SNARE-mediated membrane fusion.

Contents of This Volume

At the presynaptic terminal of chemical synapses, in response to an action potential, synaptic transmission is performed by synaptic vesicle exocytosis. This process is orchestrated by several key proteins and protein complexes. Removal of excess membrane resulted from synaptic vesicle exocytosis, and local reuse of synaptic vesicles is necessary to maintain stable neurotransmitter release in response to unceasing neuronal signals arriving at the presynaptic terminals. Several vesicle steps in this process are controlled by other key proteins and protein complexes. The functional roles of most of the key proteins and protein complexes in stepwise synaptic vesicle phases in the presynaptic terminal are discussed in the introductory Chap. 1 by Sumiko Mochida.

Presynaptic Active Zone Proteins

The active zone is a unique structure of the presynaptic terminals where neurotransmitters are released by synaptic vesicles exocytosis. For the synaptic vesicle exocytosis, a specific architecture with proteins complex is composed. The anatomy of the active zone and the composition of active zone proteins, tethering Ca^{2+} channels, and loading synaptic vesicles are demonstrated in Chap. 2 by Thomas Dresbach. Currently, several proteins unique in the active zone, including Bassoon,

Piccolo, RIM1, RIM-BP, Munc13-1, CAST/ELKS, and α-liprin, have been identified and characterized. Toshihisa Otsuka, who found CAST in 2002 and has demonstrated its roles as a core protein of the large complex composed of active zone proteins, discusses the network of protein–protein interaction at the presynaptic active zone in Chap. 3. Prior to synaptic vesicle exocytosis, synaptic vesicles loaded at the active zone are docked and primed: a phase of synaptic vesicle ready for fusion with plasma membrane. Possible molecular mechanisms for this vesicle phase are also discussed in Chaps. 2 and 3.

Synaptic Vesicle Fusion

Synaptic vesicle fusion with active zone membrane is the primary action in response to an arrival of neural signal, action potential, at presynaptic terminals. A synaptic vesicle protein, VAMP2/synaptobrevin-2, and target plasma membrane proteins, syntaxin 1 and SNAP-25, are called SNAREs [soluble NSF attachment protein (SNAP) receptors], and form a complex at the active zone and act as the vesicle fusion machinery. The details of assembly, activation, and disassembly of this fusion machinery are discussed in Chap. 4 by Ira Milosevic and Jakob B. Sørensen, specialists in the functional study of presynaptic terminal proteins.

The SNARE complex formation is regulated by several cytosolic proteins, such as tomosyn and complexin. Tomosyn was found as a syntaxin-1-binding protein and named "friend (*tomo* in Japanese) of syntaxin" in Takai's lab in 1998. In Chap. 5, the former members of the lab Yasunori Yamamoto and Toshiaki Sakiaska demonstrate roles of tomosyn in regulation of the SNARE complex-involved pre-fusion step and the Ca^{2+}-triggered synaptic vesicle fusion step. Complexin/synaphin was found in two labs in 1995. Since then Hiroshi Tokumaru has been studying its function. He discusses in Chap. 6 recent observations that complexin has at least two independent roles: clamping of the *trans*-SNARE complex and activation of Ca^{2+}-triggered synchronous release of neurotransmitter. In addition, Oussama El Far and Michael Seagar discuss in Chap. 7 the role of the V-ATPase, the V0 sector in regulating SNARE-mediated membrane fusion, in addition to the primary role of vesicle refilling with neurotransmitters.

Ca^{2+}-triggered synchronous release of neurotransmitter is mediated by a Ca^{2+}-sensor synaptotagmin expressed as a synaptic vesicle protein. Among 17 isoforms in the synaptotagmin family, synaptotagmin I is the best characterized for the synchronous fast neurotransmitter release. In Chap. 8, Tei-ichi Nishiki discusses accumulated findings for synaptotagmin I functions, including his recent studies. Ca^{2+}-triggered synchronous release of neurotransmitter is initiated by Ca^{2+} entry from Ca^{2+} channels at the active zone. Karina Leal and I demonstrate the properties of the voltage-gated Ca^{2+} channels and modulation of Ca^{2+} channels activity by other Ca^{2+}-sensor proteins in Chap. 9.

Synaptic Vesicle Endocytosis

Endocytosis is a complicated synaptic vesicle phase. There are at least three morphologically distinguished ways of endocytosis: clathrin-coated, kiss-and-run, and bulk endocytosis. These distinct pathways are triggered by different neural firing activity and mediated by different protein–protein interactions. An explanation of the detailed molecular mechanisms of each endocytosis is provided by Ira Milosevic in Chap. 10. Thanks to Ira, the reader can easily understand the function of each protein and the complicated protein complexes.

Endocytosis is important for rapid retrieval of membrane added by synaptic vesicle fusion at the active zone. Thus, it is a rate-limiting step for the next round of exocytosis, and is necessary for local recycling of synaptic vesicles to maintain stable neurotransmitter release in a limited space. Shota Tanifuji and I discuss how dynamin isoforms are key molecules to decode action potential firing for initiating fission of vesicle membrane neck in Chap. 11.

Synaptic Vesicle Refilling and Storage

Synaptic vesicles recycled into the presynaptic terminal must be refilled with neurotransmitters. In the mammalian central nervous system, the majority of neurons utilize amino acids such as glutamate, γ-aminobutyric acid (GABA), and glycine. Glutamate is the major excitatory neurotransmitter, whereas GABA and glycine are inhibitory. These amino acids are synthesized in the presynaptic cytoplasm and transported into synaptic vesicles. Shigeo Takamori overviews advances in the vesicular loading process and discusses some controversial concepts that may have important consequences for synaptic transmission in Chap. 12.

Synaptic vesicles loaded with neurotransmitters should be stored for reuse. Synapsin I was the first presynaptic protein to have its function demonstrated in synaptic vesicle storage by Llinás and Greengard in 1985. Given its ability to bind both the vesicular membrane and actin filaments in a phosphorylation-dependent manner, the classical role attributed to synapsins is the reversible tethering of synaptic vesicles to the actin cytoskeleton. In Chap. 13, Fabrizia C. Guarnieri, Fabio Benfenati, and Flavia Valtorta, specialists in synapsins, discuss recent evidence suggesting other aspects of the synaptic vesicle life cycle, such as docking, fusion, and recycling.

Synaptic Vesicle Pools

Synaptic vesicles are accumulated in the classical three-pool model; the readily releasable pool provides fast initial neurotransmitter release, the recycling pool maintains release during physiological level of neural activity, and the reserve

pool is inert in terms of neurotransmitter release under physiological activity. In Chap. 14, Sven Truckenbrodt and Silvio O. Rizzoli discuss recent findings for roles of synaptic vesicle pools in addition to their classical roles of different pools.

Together these expert contributions provide a thorough review of recent works in the high-profile and exciting field of presynaptic terminals research, and more importantly provide compelling evidence that we still have much to learn about the role of these crucial proteins and their interactions for synaptic vesicle life at presynaptic terminals. The work described here provides the basis for such research.

Tokyo, Japan Sumiko Mochida

References

Birman S, Israel M, Lesbats, B, Morel N (1986) Solubilization and partial purification of a presynaptic membrane protein ensuring calcium-dependent acetylcholine release from proteoliposomes. J Neurochem 47:433–444

Birman S, Meunier FM, Lesbats B, Le Caer JP, Rossier J, Israel M (1990) A 15 kDa proteolipid found in mediatophore preparations from Torpedo electric organ presents high sequence homology with the bovine chromaffin granule protonophore. FEBS Lett 261:303–306

del Castillo J, Katz B (1954a) Quantal components of the end-plate potential. J Physiol 124:560–573

del Castillo J, Katz B (1954b) Statistical factors involved in neuromuscular facilitation and depression. J Physiol 124:574–585

del Castillo J, Katz B (1957) La base 'quantale' de la transmission neuromusculaire. In: Microphysiologie cornparée des élérnents excitables, vol 67, C.N.R.S. Colloq. Int., Paris, pp 245–258

Dunant Y, Israël M (1995) Mediatophore and other presynaptic proteins. A cybernetic linking at the active zone. J Physiol 89:147–156

Fatt P, Katz B (1952) Spontaneous subthreshold activity at motor nerve endings. J Physiol 117:109–128

Israël M, Morel N, Lesbats B, Birman S, Manaranche R (1986) Purification of a presynaptic membrane protein that mediates a calcium-dependent translocation of acetylcholine. Proc Natl Acad Sci U S A 83:9226–9230

Katz B, Miledi R (1967) A study of synaptic transmission in the absence of nerve impulses. J Physiol 192:407–436

Llinás R, Steinberg IZ, Walton K (1981) Relationship between presynaptic calcium current and postsynaptic potential in squid giant synapse. Biophys J 33:323–351

Tauc L (1979) Are vesicles necessary for release of acetylcholine at cholinergic synapses? Biochem Pharmacol 27:3493–3498

Tauc L (1982) Nonvesicular release of neurotransmitter. Physiol Rev 62:857–893

Tauc L (1997) Quantal neurotransmitter release: vesicular or not vesicular? Neurophysiology 29:219–226

Contents

Part I Overview

1 Overview: Presynaptic Terminal Proteins Orchestrate Stepwise Synaptic Vesicle Phases 3
Sumiko Mochida

Part II Presynaptic Active Zone Proteins

2 Active Zone Assembly 47
Thomas Dresbach

3 Network of Protein-Protein Interactions at the Presynaptic Active Zone 69
Toshihisa Ohtsuka

Part III Synaptic Vesicle Fusion

4 Fusion Machinery: SNARE Protein Complex 87
Ira Milosevic and Jakob B. Sørensen

5 Roles of Tomosyn in Neurotransmitter Release 129
Yasunori Yamamoto and Toshiaki Sakisaka

6 Regulation of SNAREs: Complexin 141
Hiroshi Tokumaru

7 The Synaptic Vesicle V-ATPase: A Regulatory Link Between Loading and Fusion? 149
Oussama El Far and Michael Seagar

8 Ca^{2+} Sensors: Synaptotagmins 167
Tei-ichi Nishiki, Kentaro Kuroki, Toshio Masumoto, and Hideki Matsui

9 Regulation of Active Zone Ca^{2+} Channels 195
Karina Leal and Sumiko Mochida

Part IV Synaptic Vesicle Endocytosis

10 Endocytic Machinery at the Neuronal Synapse 223
Ira Milosevic

11 Dynamin Is a Key Molecule to Decode Action Potential Firing 257
Shota Tanifuji and Sumiko Mochida

Part V Synaptic Vesicle Refilling and Storage

12 Transport of Amino Acid Neurotransmitters into Synaptic Vesicles 275
Shigeo Takamori

13 Synapsins and Synaptic Vesicle Storage 295
Fabrizia C. Guarnieri, Fabio Benfenati, and Flavia Valtorta

Part VI Synaptic Vesicle Pools

14 Synaptic Vesicle Pools: Classical and Emerging Roles 329
Sven Truckenbrodt and Silvio O. Rizzoli

Index 361

Part I
Overview

Chapter 1
Overview: Presynaptic Terminal Proteins Orchestrate Stepwise Synaptic Vesicle Phases

Sumiko Mochida

Abstract In mammalian central and peripheral nervous systems, most of presynaptic terminals operate as follows. Synaptic vesicles docked and primed at the active zone are ready for exocytosis and form a synaptic vesicle pool, the readily releasable pool. Neural electrical signal, an action potential, reached to the presynaptic nerve terminal induces the opening of voltage-gated Ca^{2+} channels and a rapid influx of Ca^{2+} at the active zone. The Ca^{2+} transient at the active zone triggers neurotransmitters release by synaptic vesicle exocytosis and controls the release efficacy for incoming neuronal signals. After exocytosis, the synaptic vesicle membrane is recycled from the nerve terminal membrane by endocytosis. Renewal synaptic vesicles are filled with transmitter and reserved in a pool, the reserve pool. Storage of synaptic vesicles in the pools and the mobilization from the presynaptic membrane and the reserve pool are controlled by Ca^{2+} signals after action potential firing. How synaptic vesicles in each phase are controlled? Studies over the last 25 years have revealed that individual proteins in the presynaptic terminal form complexes and orchestrate each of the synaptic vesicle phases and that Ca^{2+} sensor proteins promote synaptic vesicle in a phase to a next stage of the phase along the maturation pathway. To understand presynaptic terminals, in this book, specialists for presynaptic protein(s) illustrate in detail the involvement and the regulation in each synaptic vesicle phase. This overview chapter shortly follows, step by step, the synaptic vesicle phases in mammalian presynaptic terminals.

Keywords Synaptic vesicles • Exocytosis • Endocytosis • Presynaptic proteins • Ca^{2+}-binding proteins

1.1 Introduction

Since Sir Bernard Katz demonstrated electrophysiologically synaptic transmission at the neuromuscular junction in 1950s (Fatt and Katz 1952), how the neurotransmitter release from the presynaptic terminal is regulated had been covered

S. Mochida (✉)
Department of Physiology, Tokyo Medical University, 6-1-1 Shinjuku, Shinjuku-ku, Tokyo 160-8402, Japan
e-mail: mochida@tokyo-med.ac.jp

S. Mochida (ed.), *Presynaptic Terminals*, DOI 10.1007/978-4-431-55166-9_1

with a veil. In the 1990s, a large number of proteins were found in the presynaptic terminals, including in synaptic vesicles' membrane, cytoplasm, and plasma membrane (Sudhof 1995). In 1993, the SNAREs (soluble NSF attachment protein receptors) hypothesis for synaptic vesicle fusion with plasma membrane was proposed by Dr. James Rothman (Yale University) (Sollner et al. 1993b; Rothman 1994). In addition, a Ca^{2+}-sensor protein synaptotagmin (Perin et al. 1991) that triggers synaptic vesicle exocytosis was also proposed by Dr. Thomas Südhof (Stanford University) (Geppert et al. 1994). These attractive proposals opened the curtain of the stage for the play of synaptic vesicle exocytosis in the theater named "Presynaptic terminals." The evidence of accumulated experimental results revealed that the players for the exocytosis are a part of presynaptic proteins and that other proteins play a role in different synaptic vesicle phases such as endocytosis, refilling, storage, and mobilization of synaptic vesicles. Since the proposals by Drs. James Rothman and Thomas Südhof, for two decades, we have got huge amount of information about these proteins functioning in presynaptic mechanisms. Honorably, Drs. James Rothman and Thomas Südhof were awarded the Nobel Prize in 2013. That was an exciting event for us who have been investigating presynaptic protein functions. However, we are not familiar with all of presynaptic proteins and are bewildered to understand how synaptic vesicles are regulated for stable neurotransmitter release in response to unceasing nerve signals. In this book the reader would see how synaptic vesicles in a certain phase are situated on the stages of each act (chapter) such as "docking and priming of synaptic vesicles at the active zone" (Chaps. 2 and 3), "fusion of synaptic vesicles with plasma membrane" (Chaps. 4, 5, 6, 7, 8, and 9), "endocytosis of synaptic vesicles" (Chaps. 10 and 11), "refilling and storage of synaptic vesicles" (Chaps. 12 and 13), and "synaptic vesicle pools" (Chap. 14) that are orchestrated by protein complexes. This chapter shortly describes possible mechanisms in stepwise synaptic vesicle phases following the recycling pathway (Fig. 1.1) that supports stable neurotransmitter release in response to unceasing nerve signals.

1.2 Ca^{2+} Is an Essential Messenger for Presynaptic Functional Processes

Calcium ions are essential messengers that regulate various cellular processes. At the neuronal presynaptic active zone, an action potential results in opening of calcium channels and transient increases in Ca^{2+} of up to 200 μM at domains around the channel pore (Silver et al. 1994). Calcium channels are linked to synaptic vesicles through SNAREs (Sollner et al. 1993b; Rothman 1994) and a Ca^{2+}-sensing protein synaptotagmin (Catterall 2000) that initiates synaptic vesicles exocytosis (Perin et al. 1991). The distance between presynaptic Ca^{2+} channels and synaptotagmin determines the efficacy (Mochida et al. 1996) and speed of exocytosis, suggesting that the spatial diffusion of Ca^{2+} is crucial for the regulation of synaptic

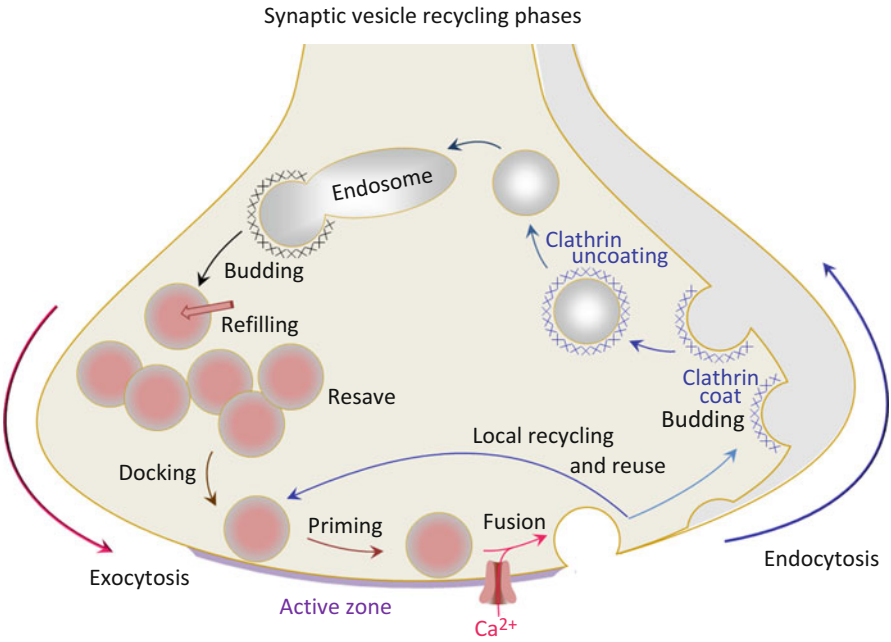

Fig. 1.1 Synaptic vesicle recycling phases at the presynaptic terminal. Synaptic vesicles in the presynaptic terminal follow the recycling pathway composed of stepwise phases of "docking and priming of synaptic vesicles at the active zone," "fusion of synaptic vesicles with the plasma membrane," "endocytosis of synaptic vesicles from the plasma membrane," "refilling and storage of synaptic vesicles," and "synaptic vesicles trafficking" to maintain stable neurotransmitter release in response to unceasing nerve signals. Each of the synaptic vesicle phases is orchestrated by individual protein complex

transmission (Neher and Sakaba 2008; Eggermann et al. 2012). Like exocytosis, presynaptic short-term plasticity (Catterall and Few 2008; Catterall et al. 2013) and vesicle pool maintenance such as the readily releasable pool (RRP) (Sakaba and Neher 2001; Junge et al. 2004) and the reserve pool (RP) (Llinás et al. 1991) are regulated in a Ca^{2+}-dependent manner. After an action potential firing, Ca^{2+} levels rapidly peak at the active zone and then gradually decline to resting levels. During this replenishment period, various Ca^{2+}-binding proteins act cooperatively to regulate synaptic vesicle competence to respond to incoming nerve signals (Mochida 2011b). Likewise, it is known that other post-exocytosis Ca^{2+} receptors in presynaptic terminals control other aspects of synaptic function such as synaptic vesicle endocytosis (Poskanzer et al. 2003; Nicholson-Tomishima and Ryan 2004) and structural remodeling of presynaptic morphology (Su et al. 2012). These Ca^{2+}-mediated regulations of synaptic vesicles phases indicate that Ca^{2+} sensor proteins play a role after action potential firing for progression of synaptic vesicles phases to support stable neurotransmitter release in response to unceasing nerve signals.

Evidence in a large presynaptic terminal of the calyx of Held, where multiple Ca_V2 channels are expressed, provides a starting point to understand roles in modulation of presynaptic Ca^{2+} channels to regulate synaptic transmission in the central neurons. Sympathetic superior cervical ganglion (SCG) neurons form a well-characterized cholinergic synapse in long-term culture (Mochida et al. 1994b; Ma and Mochida 2007), and synaptic transmission is mediated by $Ca_V2.2$ channels (Mochida et al. 1995, 2003). The SCG neurons are an ideal cell model for modulation of $Ca_V2.2$ and brain-derived $Ca_V2.1$ (Mochida et al. 1996, 2003, 2008; Stephen and Mochida 2005; Bucci et al. 2011). The large cell body and nucleus allow for the manipulation of gene expression and function in mature neurons via acute microinjection of cDNA, small interfering RNA (siRNA), dominant-negative transgenes, peptides, antibodies, and metabolites (Mochida et al. 2003, 2008; Baba et al. 2005; Krapivinsky et al. 2006; Ma and Mochida 2007), an approach not technically feasible for cultured neurons from the central nervous system. In addition, synaptic activity and short-term plasticity can be accurately monitored by recording excitatory postsynaptic potentials (EPSPs) evoked by paired or repetitive action potentials in presynaptic neurons. Using this approach, we have uncovered a critical role of cytoplasmic N-terminal and I–II loop interaction (Bucci et al. 2011) and calmodulin (CaM), CaM-like Ca^{2+} sensor proteins, and Ca^{2+}/CaM-dependent protein kinase II (CaMKII) binding to the cytoplasmic C-terminal (Mochida et al. 2008; Nakajima et al. 2009) in regulation of Ca^{2+} channel activity, thus highlighting molecular mechanisms through which presynaptic function and plasticity are regulated by Ca^{2+} channel modulation. CaM is a Ca^{2+} effector sensing residual Ca^{2+} that mediates time- and space-dependent synaptic depression and facilitation via effects on Ca_V2 channel gating (Mochida et al. 2008; Leal et al. 2012). $Ca_V2.1$ channel has an "effector checkpoint" associating with CaMKII to control channel fitness for function (Magupalli et al. 2013). In Chap. 9 Karina Leal demonstrates "regulation of Ca^{2+} channels in the active zone." Likewise, functional studies of presynaptic proteins are possible in a model system for fast cholinergic transmission between rat SCG neurons mediated by N-type Ca^{2+} channels activation (Mochida et al. 1996, 2003). In this chapter and in Chaps. 3, 5, 7, and 11, evidences observed in the model synapse for roles of presynaptic proteins in different synaptic vesicles phases are also discussed.

1.3 Docking and Priming of Synaptic Vesicles at the Active Zone

1.3.1 The SNARE Hypothesis

The SNARE hypothesis proposed as that SNAREs are involved in vesicle docking and priming (Sollner et al. 1993b; Rothman 1994). SNAREs assemble with a 1:1:1 stoichiometry into stable ternary complexes that are disassembled by NSF,

an ATPase, working together with α-SNAP (Sollner et al. 1993b; Hayashi et al. 1995). SNAREs represented by synaptobrevin (or VAMP-1, vesicle-associated membrane protein 1) (Trimble et al. 1988) are vesicle membrane, or "v-SNAREs," whereas SNAREs represented by syntaxin and SNAP-25 are target membrane, or "t-SNAREs" (Sollner et al. 1993b; Rothman 1994). As v- and t-SNAREs are predominantly located on the vesicles and target membranes, respectively, it has been proposed that the formation of SNARE complex may play a critical role in establishing and stabilizing membrane docking and priming (Sollner et al. 1993b; Rothman 1994). However, functional experiments using clostridial neurotoxins have shown that disruption of the v-SNARE, synaptobrevin (Hunt et al. 1994), or that of the t-SNARE, SNAP-25 (Banerjee et al. 1996), does not affect the docking or priming of synaptic vesicles. Genetic deletion of the t-SNARE, syntaxin, or the v-SNARE in *Drosophila* had profound effects on the function of secretory pathways, with complete loss of synaptic transmission (Broadie et al. 1995; Schulze et al. 1995; Sweeney et al. 1995; Deitcher et al. 1998). These results indicate that SNAREs do not play an essential role in synaptic vesicle docking and priming.

1.3.2 Contribution of Active Zone Proteins

The active zone beneath the presynaptic membrane is the principal site for Ca^{2+}-dependent exocytosis (Landis et al. 1988). The release probability scales with the size of the active zone (Holderith et al. 2012). The full molecular composition of the active zone is presently unclear, but many active zone proteins, including RIM1 (Rab-interacting molecule 1), Munc13, RIM-BP, α-liprin, ELKS, CAST/ERC2, Piccolo/Aczonin, and Bassoon, have been identified (Brose et al. 1995; Wang et al. 1997, 1999, 2002; tom Dieck et al. 1998; Fenster et al. 2000; Ohtsuka et al. 2002; Takao-Rikitsu et al. 2004) (see Chap. 2). Dr. Südhof (2012) reviews that among the active zone proteins, emerging evidence suggests that five evolutionarily conserved proteins, RIM, Munc13, RIM-BP, α-liprin, and ELKS proteins, form the core of active zones. The core of active zones forming a single large protein complex is thought to dock and prime synaptic vesicles, recruits Ca^{2+} channels to the docked and primed vesicles, tethers the vesicles and Ca^{2+} channels to synaptic cell-adhesion molecules, and mediates synaptic plasticity (Sudhof 2012). It should be noted that the core active zone proteins are not specific for active zones, or even neurons. ELKS and α-liprins were discovered in nonneuronal cells (Serra-Pages et al. 1995; Nakata et al. 1999), and RIMs, Munc13s, and RIM-BPs are at least partially expressed in neuroendocrine and other secretory cells, suggesting that these proteins perform additional general functions (Sudhof 2012).

In addition to five evolutionarily conserved proteins, RIM, Munc13, RIM-BP, α-liprin, and ELKS (Sudhof 2012), a highly homologous protein with ELKS called CAST/ERC2 also plays a role as a core of the active zone proteins complex in mammalian synapses, including synaptically coupled SCG neurons (Ohtsuka et al. 2002; Wang et al. 2002; Takao-Rikitsu et al. 2004; Kaeser et al. 2011;

tom Dieck et al. 2012) (see Chap. 3). In SCG neurons CAST/ERC2, but not ELKS, colocalizes with other active zone proteins to presynapses (Mochida et al. unpublished data). Disruption of CAST interaction with RIM or Bassoon reduced neurotransmitter release (Takao-Rikitsu et al. 2004). However, how this protein family regulates neurotransmitter release remains unclear. Bruchpilot, a *Drosophila* orthologue of CAST/ELKS, is required for active zone structural integrity. During synaptic plasticity, remodeling of the active zone related to Bruchpilot has been suggested (Weyhersmüller et al. 2011). Bruchpilot is also required for Ca^{2+} channel clustering (Kittel et al. 2006; Wagh et al. 2006), suggesting a potential function for CAST in the mammalian active zone (Kiyonaka et al. 2012). Indeed, CAST deletion in mice reduces the active zone size in the retina (tom Dieck et al. 2012) and impairs inhibitory neurotransmission in the hippocampus (Kaeser et al. 2009). RIM1, a binding partner of CAST/ELKS, interacts with Munc13-1 and has been implicated in docking (Wojcik and Brose 2007) and priming of synaptic vesicles (Betz et al. 2001). Another binding partner, Bassoon (Takao-Rikitsu et al. 2004), is involved in activity-dependent RRP reloading with synaptic vesicles to the release site at excitatory synapses (Hallermann et al. 2010). Therefore, several lines of evidence indicate that the active zone protein complex plays fundamental roles in synaptic vesicle docking and priming that remodel exocytosis and short-term plasticity to ensure the long-term stability of synaptic vesicle release efficacy.

1.3.3 Regulation by Presynaptic Activity

Loss of rapid Ca^{2+} signal by a Ca^{2+} chelator with rapid on-rate binding slowed the rate of recovery from RRP depletion with action potential firing train (Mori et al. 2014), suggesting a control of the kinetics of synaptic vesicle release from the RP, or synaptic vesicles priming by cytoplasmic proteins at the active zone, such as Munc13-1 and RIM1. RIM1 tethers Ca^{2+} channels (Kiyonaka et al. 2007; Kaeser et al. 2011) and binds to the synaptic vesicle proteins, Rab3 (Coppola et al. 2001) and synaptotagmin-1 (Schoch et al. 2002). The binding of RIM1 and Munc13-1 is thought to be an important component for priming of synaptic vesicles (Deng et al. 2011) (see Chap. 3). RIM1 has C_2 domains but does not bind Ca^{2+}; thus, RIM1 binding to synaptotagmin-1 possibly regulates activation of Munc13-1 during the priming process. A rapid Ca^{2+} rise may control synaptic vesicles ability to be modulated by active zone proteins.

Following firing of action potentials, residual Ca^{2+} accumulated at the active zone activates kinases (Zucker and Regehr 2002) and Ca^{2+}-binding proteins (Mochida et al. 2008; Mochida 2011a; Leal et al. 2012) that modulate synaptic transmission. Among the numerous kinases of the presynaptic terminal, protein kinase C reportedly regulates reloading of synaptic vesicles into the RRP (Gillis et al. 1996) and a short-term form of presynaptic plasticity, posttetanic potentiation (PTP) (Brager et al. 2003; Beierlein et al. 2007). For rapid remodeling of synaptic efficacy at the active zone, phosphorylation of active zone protein(s) is ideal.

An active zone-associated serine/threonine kinase, SAD, phosphorylates RIM1, and controls size of the RRP, suggesting activity-dependent phosphorylation of RIM1 and other active zone proteins, may be involved in the RRP control (Inoue et al. 2006). RIM1 is also phosphorylated by protein kinase A (PKA), another serine/threonine kinase (Lonart et al. 2003). Recent genomic and proteomic analyses have revealed more than 600 kinases in humans (Manning et al. 2002). Therefore, we are only at the starting point for understanding the phosphorylation signaling pathways that control the structure and function of the active zone.

1.4 Synaptic Vesicle Fusion

1.4.1 Fusion Machinery: SNARE Protein Complex

Membrane fusion of synaptic vesicles to the presynaptic plasma membrane is generally mediated by SNARE proteins (see Chap. 4). At the active zone, the v-SNARE protein synaptobrevin (VAMP) forms a complex with the t-SNARE proteins syntaxin-1 and SNAP-25 (Sollner et al. 1993b). v- and t-SNARE proteins in the two fusing membranes form a trans-complex that involves a progressive zippering of the four-helical SNARE complex in an N- to C-terminal direction (Hanson et al. 1997). Zippering of trans-SNARE complex forces the fusing membranes into close proximity, destabilizing their hydrophilic surfaces. Assembly of the full trans-SNARE complex opens the fusion pore. Fusion pore expansion transforms the initial "trans"-SNARE complex into "cis"-SNARE complex. The "cis"-SNARE complex is dissociated by ATPase NSF and its adaptor proteins SNAPs (Sollner et al. 1993a).

Prior to SNARE complex formation, syntaxin-1 is in closed conformation. For the SNARE complex assembly, syntaxin-1 stretches the C-terminal (Misura et al. 2000; Dulubova et al. 2002). Munc18-1 binds to the closed conformation of syntaxin-1 (Hata et al. 1993; Dulubova et al. 2002). Munc18-1 remains attached to syntaxin-1 in the assembling SNARE complex and switches its binding mode to an interaction with the SNARE complex (Dulubova et al. 2007). In a physiological context, SNARE complex assembly alone does not mediate fusion (Hata et al. 1993). Munc18-1 binding to assembling SNARE complexes is essential for synaptic vesicle fusion, whereas Munc18-1 binding to closed syntaxin-1 is not (Khvotchev et al. 2007; Gerber et al. 2008; Deák et al. 2009; Zhou et al. 2012). How SNARE complexes induce fusion pore opening is unclear, as is the role of Munc18-1 in fusion. This will be discussed in Chap. 4.

1.4.2 Regulation of SNAREs

SNARE complex formation is regulated by cytoplasmic proteins, such as tomosyn (see Chap. 5) and complexin (see Chap. 6) or synaptic vesicle membrane protein V-ATPase (see Chap. 7), through their SNARE-binding domain.

1.4.2.1 Tomosyn

Tomosyn was originally identified as a syntaxin-1-binding protein (Fujita et al. 1998), and mammals express several splice variants derived from two genes (Groffen et al. 2005). Tomosyn forms a SNARE-like complex with syntaxin-1 and SNAP-25 through the C-terminal VAMP-like domain, and the tomosyn-SNARE complex cannot be displaced by synaptobrevin-2 (Pobbati et al. 2004). Thus, the tomosyn-SNARE complex would preclude SNARE-mediated association of primed vesicles with their target membrane before transmitter release triggered by action potential firing. In contrary, inhibition of vesicles priming by tomosyn is also proposed by the evidence of increase in vesicle docking and neurotransmitter release in the absence of tomosyn-1 (McEwen et al. 2006) and reduction of the RRP reduced in tomosyn-1 overexpressed chromaffin cells (Yizhar et al. 2004).

In cultured SCG neurons, both overexpression and knockdown of tomosyn negatively affect neurotransmitter release (Baba et al. 2005). This indicates that tomosyn levels at the active zone may be critical for efficient control of transmitter release. In addition, the interaction of tomosyn with syntaxin-1 is negatively regulated by PKA phosphorylation of tomosyn. Thus, in vivo, the competition between the formation of the nonproductive tomosyn/syntaxin-1/SNAP-25 complex and the fusion competent synaptobrevin/syntaxin-1/SNAP-25 complex may be controlled by multiple regulatory processes. Furthermore, N-terminal WD-40 repeat domain is also important for the regulation of SNARE complex (Yamamoto et al. 2009) and inhibited vesicle release from the microinjected SCG neurons. Thus, tomosyn inhibits vesicle release by catalyzing oligomerization of SNARE complex through the N-terminal WD-40 repeat domain in addition to the inhibitory activity of the C-terminal VAMP-like domain. Together, tomosyn has multiple sites to inhibit SNARE complex formation.

1.4.2.2 Complexin

Complexin interacts with SNAREs as an antiparallel α-helix, through its central region (Ishizuka et al. 1995; McMahon et al. 1995; Pabst et al. 2000; Bracher et al. 2002). It interacts selectively with the ternary SNARE complex but not with monomeric SNARE proteins. Complexin promotes interaction of the transmembrane regions of syntaxin and synaptobrevin and stabilizes the C-terminal part of the SNARE complex and acts as an inhibitor of vesicle fusion. Complexin also acts as a promoter for synaptic vesicle fusion. This dual functionality is dependent on neuronal activity arriving at the synapse. By acting as a fusion clamp in inhibiting fusion, and a promoter of priming during action potential firing, complexin expression levels at the active zone regulate size of the RRP, important for presynaptic short-term plasticity (Jorquera et al. 2012). In addition, recent evidences suggest that complexin is a cofactor for synaptotagmin in Ca^{2+}-triggered fusion reactions (Reim et al. 2001; Tang et al. 2006; Cai et al. 2008; Jorquera et al. 2012;

Cao et al. 2013). The loss of function induced a decrease in Ca^{2+} triggering of release, an increase in spontaneous mini release, and a decrease in the size of the RRP.

Complexin, when bound to assembled SNARE complexes, contains two short helices flanked by flexible sequences (Chen et al. 2002). The central, more C-terminal alpha helix is bound to SNARE complex and is essential for all complexin functions (Maximov et al. 2009). The accessory, more N-terminal alpha helix is required only for the clamping but not for the activating function of complexin (Neher 2010). The flexible N-terminal sequence of complexin, conversely, mediates only the activating but not the clamping function of complexin (Xue et al. 2007; Maximov et al. 2009). The equally flexible C-terminal sequence, in turn, is required only for the clamping and priming activities of complexin but not for its Ca^{2+}-triggering activity (Kaeser-Woo et al. 2012). Thus, all three activities of complexin—clamping, priming, and activation of Ca^{2+} triggering—require distinct complexin sequences.

1.4.2.3 V-ATPase

The vacuolar proton pump (V-ATPase) is a huge multi-subunit complex composed of two distinct non-covalently associated sectors. The cytosolic V1 sector hydrolyzes ATP, providing the energy for the V0 membrane sector to translocate protons into the vesicle lumen. The proton gradient is then used by vesicular transporters to load synaptic vesicles with specific neurotransmitters. The primary role of the V-ATPase in vesicle loading is widely accepted. However, multiple studies point to an additional general role of the V0 sector in regulating SNARE-mediated membrane fusion. In a search for synaptic vesicle-binding proteins, Thomas and Betz suggested an interaction of the soluble V-ATPase V0d subunit with synaptophysin, a synaptic vesicle protein of unknown function that interacts with VAMP2 (Thomas and Betz 1990). A complex of V0a and c also shown to associate with assembled SNARE complexes, as well as with VAMP2 (Morel et al. 2003). The V0a subunit is involved in a late step of synaptic vesicle exocytosis and its deficit induces severe defects in evoked synaptic transmission in *Drosophila* photoreceptors. These effects were independent of V0a action in acidification (Hiesinger et al. 2005). The V0c subunit, the most hydrophobic component of the V-ATPase V0 sector, directly interacts with the juxtamembrane VAMP2 tryptophan residues 89 and 90 via the cytosolic loop that links TMR 3 and 4 (Di Giovanni et al. 2010). Perturbing V0c/VAMP2 binding, using interfering peptides from V0c loop 3.4 or VAMP2 juxtamembrane domain, inhibited glutamatergic as well cholinergic neurotransmitter release (Di Giovanni et al. 2010). The 1 VAMP2: 1 V0c-binding stoichiometry suggests that the c-subunit ring could organize v-SNAREs into a radial array and determine the number of SNARE complexes that assemble around the fusion pore (EL Far and Seagar 2011).

1.4.3 Ca^{2+} Sensors: Synaptotagmins

Ca^{2+} sensor synaptotagmins transduce the presynaptic Ca^{2+} signal for release, acting on a primed fusion machinery that is ready to go (see Chap. 8). Synaptotagmins are evolutionarily conserved transmembrane proteins with two cytoplasmic domains (Perin et al. 1990, 1991) that bind Ca^{2+} (Brose et al. 1992). C_2 domains were initially defined in PKC isozymes as a conserved sequence of unknown function. Studies on synaptotagmin-1 showed that C_2 domains constitute autonomously folding Ca^{2+}/phospholipid-binding domains (Perin et al. 1990; Davletov and Sudhof 1993; Sutton et al. 1995). In addition, C_2 domains constitute protein interaction domains and, in the case of synaptotagmin-1, bind to syntaxin-1 and to SNARE complexes (Bennett et al. 1992; Sollner et al. 1993a). Functional study of synaptotagmin-1 with knockout forebrain neurons revealed that synaptotagmin-1 is selectively essential for fast Ca^{2+}-triggered release (Geppert et al. 1994): Introduction into the endogenous mouse synaptotagmin-1 gene of a point mutation that decreased the synaptotagmin-1 Ca^{2+}-binding affinity ~2-fold also decreased the Ca^{2+} affinity of neurotransmitter release ~2-fold. In addition to mediating Ca^{2+} triggering of release, synaptotagmin-1 clamps mini release (Littleton et al. 1993; Xu et al. 2009), thus serving as an essential mediator of the speed and precision of release by association with SNARE complexes.

Sixteen synaptotagmins are expressed in brain, eight of which bind Ca^{2+}. Initial functional studies were carried out with synaptotagmin-1. Synaptotagmin-2 and synaptotagmin-9 also act as Ca^{2+} sensors for synchronous synaptic vesicle exocytosis with different kinetics (Xu et al. 2007). These synaptotagmins are expressed in synapses corresponding to transmission of different speed of neural signals. Synaptotagmin-2 as the fastest synaptotagmin is expressed in the neurons mediating sound localization, which requires extremely fast synaptic responses (Sun et al. 2007), whereas synaptotagmin-9 is the slowest synaptotagmin that is primarily expressed in the limbic system mediating slower emotional responses (Xu et al. 2007). Synaptotagmin-10 is a Ca^{2+} sensor for IGF-1 exocytosis that differs from the Ca^{2+} sensor function of synaptotagmin-1 in synaptic vesicle and neuropeptide vesicle exocytosis in olfactory neurons (Cao et al. 2011). Thus, even in a single neuron, different synaptotagmins can act as Ca^{2+} sensors for distinct Ca^{2+}-triggered fusion reactions. All synaptotagmin-controlled fusion reactions appear to require complexin as a cofactor (Reim et al. 2001; Cai et al. 2008; Jorquera et al. 2012; Cao et al. 2013).

1.4.4 Regulation of Ca^{2+} Channels

1.4.4.1 Interaction with Active Zone Proteins

RIM, an active zone protein that is required for vesicle docking and priming (Koushika et al. 2001; Gracheva et al. 2008; Deng et al. 2011; Han et al. 2011,

Kaeser et al. 2011) and is implicated in synaptic plasticity (Castillo et al. 2002; Schoch et al. 2002), interacts with the C-terminal cytoplasmic tails of $Ca_V2.1$ and 2.2 channels (Coppola et al. 2001; Hibino et al. 2002; Kaeser et al. 2011) (see Chap. 9). The interaction of RIM with Ca^{2+} channel is essential for recruiting Ca^{2+} channels to presynaptic active zone (Kaeser et al. 2011) and determines channel density and vesicle docking at presynaptic active zone (Han et al. 2011). RIM-binding protein, RIM-BPs, also interacts with $Ca_V2.1$ and 2.2 channels (Hibino et al. 2002). The tripartite complex composed of RIM, RIM-BPs, and C-terminal tails of the Ca_V2 channels regulates the recruitment of Ca_V2 channels to active zones. RIM also interacts with $Ca_V\beta$ subunits and shifts the voltage dependence of inactivation to more positive membrane potentials, increasing Ca^{2+} channel activity (Kiyonaka et al. 2007). Regulation of presynaptic Ca^{2+} channel function and vesicle docking and priming by RIM provides an additional potential pathway to increase the release probability of synaptic vesicles docked close to Ca_V2 channels.

1.4.4.2 Interaction with t-SNAREs

The v-SNARE synaptobrevin-2 and t-SNAREs syntaxin-1 and SNAP-25 are required for fusion of synaptic vesicle with a plasma membrane to release neurotransmitters (Sudhof 2004) (see Chap. 4). Both $Ca_V2.1$ and $Ca_V2.2$ channels colocalize densely with syntaxin-1 at the presynaptic nerve terminals (Cohen et al. 1991; Westenbroek et al. 1992, 1995). These channels can be isolated as a complex with SNAREs from central neurons (Bennett et al. 1992; Yoshida et al. 1992; Leveque et al. 1994). The t-SNARE proteins syntaxin-1A and SNAP-25, but not the v-SNARE synaptobrevin-2, specifically interact with the $Ca_V2.2$ channel by binding to the intracellular loop between domains II and III of the $\alpha_1 2.2$ subunit$_{718–963,}$ named as the synprint (synaptic protein interaction) site (Sheng et al. 1994). This interaction is Ca^{2+} dependent, with maximal binding at 20 μM Ca^{2+} and reduced binding at lower or higher Ca^{2+} concentrations (Sheng et al. 1996), suggesting sequential steps of association and dissociation of SNAREs with Ca_V2 channels as a function of Ca^{2+} concentration. Two peptide segments separated by a flexible linker within the synprint site independently bind both syntaxin-1A and SNAP-25 (Yokoyama et al. 2005). $Ca_V2.1$ channels have an analogous synprint site, and different channel isoforms have distinct interactions with syntaxin and SNAP-25 (Rettig et al. 1996; Kim and Catterall 1997), which may confer specialized regulatory properties that contribute to synaptic modulation.

Through interaction with $Ca_V2.1$ and Ca_V 2.2 channels, presynaptic t-SNAREs regulate Ca^{2+} channel function. Coexpression of syntaxin-1A and/or SNAP-25 with $Ca_V2.1$ or $Ca_V2.2$ channels shifts the voltage dependence of inactivation toward more negative membrane potentials and reduces the availability of the channels to open (Bezprozvanny et al. 1995; Wiser et al. 1996; Zhong et al. 1999). Coexpression of SNAP-25 can reverse the inhibitory effects of syntaxin on $Ca_V2.2$ channels (Wiser et al. 1996; Jarvis and Zamponi 2001). The synprint site

binds to the entire H3 helix in the cytoplasmic domain of syntaxin-1A (Sheng et al. 1994, 1996; Bezprozvanny et al. 2000). However, the transmembrane region and only a short segment within the H3 helix are critical for channel modulation (Bezprozvanny et al. 2000). Deletion of the synprint site weakened the modulation of the channels by syntaxin-1A, but did not abolish it, arguing that the synprint site acts as an anchor in facilitating channel modulation but is not required absolutely for modulatory action.

1.4.4.3 Interaction with Synaptotagmin-1

Synaptotagmin-1, synaptotagmin-2, synaptotagmin-3, and synaptotagmin-9 serve as the Ca^{2+} sensors for the fast, synchronous neurotransmitter release (Geppert et al. 1994; Sudhof 2004; Xu et al. 2007). Synaptotagmin-1 contains two homologous C_2 domains, which bind Ca^{2+} to initiate synchronous transmitter release (Sudhof 2004, 2012). The C_2B domain of synaptotagmin-1 binds to the synprint sites of both $Ca_V2.1$ and $Ca_V2.2$ channels (Sheng et al. 1997). Moreover, syntaxin-1 interacts competitively with either synprint or synaptotagmin-1 in a Ca^{2+}-dependent manner, such that at low Ca^{2+} concentrations syntaxin-1 binds synprint, whereas at higher concentrations (>30 μM) its association with synaptotagmin-1 increases. The sequential Ca^{2+}-dependent binding of syntaxin-1 to the synprint site and then to synaptotagmin-1 in vitro may reflect stepwise protein interactions that occur during exocytosis (Sheng et al. 1996). Coexpression of synaptotagmin-1 can relieve the inhibitory effects of SNAP-25 on $Ca_V2.1$ channels (Wiser et al. 1997; Zhong et al. 1999). Relief of Ca^{2+} channel inhibition by formation of a complete synaptotagmin/SNARE complex favors Ca^{2+} influx through Ca_V2 channels, thus providing a potential mechanism to increase the release probability of synaptic vesicles that are docked close to Ca_V2 channels (Catterall and Few 2008).

1.4.4.4 Regulation of Ca^{2+} Channel by Ca^{2+} and Ca^{2+} Sensor Proteins

During trains of depolarizations, P/Q-type Ca^{2+} currents increase in size during the first pulses due to facilitation and then inactivate in a pulsewise manner (Cuttle et al. 1998; Lee et al. 2000). Both facilitation and inactivation of $Ca_V2.1$ channels are dependent on CaM (calmodulin) (Lee et al. 1999, 2000; DeMaria et al. 2001). In the C-terminal domain of the $Ca_V2.1$ subunit, N-terminal lobe of CaM interacts with the IQ-like motif, while C-terminal lobe of CaM interacts with a second nearby downstream site, the CaM-binding domain (CBD) (Lee et al. 1999; DeMaria et al. 2001). Following depolarization pulses, high-affinity binding of Ca^{2+} to the C-terminal lobe of CaM and interaction with the IQ-like motif of $Ca_V2.1$ channels cause facilitation, whereas subsequent lower-affinity binding of Ca^{2+} to the N-terminal lobe of CaM and interaction with the CBD of $Ca_V2.1$ channels cause inactivation (Catterall and Few 2008).

Calmodulin-like Ca^{2+} sensor proteins (CaS), which possess four EF-hand Ca^{2+}-binding motifs organized in two lobes connected by a central α helix, are expressed in neurons. CaBP1 (calcium-binding protein 1) is a member of a subfamily of neuron-specific CaS (nCaS) highly expressed in the brain and retina (Haeseleer et al. 2000) and is colocalized with presynaptic $Ca_V2.1$ channels in some synapses (Lee et al. 2002). Like CaM, CaBP1 binds to the CBD of $Ca_V2.1$, but its binding is Ca^{2+} independent (Lee et al. 2002). CaBP1 causes rapid inactivation that is independent of Ca^{2+}, and it does not support Ca^{2+}-dependent facilitation (Lee et al. 2002). Another nCaS VILIP-2 (visinin-like protein-2) highly expressed in the neocortex and hippocampus (Burgoyne and Weiss 2001) also modulates $Ca_V2.1$ channels. VILIP-2 increases Ca^{2+}-dependent facilitation but inhibits Ca^{2+}-dependent inactivation (Lautermilch et al. 2005). CBD and IQ-like motifs of $Ca_V2.1$ are required for binding of VILIP-2. Thus, CaBP1 and VILIP-2 bind to the same site as CaM but have opposite effects on $Ca_V2.1$ channel activity.

In the presynaptic terminal, efficacy of transmitter release is regulated by Ca^{2+} just after Ca^{2+} channel open triggered by an action potential. During Ca^{2+} declining to resting level, Ca^{2+} binding to CaM, and nCaS proteins regulates presynaptic Ca^{2+} channels and causes facilitation and inactivation of the Ca^{2+} current for incoming action potential at the nerve terminal. The steep dependence of neurotransmitter release on the presynaptic Ca^{2+} current predicts that this type of regulation should profoundly alter release probability. Differential expression of these Ca^{2+}-dependent regulatory proteins may provide a means of cell-type-specific regulation of presynaptic Ca^{2+} channels. To study regulation of presynaptic $Ca_V2.1$ channels, synapses that express homogeneous $Ca_V2.1$ channels are ideal. SCG neurons have endogenous N-type but not P/Q-type Ca^{2+} currents. In the presence of N-type Ca^{2+} blocker, ω-conotoxin GVIA, P/Q-type Ca^{2+} currents are recorded from neurons transfected with cDNA encoding $Ca_V2.1$ channels by microinjection (Mochida et al. 2003, 2008). In these transfected SCG neurons, mutations in the CBD of $Ca_V2.1$ channels reduced depression of transmitter release induced by the second action potential, called paired-pulse depression (Mochida et al. 2008). In contrast, mutations in the IQ-like motif of $Ca_V2.1$ channels reduced paired-pulse facilitation and augmentation of EPSPs (Mochida et al. 2008). In the presence of endogenous CaM, the second action potential releases less amount of transmitter with the interval $\leq$50 ms and releases more with the interval $\geq$50 ms, suggesting regulation of P/Q-type Ca^{2+} channels with higher-affinity binding of Ca^{2+} to the C-terminal lobe of CaM lasts longer at the presynaptic active zone and resulting in facilitation of transmitter release. Expression of VILIP-2, which blocks Ca^{2+}-dependent inactivation of P/Q-type Ca^{2+} current, induced paired-pulse facilitation, while expression of CaBP1, which blocks Ca^{2+}-dependent activation of P/Q-type Ca^{2+} current, induced paired-pulse depression (Leal et al. 2012). Thus, CaM and VILIP-2 may respond to residual Ca^{2+} as "facilitation sensors" by binding to the IQ-like motif in the C-terminus of $Ca_V2.1$ channels and causing Ca^{2+}-dependent facilitation of the presynaptic Ca^{2+} current. Synaptic facilitation and augmentation in transfected SCG neurons share a common mechanism: activation of nCaS proteins by residual Ca^{2+} increases "instantaneous" Ca^{2+} entry via $Ca_V2.1$ channels in an activity-dependent manner, which in turn increases

neurotransmitter release according to the power law of neurotransmission. Why does different nCaS regulate $Ca_V2.1$ function at the same sites as CaM? Their affinity and binding speed to Ca^{2+} are different (Faas et al. 2011). CaM has lower affinity and higher binding speed to Ca^{2+} than nCaS proteins, suggesting temporal regulation of $Ca_V2.1$ activity by CaM and nCaS proteins (Mochida et al. 2008; Leal et al. 2012). The divergent actions of nCaS proteins on $Ca_V2.1$ channels may fine-tune the function and regulatory properties of presynaptic P/Q-type Ca^{2+} currents, allowing a greater range of input-output relationships and short-term plasticity at different synapses (Catterall and Few 2008).

1.4.4.5 Regulation of Ca^{2+} Channel by CaMKII

CaMKII is the most prominent Ca^{2+}/CaM-dependent regulator of the postsynaptic response, including long-term potentiation (Schulman and Greengard 1978; Kennedy et al. 1990; Luscher et al. 2000; Shepherd and Huganir 2007). CaMKII also regulates presynaptic function (Llinas et al. 1985, 1991), including effects on synaptic plasticity (Chapman et al. 1995; Lu and Hawkins 2006). CaMKII binds to $Ca_V2.1$ channel ($\alpha1_{1897–1912}$) and enhances activity by slowing inactivation and positively shifting the voltage dependence of inactivation (Jiang et al. 2008). Surprisingly, these effects on the function of $Ca_V2.1$ channels require binding of an autophosphorylated form of CaMKII (Magupalli et al. 2013), but do not require the catalytic activity of the enzyme (Jiang et al. 2008). Dephosphorylation of CaMKII does not reverse the binding (Magupalli et al. 2013). Furthermore, CaMKII binding to $Ca_V2.1$ C-terminus$_{1897–1912}$ increases phosphorylation of synapsin-1 and induces oligomers of synapsin-1 (Magupalli et al. 2013). It was proposed that noncatalytic regulation of $Ca_V2.1$ channels by bound CaMKII serves to enhance the activity of those channels that have the effector of the Ca^{2+} signal (i.e., CaMKII) in position to bind entering Ca^{2+} and respond to it (Jiang et al. 2008). This form of regulation is similar to regulation by SNAREs and RIM, as described above; that is, the activity of the $Ca_V2.1$ channels is increased by formation of a complete SNARE complex with synaptotagmin and RIM bound (Zhong et al. 1999; Kiyonaka et al. 2007), which serves as the effector of the Ca^{2+} signal for initiation of synaptic transmission. This “effector checkpoint” mechanism serves to focus Ca^{2+} entry through those Ca^{2+} channels whose effectors (i.e., a complete SNARE complex and CaMKII) are bound and ready to respond (Jiang et al. 2008; Magupalli et al. 2013).

1.5 Synaptic Vesicle Endocytosis

Neurons in both central and peripheral nervous systems encounter a wide range of activity in the form of action potential firing patterns. In accord, membrane transport systems, in particular, synaptic vesicle recycling pathways in synapses that mediate fast neurotransmission, must maintain the ability to rapidly react

to ongoing changes in action potential firing and adjust membrane trafficking to provide sufficient membrane replenishment. Synaptic vesicle recycling through repetitive cycles of exocytosis and endocytosis is well established to operate under different conditions of synaptic activity and are typically classified for fast synapses by imaging and electrophysiology into fast and slow modes (Royle and Lagnado 2003; Wu 2004), while morphological retrieval is usually described as "kiss-and-run" fusion/fission, full-collapse vesicle retrieval, and bulk modes (Ceccaldi et al. 1995; Heuser and Reese 1973; Royle and Lagnado 2003). The relationship between the kinetic phases with morphological retrieval is a matter of ongoing debate and may depend on the particular synapse.

1.5.1 Synaptic Activity and Synaptic Vesicle Endocytosis

The slow mode of endocytosis is mediated by the classic clathrin-mediated pathway (Heuser and Reese 1973; Granseth et al. 2006) and is responsible for refilling of the RRP via the RP (Kuromi and Kidokoro 2002; Sudhof 2004). The clathrin-mediated pathway is considered to be the main pathway in high activity hippocampal synapses (Granseth et al. 2006) where full-collapse fusion events are broadly distributed across the synaptic bouton adjacent to the release site, whereas "kiss-and-run" fusion events are concentrated near the center of the synapse close to the active zone (Park et al. 2012). In contrast, the fast mode of endocytosis involves the rapid reuse of synaptic vesicles that bypasses the RP (Artalejo et al. 1995). In hippocampal neurons, "kiss-and-run" retrieval with a time constant 1–2 s and preference for vesicles with a high release probability is increased by rapid firing and can shape the kinetics of neurotransmission over a wide range of firing patterns (Zhang et al. 2009). In contrast, low-frequency firing activates both fast (~3 s) and slow pathways (after 10 s) (Zhu et al. 2009). As firing frequency increases, slow endocytosis becomes the predominant pathway (Zhu et al. 2009). In addition to these modes of synaptic vesicle recycling, bulk endocytosis is also rapidly triggered during strong stimulation (Wu and Wu 2007; Clayton et al. 2008). The primary signal coupling activity to synaptic vesicle recycling is believed to be Ca^{2+} (Balaji et al. 2008; Hosoi et al. 2009; Wu et al. 2009; Yao et al. 2009) acting at a Ca^{2+} sensor (Poskanzer et al. 2003; Nicholson-Tomishima and Ryan 2004); however, the molecular mechanism for the selection of a specific pathway for membrane trafficking during dynamic changes in action potential properties is poorly understood.

1.5.2 Dynamin Isoform Selects Distinct SV Recycling Pathway in Response to Neural Activity

A candidate mechanism for synaptic vesicle recycling indexed to cell activity could involve a protein central to endocytosis. Most forms of endocytosis in the

physiological range requires the protein dynamin, a GTPase that mediates fission of synaptic vesicles from the presynaptic terminal membrane (Takei et al. 1995). There are three dynamin isoforms (Cao et al. 1998; Ferguson et al. 2007); dynamin-1 and -3 are highly expressed in brain, while dynamin-2 is ubiquitous (Cook et al. 1994; Cao et al. 1998). Knockout of dynamin-1 and dynamin-3 in central neurons suggests that dynamin-1 is required for fast synaptic vesicle recycling with high-frequency neuronal activity (Ferguson et al. 2007; Klingauf 2007; Hayashi et al. 2008) and dynamin-2 may control synaptic vesicle recycling after neuronal activity (Ferguson et al. 2007). In these studies, dynamin-3 has no direct effect on synaptic vesicle endocytosis, but cooperates with dynamin-1 to support optimal rates of synaptic vesicle endocytosis (Raimondi et al. 2011). In contrast to central synapses, dynamins 1, 2, and 3 are equally expressed in peripheral sympathetic neuron (see Chap. 11). Combined genetic knockdown and direct physiological measurements of synaptic transmission from paired SCG neurons show that three isoforms of dynamin work individually to match vesicle reuse pathways having distinct rate and time constants with physiological action potential frequencies. Dynamin-3 resupplied the RRP with slow kinetics independent of action potential frequency, but acted quickly, within 20 ms of the incoming action potential. Under high-frequency firing, dynamin-1 regulated synaptic vesicle recycling to the RRP with fast kinetics in a slower time window of greater than 50 ms. Dynamin-2 displayed a hybrid response between the other isoforms. Collectively, dynamin isoforms select appropriate vesicle reuse pathways associated with specific neuronal firing patterns of sympathetic neurons.

Endocytosis at many synapses requires initiation by Ca^{2+}/CaM and dynamin (Wu et al. 2009). The clathrin-mediated pathway (Heuser and Reese 1973; Granseth et al. 2006) is responsible for refilling of the RRP via the RP (Kuromi and Kidokoro 2002; Sudhof 2004). Traditionally, synaptic vesicles in the RP are thought to solely support maintenance of RRP size during high-frequency synaptic activity (Sudhof 2004). Thus, in hippocampal synapses, the clathrin pathway is considered to operate during high levels of activity (Granseth et al. 2006) and involves Ca^{2+}/CaM-dependent dynamin activation (Wu et al. 2009). In SCG neurons, synaptic vesicle recycling was blocked in the presence of slow Ca^{2+}-chelator EGTA (Mori et al. 2014). The precise Ca^{2+}-triggering pathway for endocytosis is not well established, but it is known that when isolated nerve terminals in the brain are stimulated, a group of phosphoproteins essential for synaptic vesicle endocytosis including dynamin are coordinately dephosphorylated by calcineurin, a Ca^{2+}/CaM-dependent protein phosphatase (Cousin and Robinson 2001). Thus, a candidate mechanism for the slow Ca^{2+}-mediated regulation of endocytosis involves CaM, calcineurin, and endocytic phosphoproteins representing Ca^{2+}-sensors, mediators, and effectors, respectively. In contrast, studies at the calyx of Held nerve terminal with rapid Ca^{2+}-chelator BAPTA and at cerebral cortex neurons suggest that the nanodomain Ca^{2+} simultaneously triggers exocytosis and endocytosis of synaptic vesicles (Yamashita et al. 2010), cooperating with a Ca^{2+} sensor, synaptotagmin (Nicholson-Tomishima and Ryan 2004). Furthermore, the rate of vesicle exocytosis is controlled by dynamin isoform following consecutive action potential firing

(Armbruster et al. 2013). Recent high-sensitivity pHluorin assays of vesicle endocytosis in cultured hippocampal and cortical neurons demonstrated that acceleration of endocytosis is followed by the stalling during action potential bursts. The acceleration of endocytosis was blocked by mutation of dynamin-1 at the phosphorylation site (Armbruster et al. 2013), suggesting that dynamin isoforms control optimization of the synaptic vesicle recycling rate for varying synaptic activity. However, molecular mechanisms for the selection of a Ca^{2+} sensor and an isoform of dynamin for membrane trafficking during dynamic changes in firing properties are, so far, poorly understood.

1.5.3 Time Course of Endocytosis

The time course of endocytosis varies at different synapses and with the intensity of stimulation. Endocytic styryl dyes, applied in a pulse-chase manner after electrical stimulation, revealed that membrane recovery after an exocytic burst (e.g., 20–100 action potentials at 20 Hz at hippocampal synapses in culture or 10 s at 30 Hz at the frog neuromuscular junction) required less than 1 min to complete and had a time constant in the range of 20–30 s (Ryan et al. 1996b; Wu and Betz 1996), similar to what had been predicted by earlier morphological experiments (Miller and Heuser 1984). Real-time measurements of endocytosis by the use of pHluorin confirmed that the average time constant of endocytosis is in the range of 15–20 s when the system is not saturated (e.g., 50 action potentials at 10 Hz) and subsequent studies strongly suggested that under these conditions the endocytic process is dependent on the clathrin coat (Granseth et al. 2006; Balaji and Ryan 2007; Kim and Ryan 2009). At high levels of activity, the endocytic rate slows down, indicating that under these conditions the surface accumulation of synaptic vesicle membranes exceeds the endocytic capacity (Sankaranarayanan and Ryan 2001; Granseth et al. 2006; Balaji and Ryan 2007).

In contrast to the speed of endocytosis estimated by capacity measurements and pHluorin imaging, electrophysiological measurements showed a more rapid time window of tens of milliseconds, suggesting a role for dynamin in the clearance of endocytic vesicle cargo from active zones. A requirement ($<$20 ms after an action potential) for rapid dynamin action in synaptic vesicle membrane trafficking and active zone clearance has been proposed for the *Drosophila shibire* mutant (Kawasaki et al. 2000) and at the calyx of Held after dynamin dysfunction (Hosoi et al. 2009), respectively. In SCG neurons, with three isoforms, two of them gating distinct kinetic response pathways and the third isoform shared between the two and contingent on firing pattern, the dynamin endocytosis system appears well positioned to manage replenishment of the RRP of synaptic vesicles under a range of physiological action potential firing patterns encountered by neurons in vivo (Tanifuji et al. 2013). Studies at the calyx of Held (Hosoi et al. 2009; Neher 2010) indicate that rapid dynamin action may be the rate-limiting step for the RRP replenishment suggesting that dynamin isoforms may

be crucial regulatory control points for the membrane's response to cell activity. Indeed, dynamin knockdown impaired the ability of cells to translate high-frequency action potential firing into recovery and maintenance of the RRP (Tanifuji et al. 2013) (see Chap. 11).

In addition, ultrafast endocytosis at mouse hippocampal synapses is recently demonstrated using "flash-and-freeze" electron microscopy (Watanabe et al. 2013). Docked vesicles fuse and collapse into the membrane within 30 ms of the stimulus. Compensatory endocytosis occurs within 50–100 ms at sites flanking the active zone. Invagination is blocked by inhibition of actin polymerization, and scission is blocked by inhibiting dynamin. The intact synaptic vesicles are not recovered, suggesting that this form of recycling is not compatible with kiss-and-run endocytosis; moreover, it is 200-fold faster than clathrin-mediated endocytosis. The "ultrafast endocytosis" is suggested as a membrane trafficking specialized to restore the surface area of the membrane rapidly.

Endocytosis after single-vesicle exocytosis during spontaneous release in the calyx of Held was extremely fast (56 ms time constant) using capacitance measurements (Sun et al. 2002). When ~200 vesicles were stimulated by a single action potential, or thousands of vesicles by multiple action potentials at low frequency (<2 Hz), the time constant of endocytosis was only twofold slower (~115 ms), which is not a significant increase considering that many more vesicles were endocytosed after action potential-induced release than were after spontaneous release events. However, when the stimulation frequency was increased, endocytosis slowed down dramatically. After 10 stimuli at 20 or 333 Hz, the time constants of endocytosis increased to 2.3 and 8.3 s, respectively (Sun et al. 2002). The decrease in endocytosis rate did not depend on the cytosolic Ca^{2+} concentration or number of stimuli but on the net increase in membrane area produced by the sum of exo- and endocytosis (Sun et al. 2002). It is likely that the number of unretrieved vesicles at a given time determines the speed of endocytosis.

Measurements of the endocytic recovery of synaptic vesicle membranes by capacitance recording in frog saccular hair cells, axon terminals of goldfish bipolar neurons, and calyx of Held synapses showed time scales in a similar range and a longer time course after a strong exocytic load (Parsons et al. 1994; von Gersdorff and Matthews 1994; Sun and Wu 2001; Sun et al. 2002; Wu et al. 2005). These studies also revealed the existence of an additional fast kinetic component of endocytosis (~1 s), which has been proposed to be mediated by a distinct molecular mechanism (von Gersdorff and Matthews 1994; Neves et al. 2001; Hull and von Gersdorff 2004; Jockusch et al. 2005). A fast dynamin-independent endocytosis has been observed in calyx of Held, dorsal root ganglion and SCG neurons (Zhang et al. 2004; Xu et al. 2008; Lu et al. 2009).

1.5.4 Clathrin-Mediated Endocytosis

Some general principles apply to clathrin-mediated endocytosis in all cellular contexts (Conner and Schmid 2003; Jung and Haucke 2007; Dittman and Ryan 2009;

Kirchhausen 2009; McMahon and Boucrot 2011). The nucleation of a clathrin-coated pit starts with the interaction of clathrin adaptors and/or a subset of their accessory factors with the lipid bilayer and with membrane proteins. In this nucleation stage, obligatory intrinsic components of the vesicle membrane (Koo et al. 2011; Miller et al. 2011), such as those that direct vesicle traffic, are likely to play a dominant role. Subsequently, interactions of the adaptors with each other, with other accessory factors, with cargo proteins, and with clathrin lead to the rapid growth of the coat in a feedforward, cooperative fashion (see Chap. 10). Eventually, a deeply invaginated clathrin-coated bud with a narrow neck is formed. Fission of this neck in a reaction that requires the GTPase dynamin (Koenig and Ikeda 1989; Conner and Schmid 2003; Ferguson and De Camilli 2012) leads to a free vesicle that rapidly uncoats.

1.5.4.1 Clathrin Coat

The slow mode of endocytosis is operated by the classic clathrin-coated pits (Heuser and Reese 1973; Granseth et al. 2006). The clathrin coat is composed of two layers: an inner layer of adaptors and an outer clathrin layer. The clathrin adaptors typically comprise a membrane-binding folded module and flexible arms, which may terminate in an additional small folded module (Edeling et al. 2006). The folded module binds cytoplasmically exposed domains or endocytic "motifs" of vesicle membrane proteins as well as the head group of $PI(4,5)P_2$, a phosphoinositide concentrated in the plasma membrane (Beck and Keen 1991; Traub 2003; Owen et al. 2004; Zoncu et al. 2009; Jackson et al. 2010). The arms bind clathrin heavy chain as well as other adaptors and endocytic factors (Edeling et al. 2006; Schmid and McMahon 2007). Their elongated flexible unfolded structure is optimally suited to capture these proteins in the cytosol and to help concentrate them at growing coated buds. The most abundant adaptor is AP-2, a member of a heterotetrameric family of adaptors that interacts with tyrosine-based endocytic motifs (Bonifacino and Traub 2003), such as the one present in the synaptic vesicle protein SV2 (Haucke and De Camilli 1999), and with dileucine-based motifs, such as those in vesicular neurotransmitter transporters (Bonifacino and Traub 2003; Fei et al. 2008). The other major synaptic endocytic clathrin adaptor is AP180 (Morgan et al. 2000; Ford et al. 2001). Another adaptor is stonin 2 that along with AP-2 plays a role in the internalization of synaptotagmin, the Ca^{2+} sensor of the synaptic vesicle (Zhang et al. 1994; Walther et al. 2001, 2004). Several other proteins with endocytic adaptor properties have been identified, for example, Epsin (Ford et al. 2001; Boucrot et al. 2012), Dab (Morris and Cooper 2001), ARH (He et al. 2002), β-arrestin (Lin et al. 1997), etc. However, so far these proteins have not been shown to play a major role in synaptic vesicle recycling (Yoshihara and Littleton 2002).

In addition to the core components of the coat, i.e., the adaptors and clathrin, several factors that assist and/or regulate the nucleation, loading, and dynamics of endocytic clathrin-coated pits were identified (Slepnev and De Camilli 2000;

Dittman and Ryan 2009; McPherson 2010). Some of these proteins, collectively referred to as clathrin accessory factors (Slepnev and De Camilli 2000) may be considered core components of the coat. Modules often present in such factors include SH3 domains, BAR domains, ENTH/ANTH, and PH domains, whereas short amino acid motifs include proline-containing motifs (for SH3 domain binding), NPF motifs (for EH domain binding), clathrin boxes (for clathrin binding), and several motifs that recognize the appendage domains of the clathrin adaptor AP-2 (Slepnev and De Camilli 2000; Dittman and Ryan 2009; McPherson 2010). The relatively low affinity involved in the interactions mediated by these modules and motifs (low micromolar range) in clathrin accessory factors is ideally suited to allow the rapid formation and disassembly of molecular networks (Yoshihara and Littleton 2002).

1.5.4.2 Clathrin Uncoating

After fission, newly generated clathrin-coated vesicles rapidly shed their coat proteins. Clathrin shedding is ATP dependent and requires Hsc70 ATPase and its cofactor auxilin (Guan et al. 2010; Xing et al. 2010; Yim et al. 2010), whereas shedding of the adaptors is dependent on PI(4,5)P_2 hydrolysis by the PI(4,5)P_2 phosphatase synaptojanin. Genetic disruption of synaptojanin 1, or of endophilin, produces a striking accumulation of clathrin-coated vesicles in nerve terminals and delayed recycling kinetics in mice, and also in worms and flies (Cremona et al. 1999; Schuske et al. 2003; Wu 2004; Dickman et al. 2006; Hayashi et al. 2008; Milosevic et al. 2011). The combined action of Hsc70 and synaptojanin makes the uncoating reaction irreversible (Yoshihara and Littleton 2002). How these two reactions are coordinated? Although auxilin binds clathrin and AP-2, auxilin recruitment to budding coated vesicles requires its tensin homology domain. The tensin homology domain binds membrane monophosphoinositides (Guan et al. 2010), suggesting that the catalytic action of synaptojanin 1 may mediate such coordination. Conversion of PI(4,5)P_2 to PI4P by synaptojanin would not only remove membrane-binding sites for the clathrin adaptors but also generate binding sites for auxilin (Guan et al. 2010).

1.5.5 Membrane Fission

The physical separation of the clathrin-coated bud to generate a free vesicle requires the GTPase dynamin (Praefcke and McMahon 2004; Ferguson and De Camilli 2012). Dynamin oligomerizes into spirals at bud necks and mediates fission in a process involving GTP hydrolysis (Hinshaw and Schmid 1995; Takei et al. 1995). Dynamin recruitment occurs as pits mature to their deeply invaginated state (Merrifield et al. 2002). The PH domain of dynamin, which binds acidic phospholipids, PI(4,5)P_2, and the proline-rich carboxy-terminal region, which

functions as a protein-protein interaction domain, are important for dynamin's function and recruitment (Okamoto et al. 1997; Shupliakov et al. 1997). BAR domain-containing proteins and proteins that may help coordinate formation of the vesicle neck with the recruitment of dynamin bind this region (generally through SH3 domains) (Itoh and De Camilli 2006). BAR proteins that bind dynamin often also bind the $PI(4,5)P_2$ phosphatase synaptojanin and or N-WASP, thus suggesting a functional partnership between these proteins (Itoh and De Camilli 2006; Takenawa and Suetsugu 2007). However, genetic studies in mice support a primary uncoating function of synaptojanin (Cremona et al. 1999; Hayashi et al. 2008; Milosevic et al. 2011). Concerning N-WASP, its activation in proximity of dynamin (Kessels and Qualmann 2002; Koch et al. 2011) may reflect a role of actin in neck formation/elongation or in fission, by providing tension at bud necks, or in propelling newly formed vesicles away from endocytic sites (Yoshihara and Littleton 2002).

Dynamin binds to filamentous actin (Schafer et al. 2002), suggesting possible interdependencies between dynamin and actin during clathrin-mediated endocytosis (Mooren et al. 2009). The requirement for actin during clathrin-mediated endocytosis is, however, a point of controversy (Shupliakov et al. 2002; Sankaranarayanan et al. 2003; Bourne et al. 2006; Schmid and McMahon 2007; Taylor et al. 2012). Increased membrane tension partially inhibits compensatory endocytosis, and actin appears to regulate endocytosis during changes in membrane tension (Boulant et al. 2011), suggesting that the requirement for actin during endocytosis may vary with activity (Heidelberger et al. 2002) and may be transient (Merrifield et al. 2002). Depletion of dynamin perturbed organization of the actomyosin cytoskeleton in U2-OS cells, implicating dynamin in remodeling actin filaments for orchestrating the global actomyosin cytoskeleton in vivo (Mooren et al. 2009). The capacitance measurements of presynaptic terminal membrane of calyx of Held synapses demonstrated that block of myosin II activation by myosin light chain kinase inhibitors slowed down both fast and slow forms of endocytosis but not affected exocytosis (Yue and Xu 2014), suggesting that myosin may regulate vesicle scission through actin and dynamin mediation. Furthermore, activity-dependent markers FM1-43 and horseradish peroxidase revealed that myosin IIB inactivation greatly slowed vesicular replenishment of the recycling pool, suggesting that myosin IIB-driven membrane tension or actin dynamics regulate the major pathway for synaptic vesicle retrieval (Chandrasekar et al. 2013).

1.6 Synaptic Vesicle Recycling Pathways

Two pathways for synaptic vesicles in the vesicle cycle have been defined: a pathway where the vesicles recycle via an endosomal intermediate after endocytosis (endosomal recycling) and a shorter pathway where vesicles recycle directly

without a trafficking intermediate (local recycling) (Sudhof 1995). These two cycles are sometimes presented as mutually exclusive alternatives, and the existence of the endosomal pathway has been doubted. However, strong evidence suggests that both pathways are employed in parallel, although at different rates (Sudhof 2004). Three lines of evidence implicate an endosomal intermediate in the recycling of some synaptic vesicles. First, endosomes are observed morphologically after extensive stimulation of nerve terminals, suggesting that at least a subset of vesicles fuse with endosomes after endocytosis (Sudhof 1995). Second, the majority of synaptic vesicles contain Rab5 that mediates endosome fusion (Fischer von Mollard et al. 1994), suggesting that these vesicles pass through endosomes. Third, synaptic vesicles contain high concentrations of the SNARE Vti1a, which functions in membrane fusion involving endosomes but not in membrane fusion at the plasma membrane, indicating that the majority of vesicles recycle via endosomes at some point in their lifetime (Antonin et al. 2000).

Direct local recycling of synaptic vesicles without an endosomal intermediate is also supported by multiple lines of evidence. In classical experiments, Whittaker and colleagues (Jones et al. 1982) demonstrated that after stimulation of nerve terminals, a small subset of vesicles becomes preferentially filled with newly synthesized neurotransmitters. Furthermore, this vesicle subset is localized close to the membrane and is the first to undergo exocytosis upon stimulation (Barker et al. 1972). These observations indicate that synaptic vesicles can be divided into a smaller subpopulation of active recycling vesicles and a larger population of reserve vesicles. The concept of local recycling without endosomal intermediates was proposed with the observation that synaptic vesicles labeled with FM1-43 dye during endocytosis are subject to re-exocytosis without dilution of the dye (Murthy and Stevens 1998). This result is consistent with the notion that the vesicles in the recycling pool do not pass through endosomes as an intermediate.

The third vesicle cycle in the nerve terminal called “reuse” was proposed by using FM dyes with fast and slow dissociation constants (Klingauf et al. 1998). This “reuse” pathway is that after exocytosis, the fusion pores of the vesicles in the RRP close quickly, the vesicles refill with neurotransmitters immediately, and the vesicles are then re-primed for another round of exocytosis without ever leaving the active zone (Pyle et al. 2000). This “reuse” pathway results in very fast turnaround of vesicles after exocytosis, providing an attractive idea in view of the economy. The concept of the “reuse” pathway is based on two critical findings. First, vesicles in the RRP loaded with FM dyes showed an extremely short time of fusion pore opening, so short the regular FM1-43 dye was not released, but the faster dissociating dye FM2-10 was released. In contrast to this differential destaining reaction, fusion of vesicles recruited from the RP showed much longer times of fusion pore opening and equivalent dissociation of FM1-43 and FM2-10 dyes. Second, after depletion of the RRP, this pool refills much faster than can be explained by repopulation from the reserve pool. Thus, vesicles in the RRP recover the ability to re-exocytose locally without recycling through the RP.

1.7 Synaptic Vesicle Refilling and Storage

1.7.1 Synaptic Vesicle Refilling

For recycled synaptic vesicles to be reused, vesicles must be fully refilled with neurotransmitter. At hippocampal glutamatergic synapses, vesicle refilling is completed during vesicle recycling (Zhou et al. 2000), suggesting that vesicle refilling could occur in milliseconds (Sudhof 2004). Glutamate is taken up into vesicles via vesicular glutamate transporters (VGLUTs) (see Chap. 12) using H^+ gradient (see Chap. 7) and membrane potential. The time constant for vesicle acidification estimated in hippocampal cell culture is 0.4 s (Gandhi and Stevens 2003) or 4–5 s (Atluri and Ryan 2006).

Transporters responsible for vesicular uptake of classical neurotransmitters from the cytoplasm belong to three distinct families, one for monoamines (vesicular monoamine transporter, VMAT) and acetylcholine (vesicular acetylcholine transporters, VAChT), another for transmitters such as GABA (vesicular GABA transporter, VGAT) (Reimer et al. 1998), and a third for glutamate (VGLUT) (Bellocchio et al. 2000).

There are two VMAT isoforms, VMAT1 and VMAT2, encoded by separate genes and displaying different cellular distributions and pharmacological properties (Erickson et al. 1992; Liu et al. 1992b). In rodents, VMAT1 is expressed predominantly in neuroendocrine cells (e.g., adrenal medulla, small intensely fluorescent cells), while VMAT2 is expressed in peripheral, central, and enteric neurons (Weihe et al. 1994; Peter et al. 1995). The two VMATs also differ in substrate affinity. VMAT2 has three times higher affinity for dopamine, noradrenaline, and adrenaline than VMAT1 and 100 times greater affinity for histamine. In addition, VMAT2 has a higher turnover number than VMAT1, which is especially appropriate for rapidly recycling vesicles (Peter et al. 1995; Erickson et al. 1996). Heterotrimeric G proteins have been shown to regulate the monoamine uptake and thus content of both large dense core and small synaptic vesicles. VMAT2 is more sensitive to G-protein regulation than VMAT1 (Holtje et al. 2000).

The VAChT gene is localized to the first intron of the choline acetyltransferase (ChAT) gene (Bejanin et al. 1994; Erickson et al. 1994). Both ChAT and VAChT genes are in the same transcriptional orientation and both are required to express the cholinergic phenotype. There are two classes of VAChT mRNAs in the rat, R- and V-types, encoding the same VAChT protein. The R-type VAChT mRNAs contain common 5′-noncoding sequences (exon R) with two ChAT mRNAs and may therefore be transcribed from the same promoter (Bejanin et al. 1994). The V-type mRNA species differs from the R-type mRNAs by the 5′-noncoding sequences (Bejanin et al. 1994; Erickson et al. 1994). However, the molecular mechanism by which the V-type mRNA species is produced has not been clearly elucidated.

There are four cloned isoforms of VGAT in rat and human, termed GAT1-4. Among four isoforms, GAT-1 is localized primarily on presynaptic terminals

of GABAergic neurons (Dingledine and Korn 1985; Conti et al. 1998). Each of the GABA transporter isoforms is a member of the family of Na^+/Cl^--coupled transporters. GAT-1 cotranslocates two sodium ions and one chloride ion with each uncharged GABA molecule (Dingledine and Korn 1985) and is therefore electrogenic, carrying on positive charge into the vesicle. The VGAT is also responsible for glycine uptake in glycinergic neurons (Christensen et al. 1990; Burger et al. 1991). Probably, the biosynthetic enzyme glutamic acid decarboxylase (GAD), which is concentrated on the vesicle surface (Reetz et al. 1991), creates high amounts of GABA that are preferentially loaded into the vesicles in GABAergic neurons (Angel et al. 1983). In contrast, the VGLUT transports glutamate much better than aspartate and thus discriminates between these two transmitter candidates.

Two Na^+- and Cl^--dependent glycine-specific transporters, GlyTs, were identified in rat brain vesicles (Mayor et al. 1981). These observations were confirmed by the successive cloning of two closely related members of the Na^+/Cl^--coupled transporter family (GlyT1 and GlyT2a) (Guastella et al. 1992; Liu et al. 1992a; Smith et al. 1992). Five GlyT1 variants (a, b, c, e, f) have been described (Guastella et al. 1992; Liu et al. 1992a). A second GlyT2 isoform (GlyT2b), which differs by five amino acids in the N-terminal and does not transport glycine, has also been reported (Ponce et al. 1998).

Three vesicular glutamate transporters, VGLUT1-3, are encoded by solute carrier genes *Slc17a6-8*. VGLUT1 (*Slc17a7*) and VGLUT2 (*Slc17a6*) are expressed in glutamatergic neurons, while VGLUT3 (*Slc17a8*) is expressed in neurons classically defined by their use of another transmitter, such as acetylcholine and serotonin. VGLUT1 and VGLUT2 are considered the most reliable markers for glutamatergic neurons. Their expression pattern is to a large extent complementary, with VGLUT1 mainly expressed in the cerebral and cerebellar cortex and hippocampus and VGLUT2 mainly expressed in deeper brain regions including the thalamus and the brainstem (Fremeau et al. 2001; Herzog et al. 2001; Kaneko and Fujiyama 2002). The spatial distribution is not absolute, however; VGLUT2 is, for example, expressed in subpopulations of the cerebral cortex and hippocampus throughout life. There is also a temporal difference in VGLUT isoform expression, where VGLUT2 is most abundantly expressed during embryonic and early postnatal development, where after VGLUT1 becomes the dominating isoform in certain brain areas (Miyazaki et al. 2003).

In contrast to the VMATs and VAChT, which rely chiefly on the pH gradient (ΔpH) across the vesicle membrane to drive active transport, VGLUT depends almost entirely on the electrical gradient ($\Delta\psi$), vesicular GABA transport depends equally on $\Delta\psi$ and ΔpH, and vesicular glycine transport resembles vesicular GABA transport in the bioenergetics mechanism (Fykse and Fonnum 1996, 1988; Maycox et al. 1990). However, competition studies using synaptic vesicles from different brain regions have not definitively resolved whether a single transporter packages both GABA and glycine or distinct proteins package the two transmitters (Kish et al. 1989; Christensen et al. 1990; Burger et al. 1991).

1.7.2 Synaptic Vesicle Storage

Refilled synaptic vesicles are organized in two distinct vesicle clusters, a large RP (reserve pool) and a quantitatively smaller RRP (readily releasable pool) in which synaptic vesicles contact the presynaptic membrane and eventually fuse with it upon action potential firing (Neher 1998; Sudhof 2004). In the RP synaptic vesicles are restrained by the actin-based cytoskeleton. The synapsins are members of a multigene family of synaptic vesicle-specific phosphoproteins that are implicated in the regulation of neurotransmitter release and synapse formation (De Camilli et al. 1990). Accumulated evidence has demonstrated that the synapsins are both necessary and sufficient for synaptic vesicles to bind actin filaments and are responsible for the formation and maintenance of synaptic vesicle clusters in the nerve terminal (Ceccaldi et al. 1995; Pieribone et al. 1995; Valtorta et al. 1995) (see Chap. 13). Modulation of synaptic vesicles and actin binding after synapsin phosphorylation by CaMKII (Bahler and Greengard 1987; Benfenati et al. 1992; Ceccaldi et al. 1995), PKA, and mitogen-associated protein kinase Erk 1/2 (Jovanovic et al. 1996; Nielander et al. 1997; Hosaka et al. 1999) controls the transition of synaptic vesicles from the RP to the RRP, thus regulating the efficiency of neurotransmitter release.

In hippocampal neurons, synapsin I dissociates from synaptic vesicle and disperses into the axon during firing, whereas it reclusters upon return to the resting state, and the dispersion of the proteins depends on phosphorylation by CaMK and extracellular signal-regulated kinase (Chi et al. 2001, 2003). Injection of synapsin I into developing neurons stimulated the maturation of quantal neurotransmitter release and increased clustering of synaptic vesicles (Lu et al. 1992; Valtorta et al. 1995). In contrast, dysfunction of endogenous synapsins by the intracellular injection of anti-synapsin antibodies disrupted clusters of synaptic vesicles, decreased neurotransmitter release evoked by high-frequency stimulation in the lamprey giant reticulospinal neurons (Pieribone et al. 1995), and induced the disappearance of posttetanic potentiation, appearance of posttetanic depression, and increased synaptic depression in *Aplysia* ganglion neurons (Humeau et al. 2001). Furthermore, mutant mice lacking synapsin I, synapsin II, or both decreased the number of synaptic vesicle and the maximal release of neurotransmitters, induced depression during high-frequency stimulation, and increased recovery times after synaptic depression (Li et al. 1995; Rosahl et al. 1995; Ryan et al. 1996a). Together, synapsin is responsible for formation of the storage of refilled vesicles under the control of Ca^{2+}-dependent CaMKII activity (see Chap. 13).

1.8 Synaptic Vesicle Pools and Trafficking

1.8.1 Synaptic Vesicle Pools

Recycling synaptic vesicles in the presynaptic terminal are clustered in two distinct pools, a large reserve pool (RP) and a quantitatively smaller readily releasable pool

(RRP) (Sudhof 1995, 2004; Neher 1998) (see Chap. 14). The RP size is regulated by synapsin under the control of Ca^{2+}-dependent CaMKII activity (Llinás et al. 1991) (see Sect. 1.7.2), while the RRP size is regulated in turn by active zone proteins complex (Sudhof 2004, 2012) (see Sect. 1.3.2). The RRP size estimated by a cumulative plot of the excitatory postsynaptic currents (EPSCs) amplitudes evoked by presynaptic high-frequency stimulation is smaller than that estimated by the application of a hypertonic sucrose solution or presynaptic strong depolarization (Moulder and Mennerick 2005; Sakaba 2006; Stevens and Williams 2007). This discrepancy reflects the presence of reluctant synaptic vesicles, which are scarcely released by an action potential at glutamatergic synaptic terminals, as well as at cholinergic SCG neuron terminals (Ma et al. 2009). Consistently, deconvolution analysis of EPSCs evoked by a long depolarizing pulse at the calyx of Held revealed that release-competent of synaptic vesicles can be separated into fast- and slow-releasing vesicle pools (Sakaba and Neher 2001). The vesicles in the fast-releasing pool are responsible for phasic release during a high-frequency train of action potentials, whereas vesicles in the slow-releasing pool contribute primarily to asynchronous release, when the intracellular concentration of calcium ions is increased globally during the late period of the train (Sakaba 2006). Thus, vesicles in the slow-releasing pool can be classified as reluctant synaptic vesicles at the calyx of Held. The coupling between synaptic vesicles and Ca^{2+} channel in the active zone determines the fast- and slow-releasing pools (Wadel et al. 2007).

The RRP of synaptic vesicles is the most important vesicle pool for neurotransmitter release. However, this pool constitutes only a tiny fraction of the total vesicle pool. The RRP is a component of the recycling pool of vesicles, defined as all vesicles that can be labeled with FM1-43 when synapses are stimulated extensively. After the RRP depletion with extensive stimulation, vesicles are recruited for exocytosis from the RP to the active zone. The RRP and the RP together constitute the recycling pool. The total recycling pool has been estimated at 21–25 vesicles/synapse in hippocampal cultures, with ~17–20 vesicles in the RP (Murthy and Stevens 1999). Presynaptic terminals thus contain a small number of active vesicles (the recycling pool), of which a third is in the RRP. Since morphologically the same synapses were shown to contain ~200 synaptic vesicles (Shupliakov et al. 1997), the size of the recycling pool is surprisingly small, with the majority of synaptic vesicles being inactive, a resting pool (Sudhof 2000). The smallest pool is the most active in release, while the largest pool appears to participate in release very little under regular conditions (see Sect. 1.6).

1.8.2 Synaptic Vesicle Trafficking

Synaptic vesicles trafficking into the RRP is regulated by actin and myosin II motors in response to action potential firing activity (Mochida et al. 1994a; Lee et al. 2012), in addition to the vesicle trafficking into the RP (Chandrasekar et al. 2013). Posttetanic potentiation at the calyx of Held synapse is caused by

increases not only in release probability but also in the RRP size estimated from a cumulative plot of EPSCs amplitudes (see Sect. 1.8.1), which contribute to the augmentation phase and the late phase of posttetanic potentiation, respectively, suggesting that synaptic vesicles in the fast-releasing pool was increased. Increase in the size of fast-releasing pool coupled to a decrease in the size of slow-releasing pool, indicating that the pools can be converted for each other (Sakaba and Neher 2001; Lee et al. 2012). The posttetanic complementary changes of fast- and slow-releasing pool sizes were abolished by inhibitors of myosin II or myosin light chain kinase (Lee et al. 2012). In SCG neuron, after the RRP depletion with extensive action potential firing, the rate of the RRP replenishment was slowed with myosin IIB loss of function (Hayashida and Mochida, unpublished data). Together, these data suggest that, responding to high firing activity, myosin II mediates mobilization of synaptic vesicles from the RP to the RRP, supplying active vesicles.

In SCG neuron, rapid-on-rate Ca^{2+}-chelator BAPTA reduced the RRP size in a vesicle-use-dependent manner (Mori et al. 2014), suggesting the existence of a low-affinity Ca^{2+} sensor in RRP replenishment that acts via fast endocytosis. A candidate sensor may be synaptotagmin that mediates endocytosis in *Drosophila* neuromuscular synapse and mammalian cortical synapses (Poskanzer et al. 2003; Nicholson-Tomishima and Ryan 2004). The rapid replenishment of the RRP in the SCG neurons is delayed with myosin VI loss of function (Hayashida and Mochida, unpublished data), suggesting that distinct motor protein bears specifically different synaptic vesicle trafficking pathways.

1.9 Conclusion

The presynaptic terminal is a tiny space where thousands of proteins (Takamori et al. 2006) are orchestrated by a conductor, action potential, to generate a chemical signal of neurotransmitter released from a synaptic vesicle. Between the action potential firing, presynaptic proteins complexes play a role in each synaptic vesicle phase, endocytosis, refilling of vesicles with transmitters, vesicle storage, trafficking of vesicle to the active zone, and docking and priming at the release site. The reader of this book will be impressed strongly by the attractive harmony of well-organized proteins' actions in each chapter.

References

Angel I, Fleissner A, Seifert R (1983) Synaptic vesicles from hog brain-their isolation and the coupling between synthesis and uptake of γ-aminobutyrate by glutamate decarboxylase. Neurochem Int 5:697–712

Antonin W, Riedel D, von Mollard GF (2000) The SNARE Vti1a-β is localized to small synaptic vesicles and participates in a novel SNARE complex. J Neurosci 20:5724–5732

Armbruster M, Messa M, Ferguson SM, De Camilli P, Ryan TA (2013) Dynamin phosphorylation controls optimization of endocytosis for brief action potential bursts. Elife 2:e00845

Artalejo CR, Henley JR, McNiven MA, Palfrey HC (1995) Rapid endocytosis coupled to exocytosis in adrenal chromaffin cells involves Ca^{2+}, GTP, and dynamin but not clathrin. Proc Natl Acad Sci U S A 92:8328–8332

Atluri PP, Ryan TA (2006) The kinetics of synaptic vesicle reacidification at hippocampal nerve terminals. J Neurosci 26:2313–2320

Baba T, Sakisaka T, Mochida S, Takai Y (2005) PKA-catalyzed phosphorylation of tomosyn and its implication in Ca^{2+}-dependent exocytosis of neurotransmitter. J Cell Biol 170:1113–1125

Bahler M, Greengard P (1987) Synapsin I bundles F-actin in a phosphorylation-dependent manner. Nature 326:704–707

Balaji J, Ryan TA (2007) Single-vesicle imaging reveals that synaptic vesicle exocytosis and endocytosis are coupled by a single stochastic mode. Proc Natl Acad Sci U S A 104:20576–20581

Balaji J, Armbruster M, Ryan TA (2008) Calcium control of endocytic capacity at a CNS synapse. J Neurosci 28:6742–6749

Banerjee A, Kowalchyk JA, DasGupta BR, Martin TFJ (1996) SNAP-25 is required for a late postdocking step in Ca^{2+}-dependent exocytosis. J Biol Chem 271:20227–20230

Barker LA, Dowdall MJ, Whittaker VP (1972) Choline metabolism in the cerebral cortex of guinea pigs. Stable-bound acetylcholine. Biochem J 130:1063–1075

Beck KA, Keen JH (1991) Interaction of phosphoinositide cycle intermediates with the plasma membrane-associated clathrin assembly protein AP-2. J Biol Chem 266:4442–4447

Beierlein M, Fioravante D, Regehr WG (2007) Differential expression of posttetanic potentiation and retrograde signaling mediate target-dependent short-term synaptic plasticity. Neuron 54:949–959

Bejanin S, Cervini R, Mallet J, Berrard S (1994) A unique gene organization for two cholinergic markers, choline acetyltransferase and a putative vesicular transporter of acetylcholine. J Biol Chem 269:21944–21947

Bellocchio EE, Reimer RJ, Fremeau RT Jr, Edwards RH (2000) Uptake of glutamate into synaptic vesicles by an inorganic phosphate transporter. Science 289:957–960

Benfenati F, Valtorta F, Rubenstein JL, Gorelick FS, Greengard P, Czernik AJ (1992) Synaptic vesicle-associated Ca^{2+}/calmodulin-dependent protein kinase II is a binding protein for synapsin I. Nature 359:417–420

Bennett MK, Calakos N, Scheller RH (1992) Syntaxin: a synaptic protein implicated in docking of synaptic vesicles at presynaptic active zones. Science 257:255–259

Betz A, Thakur P, Junge HJ, Ashery U, Rhee JS, Scheuss V, Rosenmund C, Rettig J, Brose N (2001) Functional interaction of the active zone proteins Munc13-1 and RIM1 in synaptic vesicle priming. Neuron 30:183–196

Bezprozvanny I, Scheller RH, Tsien RW (1995) Functional impact of syntaxin on gating of N-type and Q-type calcium channels. Nature 378:623–626

Bezprozvanny I, Zhong P, Scheller RH, Tsien RW (2000) Molecular determinants of the functional interaction between syntaxin and N-type Ca^{2+} channel gating. Proc Natl Acad Sci U S A 97:13943–13948

Bonifacino JS, Traub LM (2003) Signals for sorting of transmembrane proteins to endosomes and lysosomes. Annu Rev Biochem 72:395–447

Boucrot E, Pick A, Camdere G, Liska N, Evergren E, McMahon HT, Kozlov MM (2012) Membrane fission is promoted by insertion of amphipathic helices and is restricted by crescent BAR domains. Cell 149:124–136

Boulant S, Kural C, Zeeh JC, Ubelmann F, Kirchhausen T (2011) Actin dynamics counteract membrane tension during clathrin-mediated endocytosis. Nat Cell Biol 13:1124–1131

Bourne J, Morgan JR, Pieribone VA (2006) Actin polymerization regulates clathrin coat maturation during early stages of synaptic vesicle recycling at lamprey synapses. J Comp Neurol 497:600–609

Bracher A, Kadlec J, Betz H, Weissenhorn W (2002) X-ray structure of a neuronal complexin-SNARE complex from squid. J Biol Chem 277:26517–26523

Brager DH, Cai X, Thompson SM (2003) Activity-dependent activation of presynaptic protein kinase C mediates post-tetanic potentiation. Nat Neurosci 6:551–552

Broadie K, Prokop A, Bellen HJ, O'Kane CJ, Schulze KL, Sweeney ST (1995) Syntaxin and synaptobrevin function downstream of vesicle docking in drosophila. Neuron 15:663–673

Brose N, Petrenko AG, Sudhof TC, Jahn R (1992) Synaptotagmin: a calcium sensor on the synaptic vesicle surface. Science 256:1021–1025

Brose N, Hofmann K, Hata Y, Sudhof TC (1995) Mammalian homologues of *Caenorhabditis elegans unc-13* gene define novel family of C_2-domain proteins. J Biol Chem 270:25273–25280

Bucci G, Mochida S, Stephens GJ (2011) Inhibition of synaptic transmission and G protein modulation by synthetic $Ca_V2.2$ Ca^{2+} channel peptides. J Physiol 589:3085–3101

Burger PM, Hell J, Mehl E, Krasel C, Lottspeich F, Jahn R (1991) GABA and glycine in synaptic vesicles: storage and transport characteristics. Neuron 7:287–293

Burgoyne RD, Weiss JL (2001) The neuronal calcium sensor family of Ca^{2+}-binding proteins. Biochem J 353:1–12

Cai H, Reim K, Varoqueaux F, Tapechum S, Hill K, Sørensen JB, Brose N, Chow RH (2008) Complexin II plays a positive role in Ca^{2+}-triggered exocytosis by facilitating vesicle priming. Proc Natl Acad Sci U S A 105:19538–19543

Cao H, Garcia F, McNiven MA (1998) Differential distribution of dynamin isoforms in mammalian cells. Mol Biol Cell 9:2595–2609

Cao P, Maximov A, Sudhof TC (2011) Activity-dependent IGF-1 exocytosis is controlled by the Ca^{2+}-sensor synaptotagmin-10. Cell 145:300–311

Cao P, Yang X, Südhof TC (2013) Complexin activates exocytosis of distinct secretory vesicles controlled by different synaptotagmins. J Neurosci 33:1714–1727

Castillo PE, Schoch S, Schmitz F, Sudhof TC, Malenka RC (2002) RIM1alpha is required for presynaptic long-term potentiation. Nature 415:327–330

Catterall WA (2000) Structure and regulation of voltage-gated Ca^{2+} channels. Annu Rev Cell Dev Biol 16:521–555

Catterall WA, Few AP (2008) Calcium channel regulation and presynaptic plasticity. Neuron 59:882–901

Catterall WA, Leal K, Nanou E (2013) Calcium channels and short-term synaptic plasticity. J Biol Chem 288:10742–10749

Ceccaldi PE, Grohovaz F, Benfenati F, Chieregatti E, Greengard P, Valtorta F (1995) Dephosphorylated synapsin I anchors synaptic vesicles to actin cytoskeleton: an analysis by videomicroscopy. J Cell Biol 128:905–912

Chandrasekar I, Huettner JE, Turney SG, Bridgman PC (2013) Myosin II regulates activity dependent compensatory endocytosis at central synapses. J Neurosci 33:16131–16145

Chapman PF, Frenguelli BG, Smith A, Chen CM, Silva AJ (1995) The α-Ca^{2+}/calmodulin kinase II: a bidirectional modulator of presynaptic plasticity. Neuron 14:591–597

Chen X, Tomchick DR, Kovrigin E, Araç D, Machius M, Südhof TC, Rizo J (2002) Three-dimensional structure of the complexin/SNARE complex. Neuron 33:397–409

Chi P, Greengard P, Ryan TA (2001) Synapsin dispersion and reclustering during synaptic activity. Nat Neurosci 4:1187–1193

Chi P, Greengard P, Ryan TA (2003) Synaptic vesicle mobilization is regulated by distinct synapsin I phosphorylation pathways at different frequencies. Neuron 38:69–78

Christensen H, Fykse EM, Fonnum F (1990) Uptake of glycine into synaptic vesicles isolated from rat spinal cord. J Neurochem 54:1142–1147

Clayton EL, Evans GJO, Cousin MA (2008) Bulk synaptic vesicle endocytosis is rapidly triggered during strong stimulation. J Neurosci 28:6627–6632

Cohen MW, Jones OT, Angelides KJ (1991) Distribution of Ca^{2+} channels on frog motor nerve terminals revealed by fluorescent omega-conotoxin. J Neurosci 11:1032–1039

Conner SD, Schmid SL (2003) Regulated portals of entry into the cell. Nature 422:37–44

Conti F, Melone M, De Biasi S, Minelli A, Brecha NC, Ducati A (1998) Neuronal and glial localization of GAT-1, a high-affinity gamma-aminobutyric acid plasma membrane

transporter, in human cerebral cortex: with a note on its distribution in monkey cortex. J Comp Neurol 396:51–63
Cook TA, Urrutia R, McNiven MA (1994) Identification of dynamin 2, an isoform ubiquitously expressed in rat tissues. Proc Natl Acad Sci U S A 91:644–648
Coppola T, Magnin-Luthi S, Perret-Menoud V, Gattesco S, Schiavo G, Regazzi R (2001) Direct interaction of the Rab3 effector RIM with Ca^{2+} channels, SNAP-25, and synaptotagmin. J Biol Chem 276:32756–32762
Cousin MA, Robinson PJ (2001) The dephosphins: dephosphorylation by calcineurin triggers synaptic vesicle endocytosis. Trends Neurosci 24:659–665
Cremona O, Di Paolo G, Wenk MR, Luthi A, Kim WT, Takei K, Daniell L, Nemoto Y, Shears SB, Flavell RA, McCormick DA, De Camilli P (1999) Essential role of phosphoinositide metabolism in synaptic vesicle recycling. Cell 99:179–188
Cuttle MF, Tsujimoto T, Forsythe ID, Takahashi T (1998) Facilitation of the presynaptic calcium current at an auditory synapse in rat brainstem. J Physiol 512(Pt 3):723–729
Davletov BA, Sudhof TC (1993) A single C2 domain from synaptotagmin I is sufficient for high affinity Ca^{2+}/phospholipid binding. J Biol Chem 268:26386–26390
De Camilli P, Benfenati F, Valtorta F, Greengard P (1990) The synapsins. Annu Rev Cell Biol 6:433–460
Deák F, Xu Y, Chang W-P, Dulubova I, Khvotchev M, Liu X, Südhof TC, Rizo J (2009) Munc18-1 binding to the neuronal SNARE complex controls synaptic vesicle priming. J Cell Biol 184:751–764
Deitcher DL, Ueda A, Stewart BA, Burgess RW, Kidokoro Y, Schwarz TL (1998) Distinct requirements for evoked and spontaneous release of neurotransmitter are revealed by mutations in the D*rosophila* Gene *neuronal-synaptobrevin*. J Neurosci 18:2028–2039
DeMaria CD, Soong TW, Alseikhan BA, Alvania RS, Yue DT (2001) Calmodulin bifurcates the local Ca^{2+} signal that modulates P/Q-type Ca^{2+} channels. Nature 411:484–489
Deng L, Kaeser PS, Xu W, Südhof TC (2011) RIM proteins activate vesicle priming by reversing autoinhibitory homodimerization of Munc13. Neuron 69:317–331
Di Giovanni J, Boudkkazi S, Mochida S, Bialowas A, Samari N, Leveque C, Youssouf F, Brechet A, Iborra C, Maulet Y, Moutot N, Debanne D, Seagar M, El Far O (2010) V-ATPase membrane sector associates with synaptobrevin to modulate neurotransmitter release. Neuron 67:268–279
Dickman DK, Lu Z, Meinertzhagen IA, Schwarz TL (2006) Altered synaptic development and active zone spacing in endocytosis mutants. Curr Biol 16:591–598
Dingledine R, Korn SJ (1985) Gamma-aminobutyric acid uptake and the termination of inhibitory synaptic potentials in the rat hippocampal slice. J Physiol 366:387–409
Dittman J, Ryan TA (2009) Molecular circuitry of endocytosis at nerve terminals. Annu Rev Cell Dev Biol 25:133–160
Dulubova I, Yamaguchi T, Gao Y, Min SW, Huryeva I, Südhof TC, Rizo J (2002) How Tlg2p/syntaxin 16 'snares' Vps45. EMBO J 21:3620–3631
Dulubova I, Khvotchev M, Liu S, Huryeva I, Südhof TC, Rizo J (2007) Munc18-1 binds directly to the neuronal SNARE complex. Proc Natl Acad Sci U S A 104:2697–2702
Edeling MA, Smith C, Owen D (2006) Life of a clathrin coat: insights from clathrin and AP structures. Nat Rev Mol Cell Biol 7:32–44
Eggermann E, Bucurenciu I, Goswami SP, Jonas P (2012) Nanodomain coupling between Ca^{2+} channels and sensors of exocytosis at fast mammalian synapses. Nat Rev Neurosci 13:7–21
EL Far O, Seagar M (2011) A role for V-ATPase subunits in synaptic vesicle fusion? J Neurochem 10:1471–4159
Erickson JD, Eiden LE, Hoffman BJ (1992) Expression cloning of a reserpine-sensitive vesicular monoamine transporter. Proc Natl Acad Sci U S A 89:10993–10997
Erickson JD, Varoqui H, Schafer MK, Modi W, Diebler MF, Weihe E, Rand J, Eiden LE, Bonner TI, Usdin TB (1994) Functional identification of a vesicular acetylcholine transporter and its expression from a "cholinergic" gene locus. J Biol Chem 269:21929–21932

Erickson JD, Schafer MK, Bonner TI, Eiden LE, Weihe E (1996) Distinct pharmacological properties and distribution in neurons and endocrine cells of two isoforms of the human vesicular monoamine transporter. Proc Natl Acad Sci U S A 93:5166–5171

Faas GC, Raghavachari S, Lisman JE, Mody I (2011) Calmodulin as a direct detector of Ca^{2+} signals. Nat Neurosci 14:301–304

Fatt P, Katz B (1952) Spontaneous subthreshold activity at motor nerve endings. J Physiol 117:109–128

Fei H, Grygoruk A, Brooks ES, Chen A, Krantz DE (2008) Trafficking of vesicular neurotransmitter transporters. Traffic 9:1425–1436

Fenster SD, Chung WJ, Zhai R, Cases-Langhoff C, Voss B, Garner AM, Kaempf U, Kindler S, Gundelfinger ED, Garner CC (2000) Piccolo, a presynaptic zinc finger protein structurally related to bassoon. Neuron 25:203–214

Ferguson SM, De Camilli P (2012) Dynamin, a membrane-remodelling GTPase. Nat Rev Mol Cell Biol 13:75–88

Ferguson SM, Brasnjo G, Hayashi M, Wolfel M, Collesi C, Giovedi S, Raimondi A, Gong LW, Ariel P, Paradise S, O'Toole E, Flavell R, Cremona O, Miesenbock G, Ryan TA, De Camilli P (2007) A selective activity-dependent requirement for dynamin 1 in synaptic vesicle endocytosis. Science 316:570–574

Fischer von Mollard G, Stahl B, Walch-Solimena C, Takei K, Daniels L, Khoklatchev A, De Camilli P, Sudhof TC, Jahn R (1994) Localization of Rab5 to synaptic vesicles identifies endosomal intermediate in synaptic vesicle recycling pathway. Eur J Cell Biol 65:319–326

Ford MG, Pearse BM, Higgins MK, Vallis Y, Owen DJ, Gibson A, Hopkins CR, Evans PR, McMahon HT (2001) Simultaneous binding of PtdIns(4,5)P_2 and clathrin by AP180 in the nucleation of clathrin lattices on membranes. Science 291:1051–1055

Fremeau RT Jr, Troyer MD, Pahner I, Nygaard GO, Tran CH, Reimer RJ, Bellocchio EE, Fortin D, Storm-Mathisen J, Edwards RH (2001) The expression of vesicular glutamate transporters defines two classes of excitatory synapse. Neuron 31:247–260

Fujita Y, Shirataki H, Sakisaka T, Asakura T, Ohya T, Kotani H, Yokoyama S, Nishioka H, Matsuura Y, Mizoguchi A, Scheller RH, Takai Y (1998) Tomosyn: a syntaxin-1–binding protein that forms a novel complex in the neurotransmitter release process. Neuron 20:905–915

Fykse EM, Fonnum F (1988) Uptake of γ-aminobutyric acid by a synaptic vesicle fraction isolated from rat brain. J Neurochem 50:1237–1242

Fykse EM, Fonnum F (1996) Amino acid neurotransmission: dynamics of vesicular uptake. Neurochem Res 21:1053–1060

Gandhi SP, Stevens CF (2003) Three modes of synaptic vesicular recycling revealed by single-vesicle imaging. Nature 423:607–613

Geppert M, Goda Y, Hammer RE, Li C, Rosahl TW, Stevens CF, Südhof TC (1994) Synaptotagmin I: a major Ca^{2+} sensor for transmitter release at a central synapse. Cell 79:717–727

Gerber SH, Rah J-C, Min S-W, Liu X, de Wit H, Dulubova I, Meyer AC, Rizo J, Arancillo M, Hammer RE, Verhage M, Rosenmund C, Südhof TC (2008) Conformational switch of syntaxin-1 controls synaptic vesicle fusion. Science 321:1507–1510

Gillis KD, Mossner R, Neher E (1996) Protein kinase C enhances exocytosis from chromaffin cells by increasing the size of the readily releasable pool of secretory granules. Neuron 16:1209–1220

Gracheva EO, Hadwiger G, Nonet ML, Richmond JE (2008) Direct interactions between *C. elegans* RAB-3 and Rim provide a mechanism to target vesicles to the presynaptic density. Neurosci Lett 444:137–142

Granseth B, Odermatt B, Royle SJ, Lagnado L (2006) Clathrin-mediated endocytosis is the dominant mechanism of vesicle retrieval at hippocampal synapses. Neuron 51:773–786

Groffen AJA, Jacobsen L, Schut D, Verhage M (2005) Two distinct genes drive expression of seven tomosyn isoforms in the mammalian brain, sharing a conserved structure with a unique variable domain. J Neurochem 92:554–568

Guan R, Dai H, Harrison SC, Kirchhausen T (2010) Structure of the PTEN-like region of auxilin, a detector of clathrin-coated vesicle budding. Structure 18:1191–1198

Guastella J, Brecha N, Weigmann C, Lester HA, Davidson N (1992) Cloning, expression, and localization of a rat brain high-affinity glycine transporter. Proc Natl Acad Sci U S A 89:7189–7193

Haeseleer F, Sokal I, Verlinde CL, Erdjument-Bromage H, Tempst P, Pronin AN, Benovic JL, Fariss RN, Palczewski K (2000) Five members of a novel Ca^{2+}-binding protein (CABP) subfamily with similarity to calmodulin. J Biol Chem 275:1247–1260

Hallermann S, Fejtova A, Schmidt H, Weyhersmüller A, Silver RA, Gundelfinger ED, Eilers J (2010) Bassoon speeds vesicle reloading at a central excitatory synapse. Neuron 68:710–723

Han Y, Kaeser PS, Sudhof TC, Schneggenburger R (2011) RIM determines Ca^{2+} channel density and vesicle docking at the presynaptic active zone. Neuron 69:304–316

Hanson PI, Roth R, Morisaki H, Jahn R, Heuser JE (1997) Structure and conformational changes in NSF and its membrane receptor complexes visualized by quick-freeze/deep-etch electron microscopy. Cell 90:523–535

Hata Y, Slaughter CA, Sudhof TC (1993) Synaptic vesicle fusion complex contains *unc-18* homologue bound to syntaxin. Nature 366:347–351

Haucke V, De Camilli P (1999) AP-2 recruitment to synaptotagmin stimulated by tyrosine-based endocytic motifs. Science 285:1268–1271

Hayashi T, Yamasaki S, Nauenburg S, Binz T, Niemann H (1995) Disassembly of the reconstituted synaptic vesicle membrane fusion complex in vitro. EMBO J 14:2317–2325

Hayashi M, Raimondi A, O'Toole E, Paradise S, Collesi C, Cremona O, Ferguson SM, De Camilli P (2008) Cell- and stimulus-dependent heterogeneity of synaptic vesicle endocytic recycling mechanisms revealed by studies of dynamin 1-null neurons. Proc Natl Acad Sci U S A 105:2175–2180

He G, Gupta S, Yi M, Michaely P, Hobbs HH, Cohen JC (2002) ARH is a modular adaptor protein that interacts with the LDL receptor, clathrin, and AP-2. J Biol Chem 277:44044–44049

Heidelberger R, Zhou ZY, Matthews G (2002) Multiple components of membrane retrieval in synaptic terminals revealed by changes in hydrostatic pressure. J Neurophysiol 88:2509–2517

Herzog E, Bellenchi GC, Gras C, Bernard V, Ravassard P, Bedet C, Gasnier B, Giros B, El Mestikawy S (2001) The existence of a second vesicular glutamate transporter specifies subpopulations of glutamatergic neurons. J Neurosci 21:RC181

Heuser JE, Reese TS (1973) Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. J Cell Biol 57:315–344

Hibino H, Pironkova R, Onwumere O, Vologodskaia M, Hudspeth AJ, Lesage F (2002) RIM binding proteins (RBPs) couple Rab3-interacting molecules (RIMs) to voltage-gated Ca^{2+} channels. Neuron 34:411–423

Hiesinger PR, Fayyazuddin A, Mehta SQ, Rosenmund T, Schulze KL, Zhai RG, Verstreken P, Cao Y, Zhou Y, Kunz J, Bellen HJ (2005) The v-ATPase V_0 subunit a1 is required for a late step in synaptic vesicle exocytosis in *Drosophila*. Cell 121:607–620

Hinshaw JE, Schmid SL (1995) Dynamin self-assembles into rings suggesting a mechanism for coated vesicle budding. Nature 374:190–192

Holderith N, Lorincz A, Katona G, Rozsa B, Kulik A, Watanabe M, Nusser Z (2012) Release probability of hippocampal glutamatergic terminals scales with the size of the active zone. Nat Neurosci 15:988–997

Holtje M, von Jagow B, Pahner I, Lautenschlager M, Hortnagl H, Nurnberg B, Jahn R, Ahnert-Hilger G (2000) The neuronal monoamine transporter VMAT2 is regulated by the trimeric GTPase Go(2). J Neurosci 20:2131–2141

Hosaka M, Hammer RE, Sudhof TC (1999) A phospho-switch controls the dynamic association of synapsins with synaptic vesicles. Neuron 24:377–387

Hosoi N, Holt M, Sakaba T (2009) Calcium dependence of exo- and endocytotic coupling at a glutamatergic synapse. Neuron 63:216–229

Hull C, von Gersdorff H (2004) Fast endocytosis is inhibited by GABA-mediated chloride influx at a presynaptic terminal. Neuron 44:469–482

Humeau Y, Doussau F, Vitiello F, Greengard P, Benfenati F, Poulain B (2001) Synapsin controls both reserve and releasable synaptic vesicle pools during neuronal activity and short-term plasticity in *Aplysia*. J Neurosci 21:4195–4206

Hunt JM, Bommert K, Charlton MP, Kistner A, Habermann E, Augustine GJ, Betzt H (1994) A post-docking role for synaptobrevin in synaptic vesicle fusion. Neuron 12:1269–1279

Inoue E, Mochida S, Takagi H, Higa S, Deguchi-Tawarada M, Takao-Rikitsu E, Inoue M, Yao I, Takeuchi K, Kitajima I, Setou M, Ohtsuka T, Takai Y (2006) SAD: a presynaptic kinase associated with synaptic vesicles and the active zone cytomatrix that regulates neurotransmitter release. Neuron 50:261–275

Ishizuka T, Saisu H, Odani S, Abe T (1995) Synaphin: a protein associated with the docking/fusion complex in presynaptic terminals. Biochem Biophys Res Commun 213:1107–1114

Itoh T, De Camilli P (2006) BAR, F-BAR (EFC) and ENTH/ANTH domains in the regulation of membrane-cytosol interfaces and membrane curvature. Biochim Biophys Acta 1761:897–912

Jackson LP, Kelly BT, McCoy AJ, Gaffry T, James LC, Collins BM, Honing S, Evans PR, Owen DJ (2010) A large-scale conformational change couples membrane recruitment to cargo binding in the AP2 clathrin adaptor complex. Cell 141:1220–1229

Jarvis SE, Zamponi GW (2001) Distinct molecular determinants govern syntaxin 1A-mediated inactivation and G-protein inhibition of N-type calcium channels. J Neurosci 21:2939–2948

Jiang X, Lautermilch NJ, Watari H, Westenbroek RE, Scheuer T, Catterall WA (2008) Modulation of CaV2.1 channels by Ca^{2+}/calmodulin-dependent protein kinase II bound to the C-terminal domain. Proc Natl Acad Sci U S A 105:341–346

Jockusch WJ, Praefcke GJ, McMahon HT, Lagnado L (2005) Clathrin-dependent and clathrin-independent retrieval of synaptic vesicles in retinal bipolar cells. Neuron 46:869–878

Jones RT, Walker JH, Stadler H, Whittaker VP (1982) Immunohistochemical localization of a synaptic-vesicle antigen in a cholinergic neuron under conditions of stimulation and rest. Cell Tissue Res 223:117–126

Jorquera RA, Huntwork-Rodriguez S, Akbergenova Y, Cho RW, Littleton JT (2012) Complexin controls spontaneous and evoked neurotransmitter release by regulating the timing and properties of synaptotagmin activity. J Neurosci 32:18234–18245

Jovanovic JN, Benfenati F, Siow YL, Sihra TS, Sanghera JS, Pelech SL, Greengard P, Czernik AJ (1996) Neurotrophins stimulate phosphorylation of synapsin I by MAP kinase and regulate synapsin I-actin interactions. Proc Natl Acad Sci U S A 93:3679–3683

Jung N, Haucke V (2007) Clathrin-mediated endocytosis at synapses. Traffic 8:1129–1136

Junge HJ, Rhee J-S, Jahn O, Varoqueaux F, Spiess J, Waxham MN, Rosenmund C, Brose N (2004) Calmodulin and Munc13 form a Ca^{2+} sensor/effector complex that controls short-term synaptic plasticity. Cell 118:389–401

Kaeser PS, Deng L, Chavez AE, Liu X, Castillo PE, Sudhof TC (2009) ELKS2α/CAST deletion selectively increases neurotransmitter release at inhibitory synapses. Neuron 64:227–239

Kaeser PS, Deng L, Wang Y, Dulubova I, Liu X, Rizo J, Südhof TC (2011) RIM proteins tether Ca^{2+} channels to presynaptic active zones via a direct PDZ-domain interaction. Cell 144:282–295

Kaeser-Woo YJ, Yang X, Sudhof TC (2012) C-terminal complexin sequence is selectively required for clamping and priming but not for Ca^{2+} triggering of synaptic exocytosis. J Neurosci 32:2877–2885

Kaneko T, Fujiyama F (2002) Complementary distribution of vesicular glutamate transporters in the central nervous system. Neurosci Res 42:243–250

Kawasaki F, Hazen M, Ordway RW (2000) Fast synaptic fatigue in *shibire* mutants reveals a rapid requirement for dynamin in synaptic vesicle membrane trafficking. Nat Neurosci 3:859–860

Kennedy MB, Bennett MK, Bulleit RF, Erondu NE, Jennings VR, Miller SG, Molloy SS, Patton BL, Schenker LJ (1990) Structure and regulation of type II calcium/calmodulin-dependent protein kinase in central nervous system neurons. Cold Spring Harb Symp Quant Biol 55:101–110

Kessels MM, Qualmann B (2002) Syndapins integrate N-WASP in receptor-mediated endocytosis. EMBO J 21:6083–6094

Khvotchev M, Dulubova I, Sun J, Dai H, Rizo J, Sudhof TC (2007) Dual modes of Munc18-1/SNARE interactions are coupled by functionally critical binding to syntaxin-1 N terminus. J Neurosci 27:12147–12155

Kim DK, Catterall WA (1997) Ca^{2+}-dependent and -independent interactions of the isoforms of the α1A subunit of brain Ca^{2+} channels with presynaptic SNARE proteins. Proc Natl Acad Sci U S A 94:14782–14786

Kim SH, Ryan TA (2009) Synaptic vesicle recycling at CNS synapses without AP-2. J Neurosci 29:3865–3874

Kirchhausen T (2009) Imaging endocytic clathrin structures in living cells. Trends Cell Biol 19:596–605

Kish PE, Fischer-Bovenkerk C, Ueda T (1989) Active transport of γ-aminobutyric acid and glycine into synaptic vesicles. Proc Natl Acad Sci U S A 86:3877–3881

Kittel RJ, Wichmann C, Rasse TM, Fouquet W, Schmidt M, Schmid A, Wagh DA, Pawlu C, Kellner RR, Willig KI, Hell SW, Buchner E, Heckmann M, Sigrist SJ (2006) Bruchpilot promotes active zone assembly, Ca^{2+} channel clustering, and vesicle release. Science 312:1051–1054

Kiyonaka S, Wakamori M, Miki T, Uriu Y, Nonaka M, Bito H, Beedle AM, Mori E, Hara Y, De Waard M, Kanagawa M, Itakura M, Takahashi M, Campbell KP, Mori Y (2007) RIM1 confers sustained activity and neurotransmitter vesicle anchoring to presynaptic Ca^{2+} channels. Nat Neurosci 10:691–701

Kiyonaka S, Nakajima H, Takada Y, Hida Y, Yoshioka T, Hagiwara A, Kitajima I, Mori Y, Ohtsuka T (2012) Physical and functional interaction of the active zone protein CAST/ERC2 and the β-subunit of the voltage-dependent Ca^{2+} channel. J Biol Chem 152:149–159

Klingauf J (2007) Synaptic vesicle dynamics sans dynamin. Neuron 54:857–858

Klingauf J, Kavalali ET, Tsien RW (1998) Kinetics and regulation of fast endocytosis at hippocampal synapses. Nature 394:581–585

Koch D, Spiwoks-Becker I, Sabanov V, Sinning A, Dugladze T, Stellmacher A, Ahuja R, Grimm J, Schuler S, Muller A, Angenstein F, Ahmed T, Diesler A, Moser M, Tom Dieck S, Spessert R, Boeckers TM, Fassler R, Hubner CA, Balschun D, Gloveli T, Kessels MM, Qualmann B (2011) Proper synaptic vesicle formation and neuronal network activity critically rely on syndapin I. EMBO J 30:4955–4969

Koenig JH, Ikeda K (1989) Disappearance and reformation of synaptic vesicle membrane upon transmitter release observed under reversible blockage of membrane retrieval. J Neurosci 9:3844–3860

Koo SJ, Markovic S, Puchkov D, Mahrenholz CC, Beceren-Braun F, Maritzen T, Dernedde J, Volkmer R, Oschkinat H, Haucke V (2011) SNARE motif-mediated sorting of synaptobrevin by the endocytic adaptors clathrin assembly lymphoid myeloid leukemia (CALM) and AP180 at synapses. Proc Natl Acad Sci U S A 108:13540–13545

Koushika SP, Richmond JE, Hadwiger G, Weimer RM, Jorgensen EM, Nonet ML (2001) A post-docking role for active zone protein Rim. Nat Neurosci 4:997–1005

Krapivinsky G, Mochida S, Krapivinsky L, Cibulsky SM, Clapham DE (2006) The TRPM7 ion channel functions in cholinergic synaptic vesicles and affects transmitter release. Neuron 52:485–496

Kuromi H, Kidokoro Y (2002) Selective replenishment of two vesicle pools depends on the source of Ca^{2+} at the *Drosophila* synapse. Neuron 35:333–343

Landis DM, Hall AK, Weinstein LA, Reese TS (1988) The organization of cytoplasm at the presynaptic active zone of a central nervous system synapse. Neuron 1:201–209

Lautermilch NJ, Few AP, Scheuer T, Catterall WA (2005) Modulation of $Ca_V2.1$ channels by the neuronal calcium-binding protein visinin-like protein-2. J Neurosci 25:7062–7070

Leal K, Mochida S, Scheuer T, Catterall WA (2012) Fine-tuning synaptic plasticity by modulation of $Ca_V2.1$ channels with Ca^{2+} sensor proteins. Proc Natl Acad Sci U S A 109:17069–17074

Lee A, Wong ST, Gallagher D, Li B, Storm DR, Scheuer T, Catterall WA (1999) Ca^{2+}/calmodulin binds to and modulates P/Q-type calcium channels. Nature 399:155–159

Lee AT, Balasubramanian K, Schroit AJ (2000) β_2-Glycoprotein I-dependent alterations in membrane properties. Biochim Biophys Acta 1509:475–484

Lee A, Westenbroek RE, Haeseleer F, Palczewski K, Scheuer T, Catterall WA (2002) Differential modulation of $Ca_V2.1$ channels by calmodulin and Ca^{2+}-binding protein 1. Nat Neurosci 5:210–217

Lee JS, Ho WK, Lee SH (2012) Actin-dependent rapid recruitment of reluctant synaptic vesicles into a fast-releasing vesicle pool. Proc Natl Acad Sci U S A 109:E765–774

Leveque C, el Far O, Martin-Moutot N, Sato K, Kato R, Takahashi M, Seagar MJ (1994) Purification of the N-type calcium channel associated with syntaxin and synaptotagmin. A complex implicated in synaptic vesicle exocytosis. J Biol Chem 269:6306–6312

Li L, Chin LS, Shupliakov O, Brodin L, Sihra TS, Hvalby O, Jensen V, Zheng D, McNamara JO, Greengard P et al (1995) Impairment of synaptic vesicle clustering and of synaptic transmission, and increased seizure propensity, in synapsin I-deficient mice. Proc Natl Acad Sci U S A 92:9235–9239

Lin FT, Krueger KM, Kendall HE, Daaka Y, Fredericks ZL, Pitcher JA, Lefkowitz RJ (1997) Clathrin-mediated endocytosis of the β-adrenergic receptor is regulated by phosphorylation/dephosphorylation of β-arrestin1. J Biol Chem 272:31051–31057

Littleton JT, Stern M, Schulze K, Perin M, Bellen HJ (1993) Mutational analysis of *Drosophila* synaptotagmin demonstrates its essential role in Ca^{2+}-activated neurotransmitter release. Cell 74:1125–1134

Liu QR, Nelson H, Mandiyan S, Lopez-Corcuera B, Nelson N (1992a) Cloning and expression of a glycine transporter from mouse brain. FEBS Lett 305:110–114

Liu Y, Peter D, Roghani A, Schuldiner S, Prive GG, Eisenberg D, Brecha N, Edwards RH (1992b) A cDNA that suppresses MPP+ toxicity encodes a vesicular amine transporter. Cell 70:539–551

Llinás R, McGuinness TL, Leonard CS, Sugimori M, Greengard P (1985) Intraterminal injection of synapsin I or calcium/calmodulin-dependent protein kinase II alters neurotransmitter release at the squid giant synapse. Proc Natl Acad Sci U S A 82:3035–3039

Llinás R, Gruner JA, Sugimori M, McGuinness TL, Greengard P (1991) Regulation by synapsin I and Ca^{2+}-calmodulin-dependent protein kinase II of the transmitter release in squid giant synapse. J Physiol 436:257–282

Lonárt G, Schoch S, Kaeser PS, Larkin CJ, Sudhof TC, Linden DJ (2003) Phosphorylation of RIM1alpha by PKA triggers presynaptic long-term potentiation at cerebellar parallel fiber synapses. Cell 115:49–60

Lu FM, Hawkins RD (2006) Presynaptic and postsynaptic Ca^{2+} and CamKII contribute to long-term potentiation at synapses between individual CA3 neurons. Proc Natl Acad Sci U S A 103:4264–4269

Lu B, Greengard P, Poo MM (1992) Exogenous synapsin I promotes functional maturation of developing neuromuscular synapses. Neuron 8:521–529

Lu W, Ma H, Sheng ZH, Mochida S (2009) Dynamin and activity regulate synaptic vesicle recycling in sympathetic neurons. J Biol Chem 284:1930–1937

Luscher C, Nicoll RA, Malenka RC, Muller D (2000) Synaptic plasticity and dynamic modulation of the postsynaptic membrane. Nat Neurosci 3:545–550

Ma H, Mochida S (2007) A cholinergic model synapse to elucidate protein function at presynaptic terminals. Neurosci Res 57:491–498

Ma H, Cai Q, Lu W, Sheng ZH, Mochida S (2009) KIF5B motor adaptor syntabulin maintains synaptic transmission in sympathetic neurons. J Neurosci 29:13019–13029

Magupalli VG, Mochida S, Yan J, Jiang X, Westenbroek RE, Nairn AC, Scheuer T, Catterall WA (2013) Ca^{2+}-independent activation of Ca^{2+}/calmodulin-dependent protein kinase II bound to the C-terminal domain of $Ca_V2.1$ calcium channels. J Biol Chem 288:4637–4648

Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S (2002) The protein kinase complement of the human genome. Science 298:1912–1934

Maximov A, Tang J, Yang X, Pang ZP, Südhof TC (2009) Complexin controls the force transfer from SNARE complexes to membranes in fusion. Science 323:516–521
Maycox PR, Hell JW, Jahn R (1990) Amino acid neurotransmission: spotlight on synaptic vesicles. Trends Neurosci 13:83–87
Mayor F Jr, Marvizon JG, Aragon MC, Gimenez C, Valdivieso F (1981) Glycine transport into plasma-membrane vesicles derived from rat brain synaptosomes. Biochem J 198:535–541
McEwen JM, Madison JM, Dybbs M, Kaplan JM (2006) Antagonistic regulation of synaptic vesicle priming by Tomosyn and UNC-13. Neuron 51:303–315
McMahon HT, Boucrot E (2011) Molecular mechanism and physiological functions of clathrin-mediated endocytosis. Nat Rev Mol Cell Biol 12:517–533
McMahon HT, Missler M, Li C, Südhof TC (1995) Complexins: cytosolic proteins that regulate SNAP receptor function. Cell 83:111–119
McPherson PS (2010) Proteomic analysis of clathrin-coated vesicles. Proteomics 10:4025–4039
Merrifield CJ, Feldman ME, Wan L, Almers W (2002) Imaging actin and dynamin recruitment during invagination of single clathrin-coated pits. Nat Cell Biol 4:691–698
Miller TM, Heuser JE (1984) Endocytosis of synaptic vesicle membrane at the frog neuromuscular junction. J Cell Biol 98:685–698
Miller SE, Sahlender DA, Graham SC, Honing S, Robinson MS, Peden AA, Owen DJ (2011) The molecular basis for the endocytosis of small R-SNAREs by the clathrin adaptor CALM. Cell 147:1118–1131
Milosevic I, Giovedi S, Lou X, Raimondi A, Collesi C, Shen H, Paradise S, O'Toole E, Ferguson S, Cremona O, De Camilli P (2011) Recruitment of endophilin to clathrin-coated pit necks is required for efficient vesicle uncoating after fission. Neuron 72:587–601
Misura KM, Scheller RH, Weis WI (2000) Three-dimensional structure of the neuronal-Sec1-syntaxin 1a complex. Nature 404:355–362
Miyazaki T, Fukaya M, Shimizu H, Watanabe M (2003) Subtype switching of vesicular glutamate transporters at parallel fibre-Purkinje cell synapses in developing mouse cerebellum. Eur J Neurosci 17:2563–2572
Mochida S, Saisu H, Kobayashi H, Abe T (1995) Impairment of syntaxin by botulinum neurotoxin C1 or antibodies inhibits acetylcholine release but not Ca^{2+} channel activity. Neuroscience 65:905–915
Mochida S (2011a) Activity-dependent regulation of synaptic vesicle exocytosis and presynaptic short-term plasticity. Neurosci Res 70:16–23
Mochida S (2011b) Ca/Calmodulin and presynaptic short-term plasticity. ISRN Neurol 2011:919043
Mochida S, Kobayashi H, Matsuda Y, Yuda Y, Muramoto K, Nonomura Y (1994a) Myosin II is involved in transmitter release at synapses formed between rat sympathetic neurons in culture. Neuron 13:1131–1142
Mochida S, Nonomura Y, Kobayashi H (1994b) Analysis of the mechanism for acetylcholine release at the synapse formed between rat sympathetic neurons in culture. Microsc Res Tech 29:94–102
Mochida S, Sheng ZH, Baker C, Kobayashi H, Catterall WA (1996) Inhibition of neurotransmission by peptides containing the synaptic protein interaction site of N-type Ca^{2+} channels. Neuron 17:781–788
Mochida S, Westenbroek RE, Yokoyama CT, Itoh K, Catterall WA (2003) Subtype-selective reconstitution of synaptic transmission in sympathetic ganglion neurons by expression of exogenous calcium channels. Proc Natl Acad Sci U S A 100:2813–2818
Mochida S, Few AP, Scheuer T, Catterall WA (2008) Regulation of presynaptic $Ca_V2.1$ channels by Ca^{2+} sensor proteins mediates short-term synaptic plasticity. Neuron 57:210–216
Mooren OL, Kotova TI, Moore AJ, Schafer DA (2009) Dynamin2 GTPase and cortactin remodel actin filaments. J Biol Chem 284:23995–24005

Morel N, Dedieu J-C, Philippe J-M (2003) Specific sorting of the a1 isoform of the V-H+ ATPase a subunit to nerve terminals where it associates with both synaptic vesicles and the presynaptic plasma membrane. J Cell Sci 116:4751–4762

Morgan JR, Prasad K, Hao W, Augustine GJ, Lafer EM (2000) A conserved clathrin assembly motif essential for synaptic vesicle endocytosis. J Neurosci 20:8667–8676

Mori M, Tanufuji S, Mochida S (2014) Kinetic organization of Ca^{2+} signals that regulate synaptic release efficacy in sympathetic neurons. Mol Pharmacol 86:297–305

Morris SM, Cooper JA (2001) Disabled-2 colocalizes with the LDLR in clathrin-coated pits and interacts with AP-2. Traffic 2:111–123

Moulder KL, Mennerick S (2005) Reluctant vesicles contribute to the total readily releasable pool in glutamatergic hippocampal neurons. J Neurosci 25:3842–3850

Murthy VN, Stevens CF (1998) Synaptic vesicles retain their identity through the endocytic cycle. Nature 392:497–501

Murthy VN, Stevens CF (1999) Reversal of synaptic vesicle docking at central synapses. Nat Neurosci 2:503–507

Nakajima Y, Mochida S, Okawa K, Nakanishi S (2009) Ca^{2+}-dependent release of Munc18-1 from presynaptic mGluRs in short-term facilitation. Proc Natl Acad Sci U S A 106:18385–18389

Nakata T, Kitamura Y, Shimizu K, Tanaka S, Fujimori M, Yokoyama S, Ito K, Emi M (1999) Fusion of a novel gene, *ELKS*, to *RET* due to translocation t(10;12) (q11;p13) in a papillary thyroid carcinoma. Genes Chromosomes Cancer 25:97–103

Neher E (1998) Vesicle pools and Ca^{2+} microdomains: new tools for understanding their roles in neurotransmitter release. Neuron 20:389–399

Neher E (2010) Complexin: does it deserve its name? Neuron 68:803–806

Neher E, Sakaba T (2008) Multiple roles of calcium ions in the regulation of neurotransmitter release. Neuron 59:861–872

Neves G, Gomis A, Lagnado L (2001) Calcium influx selects the fast mode of endocytosis in the synaptic terminal of retinal bipolar cells. Proc Natl Acad Sci U S A 98:15282–15287

Nicholson-Tomishima K, Ryan TA (2004) Kinetic efficiency of endocytosis at mammalian CNS synapses requires synaptotagmin I. Proc Natl Acad Sci U S A 101:16648–16652

Nielander HB, Onofri F, Schaeffer E, Menegon A, Fesce R, Valtorta F, Greengard P, Benfenati F (1997) Phosphorylation-dependent effects of synapsin IIa on actin polymerization and network formation. Eur J Neurosci 9:2712–2722

Ohtsuka T, Takao-Rikitsu E, Inoue E, Inoue M, Takeuchi M, Matsubara K, Deguchi-Tawarada M, Satoh K, Morimoto K, Nakanishi H, Takai Y (2002) CAST: a novel protein of the cytomatrix at the active zone of synapses that forms a ternary complex with RIM1 and Munc13-1. J Cell Biol 158:577–590

Okamoto PM, Herskovits JS, Vallee RB (1997) Role of the basic, proline-rich region of dynamin in Src homology 3 domain binding and endocytosis. J Biol Chem 272:11629–11635

Owen DJ, Collins BM, Evans PR (2004) Adaptors for clathrin coats: structure and function. Annu Rev Cell Dev Biol 20:153–191

Pabst S, Hazzard JW, Antonin W, Südhof TC, Jahn R, Rizo J, Fasshauer D (2000) Selective interaction of complexin with the neuronal SNARE complex: determination of the binding regions. J Biol Chem 275:19808–19818

Park H, Li Y, Tsien RW (2012) Influence of synaptic vesicle position on release probability and exocytotic fusion mode. Science 335:1362–1366

Parsons TD, Lenzi D, Almers W, Roberts WM (1994) Calcium-triggered exocytosis and endocytosis in an isolated presynaptic cell: capacitance measurements in saccular hair cells. Neuron 13:875–883

Perin MS, Fried VA, Mignery GA, Jahn R, Sudhof TC (1990) Phospholipid binding by a synaptic vesicle protein homologous to the regulatory region of protein kinase C. Nature 345:260–263

Perin MS, Johnston PA, Ozcelik T, Jahn R, Francke U, Sudhof TC (1991) Structural and functional conservation of synaptotagmin (p65) in *Drosophila* and humans. J Biol Chem 266:615–622

Peter D, Liu Y, Sternini C, de Giorgio R, Brecha N, Edwards RH (1995) Differential expression of two vesicular monoamine transporters. J Neurosci 15:6179–6188

Pieribone VA, Shupliakov O, Brodin L, Hilfiker-Rothenfluh S, Czernik AJ, Greengard P (1995) Distinct pools of synaptic vesicles in neurotransmitter release. Nature 375:493–497

Pobbati AV, Razeto A, Böddener M, Becker S, Fasshauer D (2004) Structural basis for the inhibitory role of tomosyn in exocytosis. J Biol Chem 279:47192–47200

Ponce J, Poyatos I, Aragon C, Gimenez C, Zafra F (1998) Characterization of the 5′ region of the rat brain glycine transporter GLYT2 gene: identification of a novel isoform. Neurosci Lett 242:25–28

Poskanzer KE, Marek KW, Sweeney ST, Davis GW (2003) Synaptotagmin I is necessary for compensatory synaptic vesicle endocytosis *in vivo*. Nature 426:559–563

Praefcke GJ, McMahon HT (2004) The dynamin superfamily: universal membrane tubulation and fission molecules? Nat Rev Mol Cell Biol 5:133–147

Pyle JL, Kavalali ET, Piedras-Renteria ES, Tsien RW (2000) Rapid reuse of readily releasable pool vesicles at hippocampal synapses. Neuron 28:221–231

Raimondi A, Ferguson SM, Lou X, Armbruster M, Paradise S, Giovedi S, Messa M, Kono N, Takasaki J, Cappello V, O'Toole E, Ryan TA, De Camilli P (2011) Overlapping role of dynamin isoforms in synaptic vesicle endocytosis. Neuron 70:1100–1114

Reetz A, Solimena M, Matteoli M, Folli F, Takei K, De Camilli P (1991) GABA and pancreatic β-cells: colocalization of glutamic acid decarboxylase (GAD) and GABA with synaptic-like microvesicles suggests their role in GABA storage and secretion. EMBO J 10:1275–1284

Reim K, Mansour M, Varoqueaux F, McMahon HT, Südhof TC, Brose N, Rosenmund C (2001) Complexins regulate a late step in Ca^{2+}-dependent neurotransmitter release. Cell 104:71–81

Reimer RJ, Fon EA, Edwards RH (1998) Vesicular neurotransmitter transport and the presynaptic regulation of quantal size. Curr Opin Neurobiol 8:405–412

Rettig J, Sheng ZH, Kim DK, Hodson CD, Snutch TP, Catterall WA (1996) Isoform-specific interaction of the α1A subunits of brain Ca^{2+} channels with the presynaptic proteins syntaxin and SNAP-25. Proc Natl Acad Sci U S A 93:7363–7368

Rosahl TW, Spillane D, Missler M, Herz J, Selig DK, Wolff JR, Hammer RE, Malenka RC, Sudhof TC (1995) Essential functions of synapsins I and II in synaptic vesicle regulation. Nature 375:488–493

Rothman JE (1994) Mechanisms of intracellular protein transport. Nature 372:55–63

Royle SJ, Lagnado L (2003) Endocytosis at the synaptic terminal. J Physiol 553:345–355

Ryan TA, Li L, Chin LS, Greengard P, Smith SJ (1996a) Synaptic vesicle recycling in synapsin I knock-out mice. J Cell Biol 134:1219–1227

Ryan TA, Smith SJ, Reuter H (1996b) The timing of synaptic vesicle endocytosis. Proc Natl Acad Sci U S A 93:5567–5571

Sakaba T (2006) Roles of the fast-releasing and the slowly releasing vesicles in synaptic transmission at the calyx of held. J Neurosci 26:5863–5871

Sakaba T, Neher E (2001) Calmodulin mediates rapid recruitment of fast-releasing synaptic vesicles at a calyx-type synapse. Neuron 32:1119–1131

Sankaranarayanan S, Ryan TA (2001) Calcium accelerates endocytosis of vSNAREs at hippocampal synapses. Nat Neurosci 4:129–136

Sankaranarayanan S, Atluri PP, Ryan TA (2003) Actin has a molecular scaffolding, not propulsive, role in presynaptic function. Nat Neurosci 6:127–135

Schafer DA, Weed SA, Binns D, Karginov AV, Parsons JT, Cooper JA (2002) Dynamin2 and cortactin regulate actin assembly and filament organization. Curr Biol 2:1852–1857

Schmid EM, McMahon HT (2007) Integrating molecular and network biology to decode endocytosis. Nature 448:883–888

Schoch S, Castillo PE, Jo T, Mukherjee K, Geppert M, Wang Y, Schmitz F, Malenka RC, Sudhof TC (2002) RIM1alpha forms a protein scaffold for regulating neurotransmitter release at the active zone. Nature 415:321–326

Schulman H, Greengard P (1978) Ca^{2+}-dependent protein phosphorylation system in membranes from various tissues, and its activation by "calcium-dependent regulator". Proc Natl Acad Sci U S A 75:5432–5436

Schulze KL, Broadie K, Perin MS, Bellen HJ (1995) Genetic and electrophysiological studies of drosophila syntaxin-1A demonstrate its role in nonneuronal secretion and neurotransmission. Cell 80:311–320

Schuske KR, Richmond JE, Matthies DS, Davis WS, Runz S, Rube DA, van der Bliek AM, Jorgensen EM (2003) Endophilin is required for synaptic vesicle endocytosis by localizing synaptojanin. Neuron 40:749–762

Serra-Pages C, Kedersha NL, Fazikas L, Medley Q, Debant A, Streuli M (1995) The LAR transmembrane protein tyrosine phosphatase and a coiled-coil LAR-interacting protein co-localize at focal adhesions. EMBO J 14:2827–2838

Sheng ZH, Rettig J, Takahashi M, Catterall WA (1994) Identification of a syntaxin-binding site on N-type calcium channels. Neuron 13:1303–1313

Sheng ZH, Rettig J, Cook T, Catterall WA (1996) Calcium-dependent interaction of N-type calcium channels with the synaptic core complex. Nature 379:451–454

Sheng ZH, Yokoyama CT, Catterall WA (1997) Interaction of the synprint site of N-type Ca^{2+} channels with the C2B domain of synaptotagmin I. Proc Natl Acad Sci U S A 94:5405–5410

Shepherd JD, Huganir RL (2007) The cell biology of synaptic plasticity: AMPA receptor trafficking. Annu Rev Cell Dev Biol 23:613–643

Shupliakov O, Low P, Grabs D, Gad H, Chen H, David C, Takei K, De Camilli P, Brodin L (1997) Synaptic vesicle endocytosis impaired by disruption of dynamin-SH3 domain interactions. Science 276:259–263

Shupliakov O, Bloom O, Gustafsson JS, Kjaerulff O, Low P, Tomilin N, Pieribone VA, Greengard P, Brodin L (2002) Impaired recycling of synaptic vesicles after acute perturbation of the presynaptic actin cytoskeleton. Proc Natl Acad Sci U S A 99:14476–14481

Silver RB, Sugimori M, Lang EJ, Llinas R (1994) Time-resolved imaging of Ca^{2+}-dependent aequorin luminescence of microdomains and QEDs in synaptic preterminals. Biol Bull 187:293–299

Slepnev VI, De Camilli P (2000) Accessory factors in clathrin-dependent synaptic vesicle endocytosis. Nat Rev Neurosci 1:161–172

Smith KE, Borden LA, Hartig PR, Branchek T, Weinshank RL (1992) Cloning and expression of a glycine transporter reveal colocalization with NMDA receptors. Neuron 8:927–935

Sollner T, Bennett MK, Whiteheart SW, Scheller RH, Rothman JE (1993a) A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. Cell 75:409–418

Sollner T, Whiteheart SW, Brunner M, Erdjument-Bromage H, Geromanos S, Tempst P, Rothman JE (1993b) SNAP receptors implicated in vesicle targeting and fusion. Nature 362:318–324

Stephens GJ, Mochida S (2005) G protein βγ subunits mediate presynaptic inhibition of transmitter release from rat superior cervical ganglion neurones in culture. J Physiol 563:765–776

Stevens CF, Williams JH (2007) Discharge of the readily releasable pool with action potentials at hippocampal synapses. J Neurophysiol 98:3221–3229

Su SC, Seo J, Pan Jen Q, Samuels Benjamin A, Rudenko A, Ericsson M, Neve Rachael L, Yue David T, Tsai L-H (2012) Regulation of N-type voltage-gated calcium channels and presynaptic function by cyclin-dependent kinase 5. Neuron 75:675–687

Sudhof TC (1995) The synaptic vesicle cycle: a cascade of protein-protein interactions. Nature 375:645–653

Sudhof TC (2000) The synaptic vesicle cycle revisited. Neuron 28:317–320

Sudhof TC (2004) The synaptic vesicle cycle. Annu Rev Neurosci 27:509–547

Sudhof TC (2012) The presynaptic active zone. Neuron 75:11–25

Sun JY, Wu LG (2001) Fast kinetics of exocytosis revealed by simultaneous measurements of presynaptic capacitance and postsynaptic currents at a central synapse. Neuron 30:171–182

Sun JY, Wu XS, Wu LG (2002) Single and multiple vesicle fusion induce different rates of endocytosis at a central synapse. Nature 417:555–559

Sun J, Pang ZP, Qin D, Fahim AT, Adachi R, Sudhof TC (2007) A dual-Ca^{2+}-sensor model for neurotransmitter release in a central synapse. Nature 450:676–682

Sutton RB, Davletov BA, Berghuis AM, Sudhof TC, Sprang SR (1995) Structure of the first C_2 domain of synaptotagmin I: a novel Ca^{2+}/phospholipid-binding fold. Cell 80:929–938

Sweeney ST, Broadie K, Keane J, Niemann H, O'Kane CJ (1995) Targeted expression of tetanus toxin light chain in Drosophila specifically eliminates synaptic transmission and causes behavioral defects. Neuron 14:341–351

Takamori S, Holt M, Stenius K, Lemke EA, Gronborg M, Riedel D, Urlaub H, Schenck S, Brugger B, Ringler P, Muller SA, Rammner B, Grater F, Hub JS, De Groot BL, Mieskes G, Moriyama Y, Klingauf J, Grubmuller H, Heuser J, Wieland F, Jahn R (2006) Molecular anatomy of a trafficking organelle. Cell 127:831–846

Takao-Rikitsu E, Mochida S, Inoue E, Deguchi-Tawarada M, Inoue M, Ohtsuka T, Takai Y (2004) Physical and functional interaction of the active zone proteins, CAST, RIM1, and Bassoon, in neurotransmitter release. J Cell Biol 164:301–311

Takei K, McPherson PS, Schmid SL, De Camilli P (1995) Tubular membrane invaginations coated by dynamin rings are induced by GTP-γ-S in nerve terminals. Nature 374:186–190

Takenawa T, Suetsugu S (2007) The WASP-WAVE protein network: connecting the membrane to the cytoskeleton. Nat Rev Mol Cell Biol 8:37–48

Tang J, Maximov A, Shin O-H, Dai H, Rizo J, Südhof TC (2006) A complexin/synaptotagmin 1 switch controls fast synaptic vesicle exocytosis. Cell 126:1175–1187

Tanifuji S, Funakoshi-Tago M, Ueda F, Kasahara T, Mochida S (2013) Dynamin isoforms decode action potential firing for synaptic vesicle recycling. J Biol Chem 288:19050–19059

Taylor MJ, Lampe M, Merrifield CJ (2012) A feedback loop between dynamin and actin recruitment during clathrin-mediated endocytosis. PLoS Biol 10:e1001302

Thomas L, Betz H (1990) Synaptophysin binds to physophilin, a putative synaptic plasma membrane protein. J Cell Biol 111:2041–2052

tom Dieck S, Sanmarti-Vila L, Langnaese K, Richter K, Kindler S, Soyke A, Wex H, Smalla KH, Kampf U, Franzer JT, Stumm M, Garner CC, Gundelfinger ED (1998) Bassoon, a novel zinc-finger CAG/glutamine-repeat protein selectively localized at the active zone of presynaptic nerve terminals. J Cell Biol 142:499–509

tom Dieck S, Specht D, Strenzke N, Hida Y, Krishnamoorthy V, Schmidt K-F, Inoue E, Ishizaki H, Tanaka-Okamoto M, Miyoshi J, Hagiwara A, Brandstätter JH, Löwel S, Gollisch T, Ohtsuka T, Moser T (2012) Deletion of the presynaptic scaffold CAST reduces active zone size in rod photoreceptors and impairs visual processing. J Neurosci 32:12192–12203

Traub LM (2003) Sorting it out: AP-2 and alternate clathrin adaptors in endocytic cargo selection. J Cell Biol 163:203–208

Trimble WS, Cowan DM, Scheller RH (1988) VAMP-1: a synaptic vesicle-associated integral membrane protein. Proc Natl Acad Sci U S A 85:4538–4542

Valtorta F, Iezzi N, Benfenati F, Lu B, Poo MM, Greengard P (1995) Accelerated structural maturation induced by synapsin I at developing neuromuscular synapses of *Xenopus laevis*. Eur J Neurosci 7:261–270

von Gersdorff H, Matthews G (1994) Dynamics of synaptic vesicle fusion and membrane retrieval in synaptic terminals. Nature 367:735–739

Wadel K, Neher E, Sakaba T (2007) The coupling between synaptic vesicles and Ca^{2+} channels determines fast neurotransmitter release. Neuron 53:563–575

Wagh DA, Rasse TM, Asan E, Hofbauer A, Schwenkert I, Durrbeck H, Buchner S, Dabauvalle MC, Schmidt M, Qin G, Wichmann C, Kittel R, Sigrist SJ, Buchner E (2006) Bruchpilot, a protein with homology to ELKS/CAST, is required for structural integrity and function of synaptic active zones in *Drosophila*. Neuron 49:833–844

Walther K, Krauss M, Diril MK, Lemke S, Ricotta D, Honing S, Kaiser S, Haucke V (2001) Human stoned B interacts with AP-2 and synaptotagmin and facilitates clathrin-coated vesicle uncoating. EMBO Rep 2:634–640

Walther K, Diril MK, Jung N, Haucke V (2004) Functional dissection of the interactions of stonin 2 with the adaptor complex AP-2 and synaptotagmin. Proc Natl Acad Sci U S A 101:964–969

Wang Y, Okamoto M, Schmitz F, Hofmann K, Sudhof TC (1997) Rim is a putative Rab3 effector in regulating synaptic-vesicle fusion. Nature 388:593–598

Wang X, Kibschull M, Laue MM, Lichte B, Petrasch-Parwez E, Kilimann MW (1999) Aczonin, a 550-kD putative scaffolding protein of presynaptic active zones, shares homology regions with Rim and Bassoon and binds profilin. J Cell Biol 147:151–162

Wang Y, Liu X, Biederer T, Sudhof TC (2002) A family of RIM-binding proteins regulated by alternative splicing: implications for the genesis of synaptic active zones. Proc Natl Acad Sci U S A 99:14464–14469

Watanabe S, Rost BR, Camacho-Perez M, Davis MW, Sohl-Kielczynski B, Rosenmund C, Jorgensen EM (2013) Ultrafast endocytosis at mouse hippocampal synapses. Nature 504:242–247

Weihe E, Schafer MK, Erickson JD, Eiden LE (1994) Localization of vesicular monoamine transporter isoforms (VMAT1 and VMAT2) to endocrine cells and neurons in rat. J Mol Neurosci 5:149–164

Westenbroek RE, Hell JW, Warner C, Dubel SJ, Snutch TP, Catterall WA (1992) Biochemical properties and subcellular distribution of an N-type calcium channel α1 subunit. Neuron 9:1099–1115

Westenbroek RE, Sakurai T, Elliott EM, Hell JW, Starr TV, Snutch TP, Catterall WA (1995) Immunochemical identification and subcellular distribution of the α1A subunits of brain calcium channels. J Neurosci 15:6403–6418

Weyhersmüller A, Hallermann S, Wagner N, Eilers J (2011) Rapid active zone remodeling during synaptic plasticity. J Neurosci 31:6041–6052

Wiser O, Bennett MK, Atlas D (1996) Functional interaction of syntaxin and SNAP-25 with voltage-sensitive L- and N-type Ca^{2+} channels. EMBO J 15:4100–4110

Wiser O, Tobi D, Trus M, Atlas D (1997) Synaptotagmin restores kinetic properties of a syntaxin-associated N-type voltage sensitive calcium channel. FEBS Lett 404:203–207

Wojcik SM, Brose N (2007) Regulation of membrane fusion in synaptic excitation-secretion coupling: speed and accuracy matter. Neuron 55:11–24

Wu LG (2004) Kinetic regulation of vesicle endocytosis at synapses. Trends Neurosci 27:548–554

Wu LG, Betz WJ (1996) Nerve activity but not intracellular calcium determines the time course of endocytosis at the frog neuromuscular junction. Neuron 17:769–779

Wu W, Wu L-G (2007) Rapid bulk endocytosis and its kinetics of fission pore closure at a central synapse. Proc Natl Acad Sci U S A 104:10234–10239

Wu W, Xu J, Wu XS, Wu LG (2005) Activity-dependent acceleration of endocytosis at a central synapse. J Neurosci 25:11676–11683

Wu XS, McNeil BD, Xu J, Fan J, Xue L, Melicoff E, Adachi R, Bai L, Wu LG (2009) Ca^{2+} and calmodulin initiate all forms of endocytosis during depolarization at a nerve terminal. Nat Neurosci 12:1003–1010

Xing Y, Bocking T, Wolf M, Grigorieff N, Kirchhausen T, Harrison SC (2010) Structure of clathrin coat with bound Hsc70 and auxilin: mechanism of Hsc70-facilitated disassembly. EMBO J 29:655–665

Xu J, Mashimo T, Sudhof TC (2007) Synaptotagmin-1, -2, and -9: Ca^{2+} sensors for fast release that specify distinct presynaptic properties in subsets of neurons. Neuron 54:567–581

Xu J, McNeil B, Wu W, Nees D, Bai L, Wu LG (2008) GTP-independent rapid and slow endocytosis at a central synapse. Nat Neurosci 11:45–53

Xu J, Pang ZP, Shin OH, Sudhof TC (2009) Synaptotagmin-1 functions as a Ca^{2+} sensor for spontaneous release. Nat Neurosci 12:759–766

Xue M, Reim K, Chen X, Chao HT, Deng H, Rizo J, Brose N, Rosenmund C (2007) Distinct domains of complexin I differentially regulate neurotransmitter release. Nat Struct Mol Biol 14:949–958

Yamamoto Y, Mochida S, Kurooka T, Sakisaka T (2009) Reciprocal intramolecular interactions of tomosyn control its inhibitory activity on SNARE complex formation. J Biol Chem 284:12480–12490

Yamashita T, Eguchi K, Saitoh N, von Gersdorff H, Takahashi T (2010) Developmental shift to a mechanism of synaptic vesicle endocytosis requiring nanodomain Ca^{2+}. Nat Neurosci 13:838–844

Yao CK, Lin YQ, Ly CV, Ohyama T, Haueter CM, Moiseenkova-Bell VY, Wensel TG, Bellen HJ (2009) A synaptic vesicle-associated Ca^{2+} channel promotes endocytosis and couples exocytosis to endocytosis. Cell 138:947–960

Yim YI, Sun T, Wu LG, Raimondi A, De Camilli P, Eisenberg E, Greene LE (2010) Endocytosis and clathrin-uncoating defects at synapses of auxilin knockout mice. Proc Natl Acad Sci U S A 107:4412–4417

Yizhar O, Matti U, Melamed R, Hagalili Y, Bruns D, Rettig J, Ashery U (2004) Tomosyn inhibits priming of large dense-core vesicles in a calcium-dependent manner. Proc Natl Acad Sci U S A 101:2578–2583

Yokoyama CT, Myers SJ, Fu J, Mockus SM, Scheuer T, Catterall WA (2005) Mechanism of SNARE protein binding and regulation of Ca_V2 channels by phosphorylation of the synaptic protein interaction site. Mol Cell Neurosci 28:1–17

Yoshida A, Oho C, Omori A, Kuwahara R, Ito T, Takahashi M (1992) HPC-1 is associated with synaptotagmin and omega-conotoxin receptor. J Biol Chem 267:24925–24928

Yoshihara M, Littleton JT (2002) Synaptotagmin I functions as a calcium sensor to synchronize neurotransmitter release. Neuron 36:897–908

Yue HY, Xu J (2014) Myosin light chain kinase accelerates vesicle endocytosis at the calyx of Held synapse. J Neurosci 34:295–304

Zhang JZ, Davletov BA, Sudhof TC, Anderson RG (1994) Synaptotagmin I is a high affinity receptor for clathrin AP-2: implications for membrane recycling. Cell 78:751–760

Zhang C, Xiong W, Zheng H, Wang L, Lu B, Zhou Z (2004) Calcium- and dynamin-independent endocytosis in dorsal root ganglion neurons. Neuron 42:225–236

Zhang Q, Li Y, Tsien RW (2009) The dynamic control of kiss-and-run and vesicular reuse probed with single nanoparticles. Science 323:1448–1453

Zhong H, Yokoyama CT, Scheuer T, Catterall WA (1999) Reciprocal regulation of P/Q-type Ca^{2+} channels by SNAP-25, syntaxin and synaptotagmin. Nat Neurosci 2:939–941

Zhou Q, Petersen CC, Nicoll RA (2000) Effects of reduced vesicular filling on synaptic transmission in rat hippocampal neurones. J Physiol 525(Pt 1):195–206

Zhou P, Pang ZP, Yang X, Zhang Y, Rosenmund C, Bacaj T, Südhof TC (2012) Syntaxin-1 N-peptide and H_{abc}-domain perform distinct essential functions in synaptic vesicle fusion. EMBO J 32:159–171

Zhu Y, Xu J, Heinemann SF (2009) Two pathways of synaptic vesicle retrieval revealed by single-vesicle imaging. Neuron 61:397–411

Zoncu R, Perera RM, Balkin DM, Pirruccello M, Toomre D, De Camilli P (2009) A phosphoinositide switch controls the maturation and signaling properties of APPL endosomes. Cell 136:1110–1121

Zucker RS, Regehr WG (2002) Short-term synaptic plasticity. Annu Rev Physiol 64:355–405

Part II
Presynaptic Active Zone Proteins

Chapter 2
Active Zone Assembly

Thomas Dresbach

Abstract The formation of neurotransmitter release sites, or active zones, is a fundamental process in neuronal development. It allows neurons to communicate with their targets through the regulated exocytosis of neurotransmitter. To this end, active zones, including a complex network of presynaptic scaffolding proteins called the cytomatrix of active zones, have to be formed at defined sites inside an axon. This poses a substantial challenge to cellular transport and assembly mechanisms, because a complex network of cytomatrix proteins has to be formed exactly opposite postsynaptic specializations. A large number of proteins have been implicated in active zone assembly, but how these proteins interact to mediate the transport, recruitment, and organization of the cytomatrix network has remained unclear.

Here I will review and discuss current notions of the various steps required to ultimately form active zones in axons of vertebrate and invertebrate neurons. To execute the whole process, the cell biological machinery of a neuron appears to generate organelles designed for the transport of cytomatrix components; these organelles then undergo trafficking events that are regulated to ensure the deposition of cytomatrix material at nascent active zones. Local signals act to both capture and release cytomatrix material at active zones in a controlled manner. Cell adhesion molecules allow for transsynaptic signalling, to synchronize pre- and postsynaptic assembly. Various cell adhesion systems may cooperate to mediate the local assembly of active zones and to endow each active zone with its specific functional properties. Together, these events act to coordinate the process of active zone assembly, thus generating the output sites for synaptic signalling.

Keywords CAZ, cytomatrix of active zones • PTVs, Piccolo-Bassoon transport vesicles • Precursor trafficking • Axonal pause sites • Synaptic cell adhesion molecules • Neurexins, receptor protein tyrosine phosphatases

T. Dresbach (✉)
Institute for Anatomy and Embryology, Synaptogenesis Group, University Medical Center Goettingen, Kreuzbergring 36, 37075 Goettingen, Germany
e-mail: Thomas.dresbach@med.uni-geottingen.de

S. Mochida (ed.), *Presynaptic Terminals*, DOI 10.1007/978-4-431-55166-9_2

2.1 Introduction

Synapses are asymmetric cell-cell contact sites designed for the rapid communication between neurons, occurring on a millisecond time scale. Their structural asymmetry correlates with functional asymmetry: the presynaptic side is specialized for the regulated, depolarization- and calcium-dependent exocytosis of neurotransmitter, while the postsynaptic side is specialized for the detection of neurotransmitter by clustered neurotransmitter receptors. Neurotransmitter release occurs from axonal specializations called presynaptic boutons, which may be spaced along the length of the axon ("en passant boutons") or located at the distal ends of axonal arbors ("boutons terminaux"). Each bouton harbors the machinery required for neurotransmitter release, including as component parts (a) synaptic vesicles storing neurotransmitter; (b) a specialized, on average 500 nm diameter, area of the axonal plasma membrane called active zone (Couteaux and Pecot-Dechavassine 1970), where neurotransmitter release can occur; and (c) a network of cytoplasmic scaffolding proteins termed the *c*ytomatrix of *a*ctive *z*ones or CAZ (Dresbach et al. 2001, 2006a). This network of scaffolding proteins restricts neurotransmitter release to active zones and regulates its properties (Schoch and Gundelfinger 2006; Sudhof 2012). The active zone including its associated CAZ are situated directly opposite to an analogous specialization of the postsynaptic plasma membrane: neurotransmitter receptors are clustered in the dendritic or somatic membrane, and a network of cytoplasmic scaffolding proteins termed the *post*synaptic *d*ensity, or PSD, acts to recruit, cluster, and regulate postsynaptic receptors (Boeckers 2006). Thus, the CAZ and the PSD are aligned to each other, spaced by the synaptic cleft, and may be connected to each other by transsynaptic interactions bridging the synaptic cleft. This is also suggested by the fact that a complex of proteins of the CAZ and the PSD is preserved in biochemical preparations (Langnaese et al. 1996; Phillips et al. 2001).

The organization of the axon as a cable with multiple presynaptic boutons allows for long-range connectivity of a presynaptic cell with a large number of postsynaptic partners. This organization poses several challenges to the underlying cell biological mechanisms: first, the neuron has to transport presynaptic material into the axon and provide modes for its selective deposition in boutons. Whatever the mechanisms are, motor and adaptor proteins providing axonal transport as well as cytoskeletal elements and signals allowing for local accumulation of synaptic material in boutons must be involved (Sabo et al. 2006; Maeder et al. 2014). Second, the assembly and maintenance of the presynaptic machinery must occur directly opposite postsynaptic sites. It is likely that cell adhesion molecules and transsynaptic signalling events – including direct physical interactions and diffusible factors – have a role in local synapse assembly and maintenance (Shen and Scheiffele 2010; Brose 2013; Chia et al. 2013). In addition, increasing the half-life of protein complexes after their initial assembly – for example, by locally slowing protein degradation at active zones – may be involved in active zone maintenance (Waites et al. 2013). Third, the complex architecture of the presynaptic apparatus,

including synaptic vesicle clusters, docked synaptic vesicles, and the CAZ itself, has to be assembled within any presynaptic bouton. Current notions hold that this involves specialized precursor organelles that deliver sets of pre-assembled molecular complexes in a modular fashion (Fejtova and Gundelfinger 2006; Owald and Sigrist 2009).

2.2 Structure and Molecular Architecture of the CAZ

The CAZ is a key component of presynaptic bouton architecture. It is both an ultrastructural signature and a functional pivot: as outlined below, the proteins of the CAZ may determine the site of neurotransmitter release. In electron microscopy, the CAZ becomes visible as a prominent electron-dense meshwork, both in sections of brain tissue and in biochemical fractions of synaptic junctions. Based on its ultrastructural appearance it has also been termed the presynaptic particle web or presynaptic grid (Gray 1963; Triller and Korn 1985; Pfenninger et al. 1969; Phillips et al. 2001). This is because using aldehyde fixation and phosphotungstic acid staining, regularly spaced electron-dense projections seem to arise from the plasma membrane, with synaptic vesicles located between them in close apposition to the plasma membrane. Using quick-freeze methods, which work without aldehyde fixation and are thought to preserve synapses in a close to native state, filaments of various lengths became visible, some of them apparently cross bridging synaptic vesicles, while others seem to emerge from the plasma membrane, potentially tethering synaptic vesicles to the active zone (Landis et al. 1988; Hirokawa et al. 1989; Tatsuoka and Reese 1989; Gotow et al. 1991). Using aldehyde-free fixation and electron tomography, at least two types of material have recently been detected that may physically link synaptic vesicles to the active zone: about 50–100 nm long filaments and structures less than 20 nm long with an average length of 5 nm (Siksou et al. 2007; Fernandez-Busnadiego et al. 2010; Siksou et al. 2011). Both types of material appear to tether synaptic vesicles to the active zone, but their molecular identity has not been revealed. The presynaptic scaffolding protein Bassoon (Chap. 3, Figs. 3.1 and 3.4) may represent one of the longer filaments: using fluorescence nanoscopy Dani and colleagues found that a C-terminal epitope of Bassoon is located about 50 nm away from the synaptic cleft, while a more N-terminally located epitope is located 80 nm away, suggesting that at least part of Bassoon is filamentous (Dani et al. 2010). The shorter tethers may represent parts of the machinery that keeps a pool of synaptic vesicles docked at the active zone plasma membrane in a fusion competent state (Siksou et al. 2009, 2011; Fernandez-Busnadiego et al. 2010).

In molecular terms, the CAZ is thought to represent a supramolecular complex where all CAZ-specific proteins are directly or indirectly connected to each other (Schoch and Gundelfinger 2006; Wang et al 2009) (Chap. 3, Figs. 3.1 and 3.4). Some CAZ proteins have been shown, by electron microscopy, to be highly enriched or even exclusively localized at active zones, including RIM (Wang

et al. 1997), Bassoon (tom Dieck et al. 1998), and Piccolo/Aczonin (Cases-Langhoff et al. 1996; Wang et al. 1999; Fenster et al. 2000), CAST/ELKS2 (Ohtsuka et al. 2002), and Munc13-1 (Limbach et al. 2011), and their ultrastructural distribution relative to each other has been studied in detail (Limbach et al. 2011). Of these, Bassoon and Piccolo occur only in vertebrates, while the others occur also in invertebrates. Other proteins with important CAZ-related functions, such as RIM-BPs and Syd2/liprins, are less well characterized with respect to their exact localization. Notably, SNARE proteins (Chap. 4) and sec1/Munc18 proteins (SM proteins), which are essential for neurotransmitter release, occur within the CAZ and along non-synaptic areas of the neuronal plasma membrane (Garcia et al. 1995). How then, is neurotransmitter release restricted to active zones? One obvious answer is provided by the fact that Munc13 proteins, a family of multifunctional CAZ proteins, are essential for making synaptic vesicles fusion competent in a process called priming (Augustin et al. 1999; Brose et al. 2000; Varoqueaux et al. 2002). RIM recruits Munc13 to active zones and activates its fusion-competence conferring action (Andrews-Zwilling et al. 2006; Deng et al. 2011). Thus, although additional factors may recruit synaptic vesicles to active zones, the action of RIM as a Munc13 activator and the action of Munc13 in making synaptic vesicles fusion competent suffice to explain why synaptic vesicle exocytosis cannot occur anywhere else than at active zones. The process of priming appears to involve the formation of small, 2.5 nm bridges tethering synaptic vesicles to the plasma membrane (see above) and may thus be manifested morphologically as docking (Siksou et al. 2009, 2011; Fernandez-Busnadiego et al. 2010).

Overall, the molecules of the CAZ complex serve to (1) dock and prime synaptic vesicles through RIM and Munc13 proteins; (2) recruit voltage-gated calcium channels through RIM, RIM-binding proteins, and Bassoon; and (3) mediate pre-synaptic plasticity, e.g., through actions of RIM, Munc13, and Bassoon (Gundelfinger and Fejtova 2012; Sudhof 2012, 2013). The features and functions of CAZ proteins are described in detail in this book Chap. 3. Their pivotal importance for determining the exact site of neurotransmitter release and for regulating transmitter release in the context of synaptic plasticity raises the question as to how the CAZ is formed. This can be broken down to more specific questions, including: (a) How is CAZ material delivered to nascent synapses? (b) How is the site of CAZ assembly determined? (c) What does it take to form a fully functional CAZ?

2.3 Generation and Trafficking of CAZ Precursors

CAZ proteins do not have transmembrane regions. Thus, there is no principle need for them to be trafficked through the secretory pathway. However, there is a considerable body of evidence suggesting that at least some CAZ proteins require transport on the surface of vesicles. The delivery of CAZ proteins to the plasma

membrane of nascent synapses is an integral part of active zone assembly, because it is directly related to questions such as: Are CAZ complexes preformed and merely deposited at the active zone? How does the deposition occur? Do sizable units of CAZ proteins enter the nascent synapse or are individual CAZ molecules added in a gradual fashion? Current notion holds that CAZ proteins are transported along axons as packets detectable by fluorescence microscopy, that these packets are distinct from other packets harboring synaptic vesicle precursors, and that, despite their principally independent mobility, both types of packets intermittently "meet" at axonal pause sites before they accumulate at a nascent synapse. In mammalian neurons, Bassoon, Piccolo, and ELKS2 appear to travel on the surface of a specialized class of dense core vesicles termed Piccolo-Bassoon transport vesicles (PTVs). These vesicles differ from synaptic vesicle precursor vesicles, which carry a comprehensive set of synaptic vesicle proteins (Ahmari et al. 2000). The concept of PTVs carrying CAZ material has also been applied to explain CAZ trafficking in zebrafish (Easley-Neal et al. 2013) and invertebrate model systems such as *C. elegans* and *Drosophila* (Goldstein et al. 2008; Owald and Sigrist 2009; Maeder et al. 2014), but which CAZ proteins or sets of CAZ proteins are transported by dense core vesicles in these systems is not clear.

The largest body of data analyzing CAZ trafficking exists for mammalian PTVs. These 80 nm dense core vesicles were originally identified in embryonal rat brain through immunoisolation using anti-Piccolo antibodies (Zhai et al. 2001; Shapira et al. 2003). Axonal PTVs carry not only Piccolo and Bassoon, but also Munc13-1, ELKS2/CAST1/ERC2, and RIM1-α (Ohtsuka et al. 2002; Shapira et al. 2003; Takao-Rikitsu et al. 2004; Maas et al. 2012), suggesting that they may transport preassembled CAZ material. Bassoon, Piccolo, and ELKS2 also colocalize at the level of the Golgi apparatus and require a functional Golgi apparatus for trafficking, suggesting that PTVs are generated at Golgi compartments and that parts of the CAZ may be preassembled at this early stage of the secretory pathway (Dresbach et al. 2006a, b; Maas et al. 2012). In fact, Piccolo and Bassoon appear to be required for the generation of PTVs at the Golgi apparatus. In contrast, Munc13-1 requires a distinct type of Golgi-derived vesicle, and RIM1-α does not associate with the Golgi apparatus at all. Instead, RIM1-α binds to a post-Golgi membranous compartment (Maas et al. 2012). Nonetheless, Munc13-1 and RIM1-α are associated with Bassoon and Piccolo packets in axons, suggesting that PTV generation involves an initial step followed by maturational steps: Bassoon and Piccolo, acting at the level of the Golgi apparatus, seem to drive the biogenesis of primordial PTVs that also contain ELKS2, while Munc13-1 and RIM1-α are recruited to PTVs later.

The possibility that PTVs may undergo maturational steps, by which they acquire their full set of proteins, poses a difficulty to a clear definition for what may be termed a "PTV" in an experimental observation. However, so far, all data sets are consistent with the assumption that Bassoon – including recombinant versions of Bassoon – are associated with PTVs during all stages of PTV biogenesis, rendering Bassoon a frequently used marker for PTVs. Live imaging studies using GFP-Bassoon have revealed that mobile Bassoon puncta, presumably

PTVs, travel principally independently of synaptic vesicle precursors in axons, but frequently pause at sites where synaptic vesicle precursors pause, too (Bury and Sabo 2011). Co-trafficking and co-pausing of synaptic vesicle precursors with punctate fluorescence signals for CAZ markers UNC-10-GFP, Syd-2, and SAD-1, has also been observed in *C. elegans* axons (Wu et al. 2013). Synaptic vesicle precursor pausing at axonal "stop sites" had been discovered earlier in cultured neurons (Sabo et al. 2006) and has since been observed in axons of live zebrafish and *C. elegans*, too (Easley-Neal et al. 2013; Wu et al. 2013), indicating that this represents in vivo behavior of presynaptic trafficking organelles. The molecular composition of such stop sites has remained elusive, but it has repeatedly been observed that synaptic vesicle precursors and other presynaptic cargo co-pause at these sites, and these sites have also been called "mini synapses" (Maeder et al. 2014). Pausing at such sites may reflect some kind of "probing" of precursor organelles for synaptogenic sites, perhaps including exo- and endocytotic recycling. In cultured neurons, such pause sites frequently become synaptic junctions (Sabo et al. 2006), raising the possibility that they represent predefined specializations of the axonal membrane endowed with the competence to capture synaptic vesicle precursors and PTVs.

In live zebrafish spinal chord axons forming an axosomatic presynaptic nerve terminal, synapsin, VAMP, and N-cadherin each travel as discrete and independently moving packets toward the nerve terminal. Presynaptic accumulation of each of these proteins occurs sequentially over a time course of 90 min, with VAMP arriving first, followed by N-cadherin, and finally Synapsin, thus establishing a time course for the arrival of distinct protein classes at active zones in vivo (Easley-Neal et al. 2013). This time window resembles what has been observed in cultured neurons, where synaptic vesicles and CAZ material accumulate at nascent synapses over 1–2 h, and can be detected within 30 min of the appearance of a new synapse (Friedman et al. 2000). In the zebrafish study, VAMP was used as a marker for synaptic vesicle precursors, while N-cadherin was used as a marker for PTVs, with the caveat that in cultured rodent hippocampal neurons, only 50 percent of N-cadherin reside on PTVs, leaving the possibility open that an N-cadherin containing type of organelles distinct from PTVs was imaged. In any case, this study establishes a time course for the sequential deposition of three proteins at a defined synapse in vivo. While there was little co-trafficking of VAMP and N-cadherin (except at pause sites) in this study, significant co-trafficking of Synaptophysin and Bassoon was observed in cultured hippocampal neurons (Bury and Sabo 2011). This is consistent with electron microscopy analysis performed on the same preparation, which reveals clusters of organelles harboring both clear core vesicles immuno-positive for synaptic vesicle markers and dense core vesicles immuno-positive for Bassoon and Piccolo, presumably representing PTVs. The PTVs were usually located in the center of a cloud of synaptic vesicle precursors (Tao-Cheng 2007). Thus, both live imaging studies and ultrastructural studies suggest that there is significant co-trafficking of PTVs and synaptic vesicle precursors in cultured neurons.

2.4 Regulation of CAZ Delivery

What are the motors and adaptors that may mediate the trafficking of active zone material? *Drosophila* axons of motor neurons lacking the kinesin-3 motor *imac* grow out and contact their target muscle fibers, indicating that other motors than kinesin-3 mediate membrane addition for axonal outgrowth. But both synaptic vesicles and active zone material fail to accumulate in *imac* mutants (Pack-Chung et al. 2007), implicating kinesin-3 in the delivery of active zone material to nerve terminals. However, mechanisms for the precise regulation of anterograde and retrograde transport combined with regulated pausing at stop sites and deposition of material at synaptic sites are needed to fully describe the trafficking of presynaptic material. Dense core vesicles circulate inside the axons of *Drosophila*, in that they travel to the distal axon, return toward the proximal axon, and embark on another round of anterograde traffic. Apparently, they undergo sporadic capture at en passant boutons, and this must be finely regulated to prevent excessive accumulation at a subset of boutons and to guarantee filling of all boutons (Wong et al. 2012). Whether active zone carriers display the same circulation behavior remains to be tested. In addition, what regulates the balance of capture versus loss of material at presynaptic boutons remains to be characterized in detail. In *C. elegans* double deficient in the kinases cdk-5 and pct-1, presynaptic material is mislocalized to dendrites, although transport into the axon is maintained. This points to a role for these kinases in keeping a proper balance between anterograde and retrograde transport (Ou et al. 2010; Goodwin et al. 2012). At axonal pause sites, the monomeric GTPase ARL-8 promotes the dissociation of material, presumably by regulating the association of material with KIF1A motors, while the Jun-kinase pathway and the CAZ protein Syd-2/liprin-α promote the maintenance of material (Wu et al. 2013). Overall, these observations lead to several conclusions: synaptic vesicle precursors and active zone material (presumably carried on dense core vesicles) co-trafficking in *C. elegans* and *Drosophila* axons are captured at pause sites that may become synapses, and the balance of capturing and dissociation regulates both the amount of protein at individual boutons and the filling of en passant boutons of an entire axon. In addition, anterograde and retrograde trafficking have to be finely balanced to allow for net accumulation of presynaptic material in axons. Finally, the local accumulation of active zone material may be regulated both by kinases and by active zone proteins. The latter observation raises the possibility that some active zone proteins arrive earlier at nascent synapses and may be more stable than others. For example, in *C. elegans and Drosophila*, the CAZ proteins SYD-2/liprin-α and SYD-1 are required for active zone assembly by acting through ELKS1 (Patel et al. 2006; Dai et al. 2006). In mammals, RIM1 recruits and activates Munc13-1 (Andrews-Zwilling et al. 2006; Deng et al. 2011). Likewise, Bassoon and Piccolo qualify as early and relatively stable organizers of active zones in mammals: both proteins are among the earliest molecules to accumulate at nascent synapses (Friedman et al. 2000; Zhai et al. 2001). In addition, the exchange rates of Bassoon at synapses are significantly lower than those

observed for Munc-13 (Tsuriel et al. 2009). Thus, a combination of regulated capture, time course of arrival, binding affinities, and protein stability may allow certain CAZ proteins to create a platform for the hierarchical recruitment of further CAZ material through protein-protein interactions. It remains to be seen if such hierarchical actions are conserved or modified during evolution and how they interact with the signalling cascades outlined above.

The features of Bassoon and Piccolo can be taken to illustrate current notions of CAZ assembly as emerging for vertebrates: in rat and mouse neurons, Bassoon and Piccolo containing PTVs are transported anterogradely via the motor KIF5b and the adaptor syntabulin (Su et al. 2004; Cai et al. 2007). Syntabulin is a motor adaptor specific for PTVs (and mitochondria; Cai et al. 2005), since perturbing syntabulin function impairs PTV transport while leaving synaptic vesicle protein trafficking unaffected (Cai et al. 2007; Bury and Sabo 2011). Bassoon also binds to dynein light chain directly, and this interaction is crucial for proper trafficking of Bassoon (Fejtova et al. 2009). Thus, the minimum prerequisite for axonal circulation of PTVs, i.e., the interaction with an anterograde and a retrograde motor complex, is given. Remarkably, this scenario includes three of a very small number of vertebrate-specific presynaptic proteins, i.e., Bassoon, Piccolo, and syntabulin. To our knowledge, the only other vertebrate-specific presynaptic proteins are synuclein and the Bassoon interaction partner Mover/SVAP30/TPRGL1 (Kremer et al. 2007; Ahmed et al. 2013), both of which are synaptic vesicle-associated proteins. This raises the possibility that PTV biogenesis, PTV trafficking, and perhaps some interactions of the CAZ with synaptic vesicles may represent mechanisms unique to vertebrates. On the other hand, mammalian PTVs do contain evolutionarily conserved proteins (see below), suggesting that PTV function may build on conserved mechanisms.

2.5 Modes of CAZ Assembly from PTVs

Detailed analyses of the features of PTVs and of GFP-Bassoon yielded further insights into the mechanisms underlying mammalian active zone formation, beyond axonal trafficking and local capture. Overall, these studies suggest that (a) active zones may be assembled from the unitary insertion of a small number of PTVs into the presynaptic membrane; (b) theoretically, this assembly process could readily occur by exocytotic fusion of PTVs with the plasma membrane; and (c) Bassoon and Piccolo act to keep ubiquitin-mediated proteolysis at bay, thus stabilizing active zone material for synapse maintenance. These notions are supported by several lines of evidence: first, biochemical fractions enriched for PTVs include a remarkable set of proteins destined for the plasma membrane of active zones, including voltage-gated calcium channels, the SNAREs syntaxin-1 and SNAP-25, as well as bona fide CAZ proteins, such as RIM1, Munc13, and CAST1, and CAZ-associated proteins such as Munc18-1. In addition, a fraction of N-cadherin seems to reside on PTVs (Zhai et al. 2001; Shapira et al. 2003). This suggests that a

comprehensive set of proteins, including all categories of molecules involved in active zone function, could be delivered to nascent synapses en bloc. Second, mobile puncta of GFP-Bassoon, presumably PTVs en route toward boutons, carry unitary amounts of active zone material: active zones contain integer multiples – on average 2–3 times – of the amount of Bassoon, Piccolo, or RIM1 carried by individual PTVs (Shapira et al. 2003). Thus, active zones may be assembled through the material delivered by 2–3 PTVs. This unitary mode of delivery is distinct from the gradual accumulation of scaffolding proteins and receptors at the postsynaptic density, suggesting that presynaptic active zone assembly may be fundamentally different from the assembly of the postsynaptic machinery in this respect (Bresler et al. 2004). How exactly does the deposition of CAZ material occur? It is tempting and plausible to speculate that PTVs may deliver CAZ material by exocytotic fusion: exocytosis would – in a single step – introduce voltage-gated calcium channels, N-cadherin, and syntaxin-1 into the plasma membrane as integral membrane proteins, and the entire CAZ as well as SNAP-25 would be deposited immediately adjacent to the plasma membrane. Considering the quantitative analysis mentioned above, 2–3 PTVs could suffice to generate an average active zone. Whether this occurs indeed, and whether similar exocytotic events may occur at pause sites, has so far remained elusive due to technical obstacles. An obvious approach would be to label PTVs with a tag that should become exposed at the external cell surface during exocytosis, e.g., using a tagged luminal protein or a luminally tagged transmembrane protein. However, to date no intravesicular or transmembrane protein is known to reside exclusively on PTVs. Therefore, this approach has not been feasible to employ yet.

2.6 CAZ Stability Through Regulation of Proteolysis

Irrespective of the mode of CAZ deposition from PTVs, another feature of Bassoon and Piccolo has recently added insights into the mechanisms of CAZ assembly: both Bassoon and Piccolo were shown to bind to and downregulate the ubiquitin E3 ligase Siah1 through their N-terminal zinc-finger domains (Waites et al. 2013). Remarkably, the simultaneous knockdown of Bassoon and Piccolo leads to profound loss of a large number of proteins and ultimately to synapse disassembly. The levels of Munc13-1 and RIM1 were strongly reduced, as were the levels of synaptic vesicle proteins. By electron microscopy, a loss of postsynaptic densities was detected, indicating that in the absence of Bassoon and Piccolo a large number of pre- and postsynaptic components are degraded. The loss of all presynaptic components upon double knockdown of Bassoon and Piccolo, including synaptic vesicle proteins and CAZ proteins, could be explained by increased ubiquitination and ubiquitin-dependent degradation at the active zone. Alternatively, some components may be lost because they lack binding partners. Presumably, a combination of these two mechanisms leads to the ultimate loss of the synaptic junction. Interestingly, postsynaptic proteins and PSDs were

degraded, too, indicating that under normal conditions the CAZ stabilizes the PSD through transsynaptic interactions.

It is difficult to predict if similar mechanisms operate at invertebrate active zones due to the multiple actions of the proteasomal system. For example, loss of the invertebrate ubiquitin ligase RPM-1/Highwire increases the number of synaptic boutons and active zones (Wan et al. 2000; Zhen et al. 2000). Thus, proteolytic degradation does not necessarily lead to the loss of synapses, but instead has the capacity to regulate signalling pathways (Liao et al. 2004; Nakata et al. 2005; Yan et al. 2009). In addition, proteolysis is known to cause the elimination of transient synapses during *C. elegans* development (Ding et al. 2007). Regulated proteolysis can therefore regulate active zone assembly positively and negatively. In any case, the knockdown data obtained in cultured hippocampal neurons establish a major role for Bassoon and Piccolo in protecting mammalian synapses against degradation and suggest a model for vertebrate active zone assembly, whereby Bassoon and Piccolo generate PTVs at the Golgi apparatus, travel along axons via Syntabulin/Kif5b driven motor activity, and stabilize presynaptic architecture by downregulating ubiquitin-dependent proteolysis at active zones. It would be interesting to know if the ubiquitin-inhibiting actions of the zinc fingers of Bassoon and Piccolo are constitutively active, e.g., during PTV biogenesis and trafficking, or become activated during active zone assembly at nascent synapses, e.g., through transsynaptic signalling from the postsynaptic side.

2.7 Cell Adhesion Molecules and Transsynaptic Signalling

Local cues must exist, which govern the assembly of the CAZ at nascent synapses. In general, such cues may be provided by several modes: diffusible factors generated in a certain brain region may endow axons entering this area with the general capacity to accumulate presynaptic material. In addition, such diffusible factors could be released locally and promote presynaptic assembly in axons close to the source (Ou and Shen 2010; Jin and Garner 2008; Chia et al. 2013). Cell adhesion molecules provide another mode of synapse induction, and a large number of cell adhesion molecules have indeed been implicated in presynaptic assembly (Dalva et al. 2007; Giagtzoglou et al. 2009). Remarkably, most of these synaptic cell adhesion molecules have been implicated in psychiatric disorders such as autism and schizophrenia, suggesting that their action in coordinating synapse assembly is a crucial contribution to normal brain function (Sudhof 2008; de Wit and Ghosh 2014; Kleijer et al. 2014).

Both diffusible factors and cell adhesion molecules most likely elicit intracellular signalling cascades in axons. This may involve enzymatic signalling cascades, e.g., phosphorylation or de-phosphorylation, modification of the actin cytoskeleton, and the induction of direct protein-protein interactions. Some studies use synaptic vesicle accumulation as a readout for presynaptic assembly, i.e., they do not directly test for CAZ assembly. It is plausible to assume that a mechanism that

causes the accumulation of synaptic vesicles, in particular if they are capable of undergoing stimulated exo-/endocytotic recycling, also causes the assembly of the CAZ, because the CAZ contains proteins required for synaptic vesicle fusion and stability (see above). However, even if both synaptic vesicle recruitment and CAZ assembly are caused by a certain inductor, the exact pathways may only partially overlap, and there may be distinct pathways preferentially promoting one or the other. For example, in axons of cultured neurons that contact fibroblasts expressing the cell adhesion molecule neuroligin-1, synaptic vesicle precursors pause for longer than usual at the contact site, while the pause duration for PTVs is not changed. Yet, both types of organelles accumulate at the contact site, apparently recruited through distinct mechanisms (Bury and Sabo 2014). Thus, a certain caveat remains when either synaptic vesicle recruitment or cytomatrix protein recruitment is used as the sole readout for presynaptic assembly, and it will be interesting to dissect the respective pathways.

As outlined below, an increasing number of cell adhesion molecules have been implicated in the induction of presynaptic assembly, while the intracellular pathways elicited by their action are only beginning to be characterized. Of note, cell adhesion molecules do not only act in synapse formation but also mediate subsequent maturation steps, as well as synaptic maintenance and plasticity. In fact, some cell adhesion molecules seem to have essential roles in steps downstream of synapse formation (Dalva et al. 2007; Giagtzoglou et al. 2009; Sudhof 2008; Wittenmayer et al. 2009). In addition, most cell adhesion molecules represent large protein families including multiple isoforms. It is generally believed that this diversity may provide a "specificity code," by which combinations of cell adhesion molecules specify if and where an axon can make a synaptic connection and what the functional properties of that synapse are going to be. Thus, studying cell adhesion molecules has at least two major goals, i.e., to understand the mechanisms by which they induce synapse assembly and to understand how they confer specificity.

In the context of active zone assembly, three types of heterophilic cell adhesion molecules have received particular attention recently: (a) postsynaptic cell adhesion molecules that bind to presynaptic receptor tyrosine phosphatase proteins (RPTPs), including Slitrks, IL1RAPL1, NGL-3, and TrkC; (b) postsynaptic cell adhesion molecules that bind to presynaptic neurexins, including neuroligins, leucine-rich repeat transmembrane neuronal proteins (LRRTMs), and cerebellin precursor protein 1 (Cbln1); and (c) the postsynaptic cell adhesion molecule LRRTM4, a member of the LRRTM family mentioned above, that binds to presynaptic proteoglycans. On the presynaptic side, RPTPs and neurexins are transmembrane proteins, while the proteoglycan binding partners of LRRTM4 lack a transmembrane region (Sudhof 2008; Brose 2013; Takahashi and Craig 2013; Song and Kim 2013; de Wit and Ghosh 2014). Overall, a picture emerges in which a large number of heterophilic postsynaptic cell adhesion molecules can be categorized based on their interactions with a smaller number of presynaptic receptors.

2.7.1 Cell Adhesion Molecules Binding to RPTPs

Receptor protein tyrosine phosphatases (RPTPs) (Chap. 3, Fig. 3.3) represent a family of eight subtypes. The extracellular domains of the RPTPs LAR, PTPσ, and PTPδ have been implicated in cell adhesion and cell matrix interactions based on the structure of their extracellular domains. The intracellular domains of LAR, PTPσ, and PTPδ contain a catalytically active D1 domain and a less active or inactive D2 domain. The postsynaptic partners of these RPTPs bind their ligands with distinct affinities: Slitrks bind to PTPσ and PTPδ, TrkC to PTPσ, IL1RAPL1 to PTPδ, and NGL-3 and IL1RacP bind to all three PTPs. Thus, RPTPs are targeted by distinct but overlapping sets of postsynaptic adhesion molecules, suggesting that they may act as hubs, perhaps to fine-tune synapse assembly and synapse function according to a cell-specific or even synapse-specific interaction code (Takahashi and Craig 2013). The postsynaptic adhesion molecules targeting presynaptic PTPs all induce presynaptic assembly in a co-culture assay, in which non-neuronal cells expressing a potential inducer of synaptogenesis are co-cultured with neurons to see if the neuronal processes can be induced to form synapses. This assay was originally used to show that neuroligins are potent inducers of presynaptic differentiation (Scheiffele et al. 2000). This co-culture assay has since been instrumental in testing if a certain cell adhesion protein can induce synapse assembly. Knockout experiments – designed to test if a certain protein is essential for synapse formation – have been more difficult to interpret: no single knockout so far has abolished synapse formation, presumably due to additive actions among distinct adhesion molecules or due to some level of redundancy between isoforms of a certain gene family. In fact, if the interaction code mentioned above is designed to finely regulate connectivity, one may expect a loss of a certain subtype of synapses, rather than a global lack of synapse formation.

Nonetheless, significant progress has recently been made in understanding synapse-specific actions of PTP binding proteins: while the majority of PTP interaction partners induce presynaptic assembly in axons of excitatory neurons (and some in both excitatory and inhibitory neurons), Slitrk-3 selectively induces inhibitory synapse assembly. Slitrk-3 interacts with PTPδ to induce inhibitory synapse formation, while the Slitrk-1, Slitrk-2, Slitrk-4, and Slitrk-5 isoforms interact with PTPσ to induce excitatory synapse formation (Takahashi et al. 2011, 2012; Yim et al. 2013). Interestingly, PTPδ not only mediates inhibitory synapse formation in concert with Slitrk-3, but also excitatory synapse formation when activated by IL1RAPL1 or IL1RacP (Valnegri et al. 2011; Yoshida et al. 2011, 2012). How distinct combinations of cell adhesion molecules and PTPs govern the selective assembly of excitatory, inhibitory, or both types of synapses has yet to uncovered.

2.7.2 Cell Adhesion Molecules Binding to Neurexins

Vertebrate neurexins are encoded by three genes that contain two promoters each. Considering multiple alternative splice sites, a large number of neurexin isoforms may exist (Sudhof 2008; Reissner et al. 2013). Neurexins bind to several postsynaptic cell adhesion molecules, most notably neuroligins, LRRTMs and Cbln1. Neuroligins (Dean and Dresbach 2006; see also Chap. 3, Fig. 3.3) were the first identified binding partners for neurexins (Ichtchenko et al. 1995; Boucard et al. 2005; Chih et al. 2006), and an interaction of neuroligin-1 with neurexins was the first example of a pair of pre- and postsynaptic cell adhesion proteins inducing presynaptic assembly (Scheiffele et al. 2000; Dean et al. 2003). Like for cell adhesion molecules to date, knockout of neuroligins or neurexins did not abolish active zone formation, but revealed essential roles for synaptic transmission and pre- and postsynaptic maturation (Missler et al. 2003; Varoqueaux et al. 2006; Chubykin et al 2007; Wittenmayer et al. 2009). But their potency for inducing synapse assembly in cultured neurons suggests that they do contribute to early stages of synaptogenesis, too, albeit in a redundant or additive way. Both neuroligins and LRRTMs are postsynaptic type I transmembrane proteins with a C-terminal, i.e., intracellular, PDZ binding site. In cultured neurons, overexpression of neuroligin-1, neuroligin-2, and neuroligin-3 induces both excitatory and inhibitory synapse formation, with neuroligin-2 preferentially inducing inhibitory synapses (Graf et al. 2004; Prange et al. 2004; Chih et al. 2005; Levinson et al. 2005), while LRRTM2 is specific for excitatory synapses (de Wit et al. 2009; Ko et al. 2009).

Unlike neuroligins and LRRTMs, Cbln1 is a secreted glycoprotein. Cbln1 is produced by cerebellar granule cells, which give rise to parallel fiber axons in the cerebellum, and links the postsynaptic glutamate receptor GluRδ2 to presynaptic neurexins. Parallel fibers express neurexins and secrete Cbln1, while their postsynaptic targets, the Purkinje cells, are the only cells expressing GluRδ2. Moreover, GluRδ2 is exclusively localized to parallel fiber – Purkinje cell synapses. This layout provides an example of defined connectivity and biochemical specialization in an intact circuit that can be experimentally addressed. Knockout of either Cbln1 or GluRδ2 results in Purkinje cell spines lacking presynaptic inputs as well as mismatched synapses with misaligned active zones. Apparently, GluRδ2 induces active zone assembly by clustering four neurexin molecules in incoming axons through the action of Cbln1 (Uemura et al. 2010; Lee et al. 2012; Mishina et al. 2012).

2.7.3 LRRTM4 Binding to Proteoglycans

LRRTM4 is preferentially expressed in the molecular layer of the dentate gyrus and localized to excitatory postsynaptic sites. It interacts with presynaptic heparan

sulfate proteoglycans called glypicans and syndecans, and these interactions mediate the synaptogenic action of LRRTM4. Thus, this LRRTM isoform does not act through neurexins, but instead through binding to proteoglycans. Moreover, the restricted expression of LRRTM4 is another example of cell-type specific actions of a synaptogenic protein (see GluRδ2 in Purkinje cells) and raises hopes for eventually understanding the putative "code" provided by synaptogenic cell adhesion molecules.

2.7.4 Cell Adhesion Systems and Presynaptic Signalling

Overall, these observations raise at least two prominent questions: first, what are the modes of interaction between the distinct cell adhesion systems described above? So far, there is no satisfying answers to this question, but some observations provide interesting perspectives. For instance, glypicans do not have a transmembrane region, yet they mediate presynaptic assembly induced by LRRTM4. The *Drosophila* glypican Dally-like interacts with the RPTP dLar, raising the possibility that at this point LRRTM4 signalling is directed toward the RPTP pathway (Song and Kim 2013). The RPTP pathway in turn may share targets with the neurexin pathway, since both RPTPs and neurexins directly or indirectly bind to liprin-α (Takahashi and Craig 2013). However, a recent study reveals that the intracellular (presynaptic) domain of neurexins is not required to mediate synapse assembly induced by neuroligin-1 or LRRTM2, suggesting that the extracellular part of neurexins binds to a yet-to-be identified presynaptic interaction partner (Gokce and Sudhof 2013). Finally, further interactions with other cell adhesion systems and intracellular may come into play, such as Salms, SynCAMs, Farp1 (Mah et al. 2010; Fogel et al. 2007; Cheadle and Biederer 2012), and the N-cadherin/β-catenin system, which appears to cooperate with the neuroligin system (Stan et al. 2010; Aiga et al. 2011).

Second, what are the adhesion-induced intracellular signalling pathways elicited in axons? Currently, it is unclear to what extent the tyrosine phosphatase activity of RPTPs is involved. Likewise, it is not clear how neurexins and how proteoglycans signal into the presynaptic terminal. A pathway involving direct protein-protein interactions seems to emerge from studies in invertebrates: at the *Drosophila* neuromuscular junction, neurexin forms a complex with the presynaptic CAZ protein SYD-1, which mediates local accumulation of neurexin at nascent active zones. SYD-1 driven presynaptic accumulation of neurexin in turn increases the clustering of postsynaptic neuroligin. This process may allow for synchronous assembly of the pre- and postsynaptic site and act as a positive feedback in which the nascent active zone and the postsynaptic density stabilize each other (Owald et al. 2012). SYD-1 arrives early at nascent synapses and recruits liprin-α to a peripheral area of the active zone. SYD-1 mutants have fewer active zones, and the remaining active zone material is mistargeted. Under normal conditions, the *Drosophila* CAZ protein Bruchpilot, a member of the ELKS family of CAZ proteins,

concludes CAZ biogenesis, but in SYD-1 mutants Bruchpilot forms ectopic aggregates (Owald et al. 2010). Together, these observations suggest a pathway in which an interaction of neurexin with the CAZ protein SYD-1 initiates the synchronous recruitment of pre- and postsynaptic material, followed by the recruitment of liprin-α to SYD-1. Liprin-α interacts with a number of CAZ proteins, including the *Drosophila* RPTP LAR and RIM1, and SYD-1 interacts with Bruchpilot. Thus, a cascade of protein-protein interactions downstream of SYD-1 may explain active zone assembly at the *Drosophila* neuromuscular junction. A similar intracellular pathway seems to operate at the HSNL neuromuscular junction in *C. elegans*, where SYD-1 causes the hierarchical recruitment of SYD-2/liprin-α, followed by the recruitment of ELKS and RIM. At this synapse, the cell adhesion tandem initiating this process may consist of SYG-2, an epithelial transmembrane protein expressed on so-called guidepost cells, and SYG-1, a HSNL neuron transmembrane protein (Hallam et al. 2002; Dai et al. 2006; Patel et al. 2006). SYG-1 somehow induces local F-actin formation to recruit an F-actin binding protein called NAB-1, which in turn recruits SYD-1 to initiate the downstream cascade of events (Chia et al. 2012).

To what extent this cascade of intracellular protein-protein interactions is conserved in vertebrates remains to be determined. Little is known about the function of vertebrate liprins, but a distant mammalian SYD-1 orthologue, called mSYD-1, has recently been identified. Unlike the invertebrate scenario, but like for virtually all mammalian proteins involved in synaptogenesis, reducing the levels of mSYD-1 does not prevent active zone formation. However, knockdown of mSYD-1 does reduce synaptic vesicle docking to active zones and inhibits presynaptic assembly induced by overexpressing postsynaptic neuroligin-1 or NGL-3. Thus, mSYD-1 appears to be important for presynaptic assembly induced both via neurexin and via RPTPs. Finally, mSYD-1 binds to Munc18 and to an isoform of liprins, called liprin-α2, and overexpression of mSYD-1 in cultured cerebellar granule cells increases the number of synapses. Structurally, mSYD-1 displays both similarities and differences compared with its invertebrate orthologues. Thus, a number of features are evolutionarily conserved in mSYD-1, while others seem to be vertebrate specific (Wentzel et al. 2013). In any case, the hierarchy of intracellular interactions suggested by work in vertebrates and invertebrates provides a framework for further analysis of the interactions occurring downstream of axonal transmembrane receptor signalling.

Of note, the above discussion focussed on direct protein-protein interactions, but the regulation of such interactions by posttranslational modifications, in particular phosphorylation, likely has a role in active zone assembly. The tyrosine kinase Fer, the tyrosine phosphatase SHP-2, and the N-cadherin interaction partner β-catenin interact to recruit synaptic vesicles and Bassoon to synapses. Presynaptic depletion of Fer elevates tyrosine phosphorylation of β-catenin and prevents the local recruitment of Bassoon (Lee et al. 2008). How these phosphorylation events link to the protein-protein interaction pathways described above has yet to be unraveled.

2.8 Perspectives and Open Questions

Presynaptic specializations allow neurons to convey signals to their postsynaptic targets. Active zones define the exact location and the functional properties of synaptic signal transmission by locally assembling the CAZ, i.e., a complex network of multidomain scaffolding proteins. Why does none of these CAZ proteins have a transmembrane region? Irrespective of the possible answer to this question, the nature of CAZ proteins poses a fundamental challenge to the cellular machinery: these proteins have to be delivered with exquisite precision to defined sites along the axonal membrane and have to be arranged to provide the functional architecture for the presynaptic neurotransmitter release machinery. As detailed in this chapter, a large number of cellular and molecular mechanisms are harnessed to guarantee CAZ assembly, starting with the formation of CAZ precursors at the Golgi apparatus, involving regulated trafficking, capturing and dissociation events during axonal transport, and culminating in the local deposition and organization of CAZ material under the control of transsynaptic signalling.

Our insight into these mechanisms obviously generates further questions, for example:

- How do protein-protein interactions and enzymatic actions act together in active zone assembly? Which events regulate the balance of capture versus dissociation at axonal stop sites, and which ones organize the incorporation of captured components into the nascent CAZ? What is the molecular composition of axonal stop sites?
- Do PTVs fuse with the plasma membrane to deposit active zone material? Do they carry luminal proteins that signal across the synaptic cleft?
- How do synaptic cell adhesion systems interact with each other during active zone assembly? Does an interaction code exist that guides the specification of synaptic connectivity and function? Does the increase in isoform number of cell adhesion molecules from invertebrates to vertebrates reflect a need for an expansion of the code in more complex brains?
- How do mutations in synaptic cell adhesion molecules contribute to psychiatric disorders? What exactly is “aberrant” in nervous systems that form in the presence of such mutations? Is it an imbalance between excitation and inhibition, and how exactly does it arise? Does it involve impaired synaptic “maturation,” and what is the contribution of active zones to synaptic maturation?

Addressing such topical questions is an exciting challenge and should help us understand one of the most fundamental questions in neuroscience: how do neurons connect to form a functional network?

References

Ahmari SE, Buchanan J, Smith SJ (2000) Assembly of presynaptic active zones from cytoplasmic transport packets. Nat Neurosci 3:445–451

Ahmed S, Wittenmayer N, Kremer T, Hoeber J, Kiran Akula A et al (2013) Mover is a homomeric phospho-protein present on synaptic vesicles. PLoS One 8:e63474

Aiga M, Levinson JN, Bamji SX (2011) N-cadherin and neuroligins cooperate to regulate synapse formation in hippocampal cultures. J Biol Chem 286:851–858

Andrews-Zwilling YS, Kawabe H, Reim K, Varoqueaux F, Brose N (2006) Binding to Rab3A-interacting molecule RIM regulates the presynaptic recruitment of Munc13-1 and ubMunc13-2. J Biol Chem 281:19720–19731

Augustin I, Rosenmund C, Sudhof TC, Brose N (1999) Munc13-1 is essential for fusion competence of glutamatergic synaptic vesicles. Nature 400:457–461

Boeckers TM (2006) The postsynaptic density. Cell Tissue Res 326:409–422

Boucard AA, Chubykin AA, Comoletti D, Taylor P, Sudhof TC (2005) A splice code for trans-synaptic cell adhesion mediated by binding of neuroligin 1 to α- and β-neurexins. Neuron 48:229–236

Bresler T, Shapira M, Boeckers T, Dresbach T, Futter M et al (2004) Postsynaptic density assembly is fundamentally different from presynaptic active zone assembly. J Neurosci 24:1507–1520

Brose N (2013) Why we need more synaptogenic cell-adhesion proteins. Proc Natl Acad Sci U S A 110:3717–3718

Brose N, Rosenmund C, Rettig J (2000) Regulation of transmitter release by Unc-13 and its homologues. Curr Opin Neurobiol 10:303–311

Bury LA, Sabo SL (2011) Coordinated trafficking of synaptic vesicle and active zone proteins prior to synapse formation. Neural Dev 6:24

Bury LA, Sabo SL (2014) Dynamic mechanisms of neuroligin-dependent presynaptic terminal assembly in living cortical neurons. Neural Dev 9:13

Cai Q, Gerwin C, Sheng ZH (2005) Syntabulin-mediated anterograde transport of mitochondria along neuronal processes. J Cell Biol 170:959–969

Cai Q, Pan PY, Sheng ZH (2007) Syntabulin-kinesin-1 family member 5B-mediated axonal transport contributes to activity-dependent presynaptic assembly. J Neurosci 27:7284–7296

Cases-Langhoff C, Voss B, Garner AM, Appeltauer U, Takei K et al (1996) Piccolo, a novel 420 kDa protein associated with the presynaptic cytomatrix. Eur J Cell Biol 69:214–223

Cheadle L, Biederer T (2012) The novel synaptogenic protein Farp1 links postsynaptic cytoskeletal dynamics and transsynaptic organization. J Cell Biol 199:985–1001

Chia PH, Patel MR, Shen K (2012) NAB-1 instructs synapse assembly by linking adhesion molecules and F-actin to active zone proteins. Nat Neurosci 15:234–242

Chia PH, Li P, Shen K (2013) Cell biology in neuroscience: cellular and molecular mechanisms underlying presynapse formation. J Cell Biol 203:11–22

Chih B, Engelman H, Scheiffele P (2005) Control of excitatory and inhibitory synapse formation by neuroligins. Science 307:1324–1328

Chih B, Gollan L, Scheiffele P (2006) Alternative splicing controls selective trans-synaptic interactions of the neuroligin-neurexin complex. Neuron 51:171–178

Chubykin AA, Atasoy D, Etherton MR, Brose N, Kavalali ET et al (2007) Activity-dependent validation of excitatory versus inhibitory synapses by neuroligin-1 versus neuroligin-2. Neuron 54:919–931

Couteaux R, Pecot-Dechavassine M (1970) Synaptic vesicles and pouches at the level of "active zones" of the neuromuscular junction. C R Acad Sci Hebd Seances Acad Sci D Sci Nat 271:2346–2349

Dai Y, Taru H, Deken SL, Grill B, Ackley B et al (2006) SYD-2 Liprin-α organizes presynaptic active zone formation through ELKS. Nat Neurosci 9:1479–1487

Dalva MB, McClelland AC, Kayser MS (2007) Cell adhesion molecules: signalling functions at the synapse. Nat Rev Neurosci 8:206–220
Dani A, Huang B, Bergan J, Dulac C, Zhuang X (2010) Superresolution imaging of chemical synapses in the brain. Neuron 68:843–856
de Wit J, Ghosh A (2014) Control of neural circuit formation by leucine-rich repeat proteins. Trends Neurosci 37:539–550
de Wit J, Sylwestrak E, O'Sullivan ML, Otto S, Tiglio K et al (2009) LRRTM2 interacts with Neurexin1 and regulates excitatory synapse formation. Neuron 64:799–806
Dean C, Dresbach T (2006) Neuroligins and neurexins: linking cell adhesion, synapse formation and cognitive function. Trends Neurosci 29:21–29
Dean C, Scholl FG, Choih J, DeMaria S, Berger J et al (2003) Neurexin mediates the assembly of presynaptic terminals. Nat Neurosci 6:708–716
Deng L, Kaeser PS, Xu W, Sudhof TC (2011) RIM proteins activate vesicle priming by reversing autoinhibitory homodimerization of Munc13. Neuron 69:317–331
Ding M, Chao D, Wang G, Shen K (2007) Spatial regulation of an E3 ubiquitin ligase directs selective synapse elimination. Science 317:947–951
Dresbach T, Qualmann B, Kessels MM, Garner CC, Gundelfinger ED (2001) The presynaptic cytomatrix of brain synapses. Cell Mol Life Sci 58:94–116
Dresbach T, Fejtova A, Gundelfinger ED (2006a) Assembly of presynaptic active zones. In: Dityatev A, El-Husseini A (eds) Molecular mechanisms of synaptogenesis. Springer, Berlin/Heidelberg, pp 235–244
Dresbach T, Torres V, Wittenmayer N, Altrock WD, Zamorano P et al (2006b) Assembly of active zone precursor vesicles: obligatory trafficking of presynaptic cytomatrix proteins bassoon and piccolo via a trans-golgi compartment. J Biol Chem 281:6038–6047
Easley-Neal C, Fierro J Jr, Buchanan J, Washbourne P (2013) Late recruitment of synapsin to nascent synapses is regulated by Cdk5. Cell Rep 3:1199–1212
Fejtova A, Gundelfinger ED (2006) Molecular organization and assembly of the presynaptic active zone of neurotransmitter release. Results Probl Cell Differ 43:49–68
Fejtova A, Davydova D, Bischof F, Lazarevic V, Altrock WD et al (2009) Dynein light chain regulates axonal trafficking and synaptic levels of Bassoon. J Cell Biol 185:341–355
Fenster SD, Chung WJ, Zhai R, Cases-Langhoff C, Voss B et al (2000) Piccolo, a presynaptic zinc finger protein structurally related to bassoon. Neuron 25:203–214
Fernandez-Busnadiego R, Zuber B, Maurer UE, Cyrklaff M, Baumeister W, Lucic V (2010) Quantitative analysis of the native presynaptic cytomatrix by cryoelectron tomography. J Cell Biol 188:145–156
Fogel AI, Akins MR, Krupp AJ, Stagi M, Stein V, Biederer T (2007) SynCAMs organize synapses through heterophilic adhesion. J Neurosci 27:12516–12530
Friedman HV, Bresler T, Garner CC, Ziv NE (2000) Assembly of new individual excitatory synapses: time course and temporal order of synaptic molecule recruitment. Neuron 27:57–69
Garcia EP, McPherson PS, Chilcote TJ, Takei K, De Camilli P (1995) rbSec1A and B colocalize with syntaxin 1 and SNAP-25 throughout the axon, but are not in a stable complex with syntaxin. J Cell Biol 129:105–120
Giagtzoglou N, Ly CV, Bellen HJ (2009) Cell adhesion, the backbone of the synapse: "vertebrate" and "invertebrate" perspectives. Cold Spring Harb Perspect Biol 1:a003079
Gokce O, Sudhof TC (2013) Membrane-tethered monomeric neurexin LNS-domain triggers synapse formation. J Neurosci 33:14617–14628
Goldstein AY, Wang X, Schwarz TL (2008) Axonal transport and the delivery of pre-synaptic components. Curr Opin Neurobiol 18:495–503
Goodwin PR, Sasaki JM, Juo P (2012) Cyclin-dependent kinase 5 regulates the polarized trafficking of neuropeptide-containing dense-core vesicles in *Caenorhabditis elegans* motor neurons. J Neurosci 32:8158–8172
Gotow T, Miyaguchi K, Hashimoto PH (1991) Cytoplasmic architecture of the axon terminal: filamentous strands specifically associated with synaptic vesicles. Neuroscience 40:587–598

Graf ER, Zhang X, Jin SX, Linhoff MW, Craig AM (2004) Neurexins induce differentiation of GABA and glutamate postsynaptic specializations via neuroligins. Cell 119:1013–1026

Gray EG (1963) Electron microscopy of presynaptic organelles of the spinal chord. J Anat 97:101–106

Gundelfinger ED, Fejtova A (2012) Molecular organization and plasticity of the cytomatrix at the active zone. Curr Opin Neurobiol 22(3):423–430

Hallam SJ, Goncharov A, McEwen J, Baran R, Jin Y (2002) SYD-1, a presynaptic protein with PDZ, C2 and rhoGAP-like domains, specifies axon identity in *C. elegans*. Nat Neurosci 5:1137–1146

Hirokawa N, Sobue K, Kanda K, Harada A, Yorifuji H (1989) The cytoskeletal architecture of the presynaptic terminal and molecular structure of synapsin 1. J Cell Biol 108:111–126

Ichtchenko K, Hata Y, Nguyen T, Ullrich B, Missler M et al (1995) Neuroligin 1: a splice site-specific ligand for β-neurexins. Cell 81:435–443

Jin Y, Garner CC (2008) Molecular mechanisms of presynaptic differentiation. Annu Rev Cell Dev Biol 24:237–262

Kleijer KT, Schmeisser MJ, Krueger DD, Boeckers TM, Scheiffele P et al (2014) Neurobiology of autism gene products: towards pathogenesis and drug targets. Psychopharmacology 231:1037–1062

Ko J, Fuccillo MV, Malenka RC, Sudhof TC (2009) LRRTM2 functions as a neurexin ligand in promoting excitatory synapse formation. Neuron 64:791–798

Kremer T, Kempf C, Wittenmayer N, Nawrotzki R, Kuner T et al (2007) Mover is a novel vertebrate-specific presynaptic protein with differential distribution at subsets of CNS synapses. FEBS Lett 581:4727–4733

Landis DM, Hall AK, Weinstein LA, Reese TS (1988) The organization of cytoplasm at the presynaptic active zone of a central nervous system synapse. Neuron 1:201–209

Langnaese K, Seidenbecher C, Wex H, Seidel B, Hartung K et al (1996) Protein components of a rat brain synaptic junctional protein preparation. Brain Res Mol Brain Res 42:118–122

Lee SH, Peng IF, Ng YG, Yanagisawa M, Bamji SX et al (2008) Synapses are regulated by the cytoplasmic tyrosine kinase Fer in a pathway mediated by p120catenin, Fer, SHP-2, and β-catenin. J Cell Biol 183:893–908

Lee SJ, Uemura T, Yoshida T, Mishina M (2012) GluRδ2 assembles four neurexins into trans-synaptic triad to trigger synapse formation. J Neurosci 32:4688–4701

Levinson JN, Chery N, Huang K, Wong TP, Gerrow K et al (2005) Neuroligins mediate excitatory and inhibitory synapse formation: involvement of PSD-95 and neurexin-1β in neuroligin-induced synaptic specificity. J Biol Chem 280:17312–17319

Liao EH, Hung W, Abrams B, Zhen M (2004) An SCF-like ubiquitin ligase complex that controls presynaptic differentiation. Nature 430:345–350

Limbach C, Laue MM, Wang X, Hu B, Thiede N et al (2011) Molecular in situ topology of Aczonin/Piccolo and associated proteins at the mammalian neurotransmitter release site. Proc Natl Acad Sci U S A 108:E392–E401

Maas C, Torres VI, Altrock WD, Leal-Ortiz S, Wagh D et al (2012) Formation of Golgi-derived active zone precursor vesicles. J Neurosci 32:11095–11108

Maeder CI, Shen K, Hoogenraad CC (2014) Axon and dendritic trafficking. Curr Opin Neurobiol 27:165–170

Mah W, Ko J, Nam J, Han K, Chung WS, Kim E (2010) Selected SALM (synaptic adhesion-like molecule) family proteins regulate synapse formation. J Neurosci 30:5559–5568

Mishina M, Uemura T, Yasumura M, Yoshida T (2012) Molecular mechanism of parallel fiber-Purkinje cell synapse formation. Front Neural Circuits 6:90

Missler M, Zhang W, Rohlmann A, Kattenstroth G, Hammer RE et al (2003) Alpha-neurexins couple Ca^{2+} channels to synaptic vesicle exocytosis. Nature 423:939–948

Nakata K, Abrams B, Grill B, Goncharov A, Huang X et al (2005) Regulation of a DLK-1 and p38 MAP kinase pathway by the ubiquitin ligase RPM-1 is required for presynaptic development. Cell 120:407–420

Ohtsuka T, Takao-Rikitsu E, Inoue E, Inoue M, Takeuchi M et al (2002) Cast: a novel protein of the cytomatrix at the active zone of synapses that forms a ternary complex with RIM1 and munc13-1. J Cell Biol 158:577–590

Ou CY, Shen K (2010) Setting up presynaptic structures at specific positions. Curr Opin Neurobiol 20:489–493

Ou CY, Poon VY, Maeder CI, Watanabe S, Lehrman EK et al (2010) Two cyclin-dependent kinase pathways are essential for polarized trafficking of presynaptic components. Cell 141:846–858

Owald D, Sigrist SJ (2009) Assembling the presynaptic active zone. Curr Opin Neurobiol 19:311–318

Owald D, Fouquet W, Schmidt M, Wichmann C, Mertel S et al (2010) A Syd-1 homologue regulates pre- and postsynaptic maturation in Drosophila. J Cell Biol 188:565–579

Owald D, Khorramshahi O, Gupta VK, Banovic D, Depner H et al (2012) Cooperation of Syd-1 with Neurexin synchronizes pre- with postsynaptic assembly. Nat Neurosci 15:1219–1226

Pack-Chung E, Kurshan PT, Dickman DK, Schwarz TL (2007) A Drosophila kinesin required for synaptic bouton formation and synaptic vesicle transport. Nat Neurosci 10:980–989

Patel MR, Lehrman EK, Poon VY, Crump JG, Zhen M et al (2006) Hierarchical assembly of presynaptic components in defined *C. elegans* synapses. Nat Neurosci 9:1488–1498

Pfenninger K, Sandri C, Akert K, Eugster CH (1969) Contribution to the problem of structural organization of the presynaptic area. Brain Res 12:10–18

Phillips GR, Huang JK, Wang Y, Tanaka H, Shapiro L et al (2001) The presynaptic particle web: ultrastructure, composition, dissolution, and reconstitution. Neuron 32:63–77

Prange O, Wong TP, Gerrow K, Wang YT, El-Husseini A (2004) A balance between excitatory and inhibitory synapses is controlled by PSD-95 and neuroligin. Proc Natl Acad Sci U S A 101:13915–13920

Reissner C, Runkel F, Missler M (2013) Neurexins. Genome Biol 14:213

Sabo SL, Gomes RA, McAllister AK (2006) Formation of presynaptic terminals at predefined sites along axons. J Neurosci 26:10813–10825

Scheiffele P, Fan J, Choih J, Fetter R, Serafini T (2000) Neuroligin expressed in nonneuronal cells triggers presynaptic development in contacting axons. Cell 101:657–669

Schoch S, Gundelfinger ED (2006) Molecular organization of the presynaptic active zone. Cell Tissue Res 326:379–391

Shapira M, Zhai RG, Dresbach T, Bresler T, Torres VI et al (2003) Unitary assembly of presynaptic active zones from Piccolo-Bassoon transport vesicles. Neuron 38:237–252

Shen K, Scheiffele P (2010) Genetics and cell biology of building specific synaptic connectivity. Annu Rev Neurosci 33:473–507

Siksou L, Rostaing P, Lechaire JP, Boudier T, Ohtsuka T et al (2007) Three-dimensional architecture of presynaptic terminal cytomatrix. J Neurosci 27:6868–6877

Siksou L, Varoqueaux F, Pascual O, Triller A, Brose N, Marty S (2009) A common molecular basis for membrane docking and functional priming of synaptic vesicles. Eur J Neurosci 30:49–56

Siksou L, Triller A, Marty S (2011) Ultrastructural organization of presynaptic terminals. Curr Opin Neurobiol 21:261–268

Song YS, Kim E (2013) Presynaptic proteoglycans: sweet organizers of synapse development. Neuron 79:609–611

Stan A, Pielarski KN, Brigadski T, Wittenmayer N, Fedorchenko O et al (2010) Essential cooperation of N-cadherin and neuroligin-1 in the transsynaptic control of vesicle accumulation. Proc Natl Acad Sci U S A 107:11116–11121

Su Q, Cai Q, Gerwin C, Smith CL, Sheng ZH (2004) Syntabulin is a microtubule-associated protein implicated in syntaxin transport in neurons. Nat Cell Biol 6:941–953

Sudhof TC (2008) Neuroligins and neurexins link synaptic function to cognitive disease. Nature 455:903–911

Sudhof TC (2012) The presynaptic active zone. Neuron 75:11–25

Sudhof TC (2013) Neurotransmitter release: the last millisecond in the life of a synaptic vesicle. Neuron 80:675–690

Takahashi H, Craig AM (2013) Protein tyrosine phosphatases PTPδ, PTPσ, and LAR: presynaptic hubs for synapse organization. Trends Neurosci 36:522–534

Takahashi H, Arstikaitis P, Prasad T, Bartlett TE, Wang YT et al (2011) Postsynaptic TrkC and presynaptic PTPσ function as a bidirectional excitatory synaptic organizing complex. Neuron 69:287–303

Takahashi H, Katayama K, Sohya K, Miyamoto H, Prasad T, Matsumoto Y, Ota M, Yasuda H, Tsumoto T, Aruga J, Craig AM (2012) Selective control of inhibitory synapse development by Slitrk3-PTPδ trans-synaptic interaction. Nat Neurosci 15:389–398

Takao-Rikitsu E, Mochida S, Inoue E, Deguchi-Tawarada M, Inoue M et al (2004) Physical and functional interaction of the active zone proteins, CAST, RIM1, and Bassoon, in neurotransmitter release. J Cell Biol 164:301–311

Tao-Cheng JH (2007) Ultrastructural localization of active zone and synaptic vesicle proteins in a preassembled multi-vesicle transport aggregate. Neuroscience 150:575–584

Tatsuoka H, Reese TS (1989) New structural features of synapses in the anteroventral cochlear nucleus prepared by direct freezing and freeze-substitution. J Comp Neurol 290:343–357

tom Dieck S, Sanmarti-Vila L, Langnaese K, Richter K, Kindler S et al (1998) Bassoon, a novel zinc-finger CAG/glutamine-repeat protein selectively localized at the active zone of presynaptic nerve terminals. J Cell Biol 142:499–509

Triller A, Korn H (1985) Activity-dependent deformations of presynaptic grids at central synapses. J Neurocytol 14:177–192

Tsuriel S, Fisher A, Wittenmayer N, Dresbach T, Garner CC, Ziv NE (2009) Exchange and redistribution dynamics of the cytoskeleton of the active zone molecule bassoon. J Neurosci 29:351–358

Uemura T, Lee SJ, Yasumura M, Takeuchi T, Yoshida T et al (2010) Trans-synaptic interaction of GluRδ2 and Neurexin through Cbln1 mediates synapse formation in the cerebellum. Cell 141:1068–1079

Valnegri P, Montrasio C, Brambilla D, Ko J, Passafaro M, Sala C (2011) The X-linked intellectual disability protein IL1RAPL1 regulates excitatory synapse formation by binding PTPδ and RhoGAP2. Hum Mol Genet 20:4797–4809

Varoqueaux F, Sigler A, Rhee JS, Brose N, Enk C et al (2002) Total arrest of spontaneous and evoked synaptic transmission but normal synaptogenesis in the absence of Munc13-mediated vesicle priming. Proc Natl Acad Sci U S A 99:9037–9042

Varoqueaux F, Aramuni G, Rawson RL, Mohrmann R, Missler M et al (2006) Neuroligins determine synapse maturation and function. Neuron 51:741–754

Vaughn JE (1989) Fine structure of synaptogenesis in the vertebrate central nervous system. Synapse 3:255–285

Waites CL, Leal-Ortiz SA, Okerlund N, Dalke H, Fejtova A et al (2013) Bassoon and Piccolo maintain synapse integrity by regulating protein ubiquitination and degradation. EMBO J 32:954–969

Wan HI, DiAntonio A, Fetter RD, Bergstrom K, Strauss R, Goodman CS (2000) Highwire regulates synaptic growth in Drosophila. Neuron 26:313–329

Wang Y, Okamoto M, Schmitz F, Hofmann K, Sudhof TC (1997) Rim is a putative Rab3 effector in regulating synaptic-vesicle fusion. Nature 388:593–598

Wang X, Kibschull M, Laue MM, Lichte B, Petrasch-Parwez E, Kilimann MW (1999) Aczonin, a 550-kD putative scaffolding protein of presynaptic active zones, shares homology regions with Rim and Bassoon and binds profilin. J Cell Biol 147:151–162

Wang X, Hu B, Zieba A, Neumann NG, Kasper-Sonnenberg M et al (2009) A protein interaction node at the neurotransmitter release site: domains of Aczonin/Piccolo, Bassoon, CAST, and rim converge on the N-terminal domain of Munc13-1. J Neurosci 29:12584–12596

Wentzel C, Sommer JE, Nair R, Stiefvater A, Sibarita JB, Scheiffele P (2013) mSYD1A, a mammalian synapse-defective-1 protein, regulates synaptogenic signaling and vesicle docking. Neuron 78:1012–1023

Wittenmayer N, Korber C, Liu H, Kremer T, Varoqueaux F et al (2009) Postsynaptic Neuroligin1 regulates presynaptic maturation. Proc Natl Acad Sci U S A 106:13564–13569

Wong MY, Zhou C, Shakiryanova D, Lloyd TE, Deitcher DL, Levitan ES (2012) Neuropeptide delivery to synapses by long-range vesicle circulation and sporadic capture. Cell 148:1029–1038

Wu YE, Huo L, Maeder CI, Feng W, Shen K (2013) The balance between capture and dissociation of presynaptic proteins controls the spatial distribution of synapses. Neuron 78:994–1011

Yan D, Wu Z, Chisholm AD, Jin Y (2009) The DLK-1 kinase promotes mRNA stability and local translation in *C. elegans* synapses and axon regeneration. Cell 138:1005–1018

Yim YS, Kwon Y, Nam J, Yoon HI, Lee K et al (2013) Slitrks control excitatory and inhibitory synapse formation with LAR receptor protein tyrosine phosphatases. Proc Natl Acad Sci U S A 110:4057–4062

Yoshida T, Yasumura M, Uemura T, Lee SJ, Ra M et al (2011) IL-1 receptor accessory protein-like 1 associated with mental retardation and autism mediates synapse formation by trans-synaptic interaction with protein tyrosine phosphatase δ. J Neurosci 31:13485–13499

Yoshida T, Shiroshima T, Lee SJ, Yasumura M, Uemura T et al (2012) Interleukin-1 receptor accessory protein organizes neuronal synaptogenesis as a cell adhesion molecule. J Neurosci 32:2588–2600

Zhai RG, Vardinon-Friedman H, Cases-Langhoff C, Becker B, Gundelfinger ED et al (2001) Assembling the presynaptic active zone: a characterization of an active one precursor vesicle. Neuron 29:131–143

Zhen M, Huang X, Bamber B, Jin Y (2000) Regulation of presynaptic terminal organization by *C. elegans* RPM-1, a putative guanine nucleotide exchanger with a RING-H2 finger domain. Neuron 26:331–343

Chapter 3
Network of Protein-Protein Interactions at the Presynaptic Active Zone

Toshihisa Ohtsuka

Abstract Presynaptic active zone is a slightly electron dense region beneath the presynaptic plasma membrane, where synaptic vesicles, containing neurotransmitters, dock, fuse, and release the content into the synaptic cleft in a Ca^{2+}-dependent manner. This highly ordered regulation of neurotransmitter release from the presynaptic active zone is crucial for normal brain functions such as learning and memory, emotion, and consciousness. Currently, a few active zone-specific proteins have been identified and characterized, including Bassoon, Piccolo/Aczonin, RIM1, Munc13-1, CAST/ERC2, and ELKS. These relatively large proteins with significant domain structures have been shown to interact with each other, forming a large macromolecular complex, and play pivotal roles in the structure and function of the presynaptic active zone. In this chapter, I would like to mainly describe and focus on protein-protein interactions among these active zone proteins and attempt to correlate the disruption of some of these interactions with deficits in synaptic functions such as neurotransmitter release and synaptic plasticity.

Keywords CAST/ERC2 • ELKS • RIMs • Munc13s • Bassoon • Piccolo/Aczonin

3.1 Introduction

Neurons in the brain communicate with each other at synapses, which are considered to be molecular and cellular basis for homeostasis of neural network and higher brain functions such as learning and memory. Synapses consist of three major regions: presynaptic apparatus, synaptic cleft, and postsynaptic region. In the presynaptic region, it is characterized that neurotransmitter-containing synaptic vesicles are clustered and mitochondria essential for ATP supply are present (Südhof 1995; Sheng 2014). In addition, there is a slightly electron dense region beneath the presynaptic plasma membrane, also known as the active zone, where some of the synaptic vesicle clusters are directly associated with the membrane (Landis et al. 1988; Südhof 1995). Upon elevation of intracellular Ca^{2+}

T. Ohtsuka (✉)
Department of Biochemistry, Graduate School of Medicine/Faculty of Medicine, University of Yamanashi, 1110 Shimokato, Chuo, Yamanashi 409-3898, Japan
e-mail: tohtsuka@yamanashi.ac.jp

S. Mochida (ed.), *Presynaptic Terminals*, DOI 10.1007/978-4-431-55166-9_3

concentration after the arrival of action potentials, primed vesicles rapidly undergo fused with the membrane at the active zone and release neurotransmitters into the synaptic cleft, which subsequently bind to and activate appropriate neurotransmitter receptors located in postsynaptic plasma membrane, resulting in prompt firing of postsynaptic neurons (Burns and Augustine 1995). Over 50 years ago, electron microscopic analyses revealed the existence of the active zone at subsynaptic level (Gray 1963; Couteaux and Pecot-Dechavassine 1970; Landis et al. 1988), but its molecular composition of the active zone had been obscure until recently. In contrast to the postsynaptic density (PSD), the active zone is relatively small and it has been difficult to isolate the active zone fraction separated from the PSD fraction, which thus have become an obstacle to systematically and biochemically identify active zone-specific proteins so far. Nevertheless, in the last two decades, the development of molecular biology and biochemistry (ex. mass spectrometry) allowed researchers to identify active zone-specific proteins in mammals such as rat and mice, including RIM1, Munc13-1, Bassoon, Piccolo/Aczonin, CAST/ERC2, and ELKS (Hida and Ohtsuka 2010; Südhof 2012). Some of these proteins are also found in invertebrates including *Drosophila* and *C. elegans* (Kittel et al. 2006; Wagh et al. 2006; Deken et al. 2005). Genetic studies especially in mice and flies demonstrate that these active zone proteins play pivotal roles in the structure and function of the presynaptic active zone (Südhof 2012; Kittel et al. 2006). However, the mode of interactions among active zone proteins and their physiological relevance are not fully understood and then much more complicated than previously envisaged. In this chapter, mainly focusing on protein-protein interactions among active zone proteins and related proteins, I would like to provide with a current overview of structural integrity of the presynaptic active zone.

3.2 Active Zone-Specific Proteins

3.2.1 Bassoon and Piccolo

Bassoon and Piccolo are well-established active zone proteins which were originally identified by use of classic biochemistry and molecular expression cloning (tom Dieck et al. 1998; Cases-Langhoff et al. 1996). They are quite large (420 kDa for Bassoon and ~500 kDa for Piccolo), containing some similar and/or distinct domains along the entire length (see Fig. 3.1) (Fenster et al. 2000; Schoch and Gundelfinger 2006). It is N-terminal zinc finger domains and two C_2-domains (C_2A and C_2B) that Piccolo specifically contains; the former is suggested to bind to a small G protein rab3A binding partner, PRA1, and the latter C_2A-domain is known to bind to Ca^{2+} (see Table 3.1; Fenster et al. 2000; Gerber et al. 2001). Its binding ability to PRA1 suggests that Piccolo is involved in intracellular trafficking presumably mediated by Rab3, but so far it has been unclear. The mode of binding between the C_2A-domain and Ca^{2+} is resolved by a crystal structure analysis using NMR spectroscopy (Gerber et al. 2001). The C_2A-domain

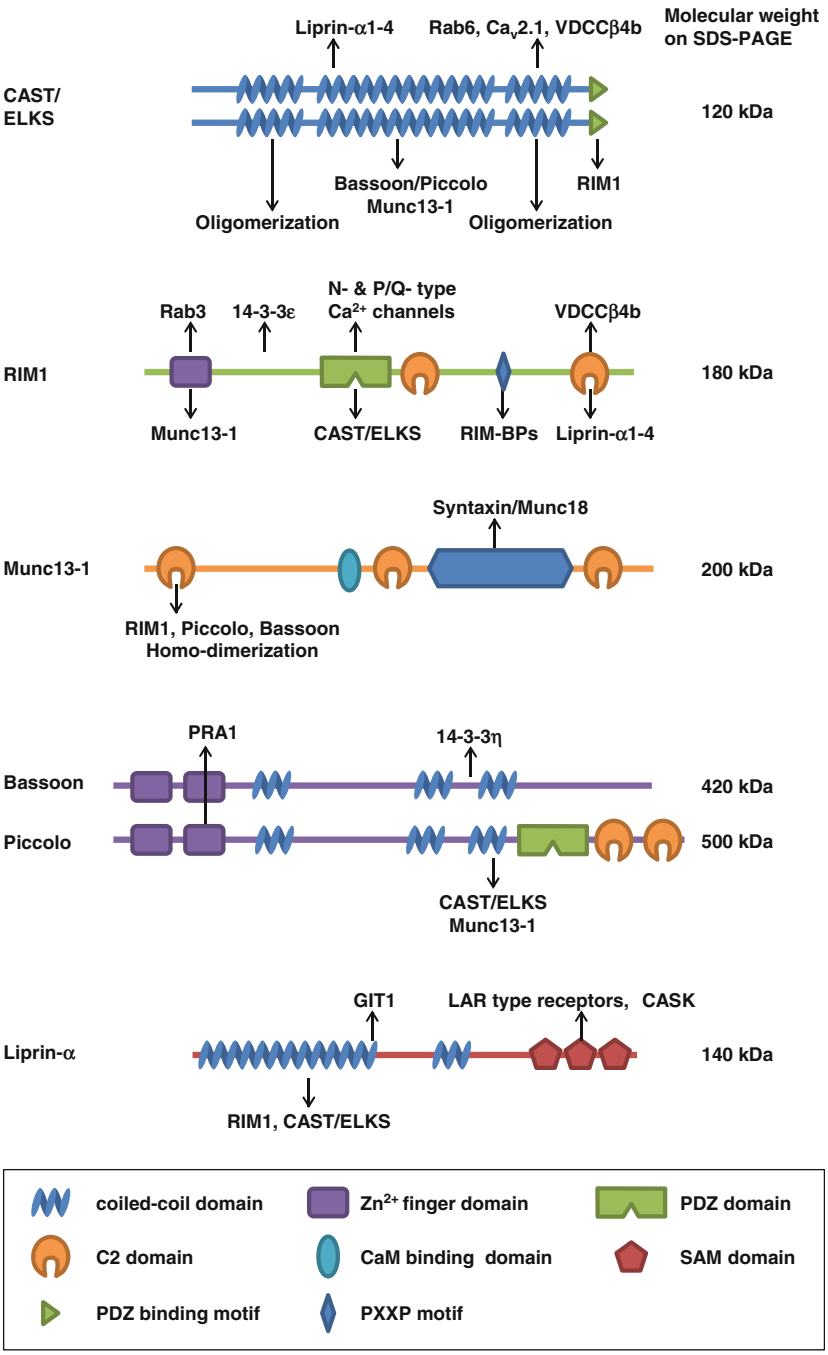

Fig. 3.1 Active zone proteins and their binding partners. RIM1 and Munc13-1 are representatives of each protein family member. For detail, see the ref (Schoch and Gundelfinger 2006)

Table 3.1 List of protein-protein interactions

Interaction	Preparation	Methods	Results	References
CAST-Liprin-α	Cultured hippocampal neurons	Co-expression	Increased synaptic localization of liprin-α1	Ko et al. (2003b)
CAST-Ca^{2+} channels	BHK6 cells	Co-expression	Shifted toward the hyperpolarizing direction	Kiyonaka et al. (2012)
CAST-RIM1	Cultured SCG neuron	Peptide inhibition	Reduced EPSP amplitude	Takao-Rikitsu et al. (2004)
CAST-Bassoon	Cultured SCG neuron	Binding inhibition	Reduced EPSP amplitude	Takao-Rikitsu et al. (2004)
RIM-Rab3	INS-1E cells	RIM overexpression	Enhanced glucose-stimulated secretion	Iezzi et al. (2000)
RIM-Ca^{2+} channel	RIM knockout mice	Electrophysiology	Rescued the decreased Ca^{2+} channel localization	Kaeser et al. (2011)
RIM-VDCCβ4b	PC12 cells	Overexpression	Increased acetylcholine release	Kiyonaka et al. (2007)
RIM-Munc13-1	Cultured hippocampal neurons	Dominant-negative overexpression	Decreased EPSC amplitude	Betz et al. (2001)

This does not cover all the interactions, but focuses on CAST, RIM, and Bassoon. For more detail, see the refs (Schoch and Gundelfinger 2006; Südhof 2012) and elsewhere

of Piccolo is mechanistically flexible and shows a relatively low affinity for Ca^{2+} (100–200 μM), suggesting that Piccolo functions in presynaptic short-term plasticity when concentration of Ca^{2+} increase during repetitive stimulation (Gerber et al. 2001). On the other hand, since the C_2B-domain of Piccolo lacks four of the five canonical aspartate residues essential for Ca^{2+} binding in C_2-domains, it seems that the Piccolo C_2B-domain does not function as a Ca^{2+}-binding domain (Gerber et al. 2001).

Bassoon and Piccolo each has several coiled-coil domains, which are composed of α-helices and involved in protein-protein interactions (tom Dieck et al. 1998). Especially, three coiled-coil regions are highly conserved and then the third coiled-coil domain of Bassoon and Piccolo directly binds to the second coiled-coil domain of CAST and ELKS (Fig. 3.1; Takao-Rikitsu et al. 2004). They share the same amino acids sequence in CAST for the binding site, and they may compete with each other to bind to CAST in vivo.

Bassoon has another binding protein, 14-3-3 adaptor protein, at the region between second and third coiled-coil domains (Fig. 3.1: Schröder et al. 2013). 14-3-3 proteins are known to be abundant in various types of cells with versatile cellular functions such as the control of signal transduction, cell proliferation and differentiation (Mackintosh 2004). They bind to phosphoserine-based motifs in their target molecules, and eventually the family member 14-3-3ε directly binds

to the region containing critical Ser residue at amino acids 2845 (rat Bassoon) in the phosphorylation-dependent manner. Elimination of this interaction indeed causes the significant decrease in molecular exchange rate of Bassoon in primary cultured hippocampal neurons (Schröder et al. 2013). Thus, phosphorylation-dependent complex formation by adaptor protein 14-3-3 may regulate the homeostasis of the presynaptic active zone partly through the control of the molecular exchange rate of Bassoon.

Bassoon also binds to RIM-binding proteins and in turn controls presynaptic P/Q-type Ca^{2+} channels (Davydova et al. 2014). Bassoon deletion or disruption between Bassoon and RIM-binding protein interaction causes the reduction of synaptic P/Q-type Ca^{2+} channels, suggesting that Bassoon specifically targets P/Q-type Ca^{2+} channels to the presynaptic active zone. As described below, RIM1 also binds to Ca^{2+} channels and possibly scaffolds both P/Q- and N-type Ca^{2+} channels (Kaeser et al. 2011). Thus, it is of interest how Ca^{2+} channels use differently these active proteins for neurotransmitter release at the presynaptic active zone.

3.2.2 RIM1

RIM1 was originally identified as a target molecule for the small G protein Rab3A (Wang et al. 1997) and is the most studied active zone protein, in particular essential for priming step of neurotransmitter release (Deng et al. 2011). As a scaffold molecule at the active zone, RIM1 has a number of binding partners such as Munc13-1, CAST/ELKS, RIM-BP2, Liprin-αs, 14-3-3, and Ca^{2+} channels (α and β subunits) (Betz et al. 2001; Ohtsuka et al. 2002; Wang et al. 2000, 2002; Schoch et al. 2002; Kaeser et al. 2008; Kiyonaka et al. 2007; Kaeser et al. 2011). Rab3A directly binds to the zinc finger domain of RIM1 in a GTP-form-dependent manner (Wang et al. 1997). This zinc finger domain is shared with another active zone protein Munc13-1, which is involved in the control of priming step of vesicle cycling (Deng et al. 2011). 14-3-3 is known to bind to phosphorylated residues as described above (Mackintosh 2004) and then bind to a region between the zinc finger and PDZ domain (Kaeser et al. 2008). This region is phosphorylated by PKA, but its physiological significance is currently unknown (Lonart et al. 2003; Kaeser et al. 2008). The first protein that directly binds to the RIM1 PDZ domain is a family of CAST/ERC2 and ELKS (Ohtsuka et al. 2002; Wang et al. 2002), and more recently α1 subunits of Ca^{2+} channels are identified as the PDZ domain-binding protein by the use of yeast two-hybrid method (Kaeser et al. 2011). Voltage-dependent Ca^{2+} channels consist of α1 subunit and auxiliary β and α2δ subunits (Catterall 2005). N- and P/Q-type Ca^{2+} channels largely control neurotransmitter release from the presynaptic active zone and the R-type also contributes, whereas L- and T-type channels do not (Wu et al. 1999). The C-terminal region of N- and P/Q-type Ca^{2+} channels selectively binds to the PDZ domain of RIM1, but L- and T-type channels do not (Kaeser et al. 2011). This is quite intriguing that CAST also directly binds to the PDZ domain of RIM1 (Ohtsuka et al. 2002; Wang et al. 2002).

Then, it would be of great interest to clarify whether and how CAST competes or simultaneously binds to the PDZ domain of RIM1 with Ca^{2+} channels. While the Kd value for binding between Ca^{2+} channel and the PDZ domain is 10.3 μM (P/Q-type) and 23.4 μM (N-type) (Kaeser et al. 2011), that of binding between the ELKS C-terminal peptide and the RIM PDZ domain is 0.27 μM (Lu et al. 2005). Since the C-terminal sequences of ELKS and CAST are almost identical, CAST probably has the similar high affinity for the RIM PDZ domain. Thus, it is likely that the protein complex of the CAST/ELKS family and RIM is much more stable in the active zones in vivo than that of the Ca^{2+} channel and RIM. Further biochemical studies and high-resolution electron microscopy should uncover the more precise mode of interaction among RIM1, CAST/ELKS, and Ca^{2+} channels at the active zones, which may contribute to our exact understanding of the relationship of active zone proteins' function and fast Ca^{2+}-dependent neurotransmitter release. Additionally, the β-subunit of Ca^{2+} channels binds to the C-terminal C2B domain of RIM1, which affects the inactivation of Ca^{2+} channels at least in vitro (Kiyonaka et al. 2007). This interaction is not required for clustering of Ca^{2+} channels (Kiyonaka et al. 2007). RIM-BPs bind to the region containing PXXP motif in RIM1 and are also shown to interact with L-, N-, and P/Q-type Ca^{2+} channels (Hibino et al. 2002). Thus, RIM1 has the ability to directly bind to the Ca^{2+} channels and indirectly bind to them at least through its binding to RIM-BPs.

3.2.3 Munc13-1

Munc13 is a mammalian orthologue of *C. elegans* UNC-13 that is one of the uncoordinated (UNC) mutants defective for locomotor function and perception (Brenner 1974; Brose et al. 1995). Munc13 consists of a protein family including Munc13-1, Munc13-2, Munc13-3, and Munc13-4 (Brose et al. 1995; Schoch and Gundelfinger 2006). Alternative splicing of the Munc13-2 gene further produces two isoforms: ubMunc13-2 and bMunc13-2 of which the former is ubiquitously expressed, but the latter is specific to the brain (Betz et al. 2001). Among them, Munc13-1 was the first to be localized to the presynaptic active zone (Brose et al. 1995). Munc13-1 plays an essential role in the release of neurotransmitter, examined by electrophysiological analyses using Munc13-1 KO mice in which the release of neurotransmitter is completely abolished and the KO mice die immediately after birth possibly due to deficits in neuromuscular junction (NMJ) function in the lung (Augustin et al. 1999). Munc13-1 directly interacts with Syntaxin, which could correlate Munc13-1 with SNARE system through Syntaxin (Betz et al. 1997). For example, in *C. elegans*, Munc13 binding is thought to induce the open conformation of Syntaxin from the closed form, following the formation of a loose SNARE complex (Richmond et al. 2001). The N-terminal region of Munc13-1 moreover has several other interacting proteins such as RIM1 (Betz et al. 2001), Piccolo/Bassoon (Wang et al. 2009), Munc13-1 itself (Lu et al. 2006), and calmodulin (Junge et al. 2004), and the C-terminal has DOC2α (Orita et al. 1997) and msec7-1 ARF GEF (Neeb et al. 1999).

3.2.4 CAST/ERC2 and ELKS

CAST (cytomatrix at the active zone-associated structural protein) was first purified and identified from the rat brain (Ohtsuka et al. 2002) and subsequently isolated by yeast two-hybrid method as ERC2 (Wang et al. 2002). CAST/ERC2 has many coiled-coil regions over its entire sequence and a unique three amino acids IWA at its C terminus. The IWA motif is essential for CAST/ERC2 binding to the PDZ domain of RIM as described above (Ohtsuka et al. 2002; Wang et al. 2002; Fig. 3.1). Other active zone proteins Bassoon, Piccolo, and Munc13-1 also directly bind to the middle coiled-coil region of CAST (Takao-Rikitsu et al. 2004; Wang et al. 2009). CAST has a family member, named ELKS, which was originally identified as a gene product translocated in the different chromosome in a thyroid carcinoma (Nakata et al. 1999). CAST and ELKS show a relatively high homology (~70 % amino acids identity) and presumably form hetero- and/or homo-oligomers through their N- and C-terminal coiled-coil regions (Deguchi-Tawarada et al. 2004). In addition to these active zone proteins, CAST has other synaptic proteins as binding partners such as liprins, Rab6, and Ca^{2+} channels (Ko et al. 2003b; Monier et al. 2002; Kiyonaka et al. 2012). Liprin, a mammalian homologue of *C. elegans* SYD-2, binds to the middle coiled-coil region of CAST (Ko et al. 2003b). Rab6, a subfamily member of small GTP binding protein, and Ca^{2+} channels directly bind to the C-terminal coiled-coil regions of ELKS and CAST, respectively (Monier et al. 2002; Kiyonaka et al. 2012). Although not all the interactions are examined, it is likely that ELKS has the same ability to bind to these active zone and synapse-related proteins (Fig. 3.1).

CAST and ELKS have distinct biochemical features in that CAST is highly resistant to extraction with nonionic detergents, and then tightly associated with the active zone cytomatrix, while ELKS is more soluble with the detergents (Ohtsuka et al. 2002; Deguchi-Tawarada et al. 2004). Currently, it is still obscure why these closely related proteins have distinct localization and solubility in neurons, but it seems likely that they have the same and distinct binding partners which may affect their biochemical features.

3.2.5 Liprin

Proteins of liprin α-family were originally identified by the yeast hybrid method, which shows that they interact with the cytoplasmic regions of leukocyte common antigen related (LAR) (Serra-Pages et al. 1995). Liprin proteins consist of N-terminal coiled-coil domains, one C-terminal liprin homology (LH) domain, and three sterile alpha motifs (SAMs) (Serra-Pages et al. 1998). Vertebrate has at least four liprin α-family members (liprin α1-4), while *Drosophila* and *C. elegans* have one homologue, liprin-α, and Syd-2, respectively (Zhen and Jin 1999; Dai et al. 2006). Syd-2 was first identified and characterized in a genetic screening to

find synaptic deficient mutants in *C. elegans* (Zhen and Jin 1999). In the Syd-2 mutant animals, the length of the active zone became longer than wild types. Liprin-α has some binding partners such as LAR, GIT1, CASK, RIM1, and CAST/ELKS (see Fig. 3.1; Ko et al. 2003a; Olsen et al. 2005; Stryker and Johnson 2007; Schoch et al. 2002; Ko et al. 2003b).

3.3 Physiological Significance of Active Zone Protein-Protein Interactions

The interaction of RIM1 and Munc13-1 was first reported that its interaction is involved in a priming step of neurotransmitter release (Betz et al. 2001), which is mediated by the interaction of zinc-finger domain of RIM1 and C1 domain of Munc13-1 as described above (Betz et al. 2001; Dulubova et al. 2005). Consistent with these observations, in the RIM1 knockout mice, the expression level of Munc13-1 is significantly reduced to ~50 % compared to that of wild-type mice (Schoch et al. 2002). Thus, this interaction may also affect the homeostasis of Munc13-1 in vivo. The C1 domains are well conserved among Munc13 family members, but Munc13-4 lacks this domain (Schoch and Gundelfinger 2006), suggesting that these members may have different mechanisms for their stability in the nerve terminals. Mechanistically, this N-terminal C2A domain can homodimerize, which then disturbs the priming function of Munc13-1 via the MUN domain (Deng et al. 2011).

Another example is the interaction among CAST and RIM1 or Bassoon/Piccolo (Takao-Rikitsu et al. 2004). Microinjection of a peptide encoding C-terminus of CAST, which disrupts interaction between CAST and RIM1, significantly impairs EPSC examined in primary cultured superior cervical ganglion neurons of rat (Fig. 3.2c, d) (Takao-Rikitsu et al. 2004). Conversely, the microinjection of the CAST-binding domain of RIM1 also impairs EPSC (Takao-Rikitsu et al. 2004). The exact mechanism for this impairment is not clear, but it is possible that this disruption causes RIM1 to be mislocalized in the nerve terminals. This idea may be supported by the following observations: (1) In CAST/ERC2 KO mice, RIM1 level associated with presynaptic membranes is significantly reduced (Kaeser et al. 2009); (2) exogenously expressed RIM1, lacking its PDZ domain (CAST-binding domain), is diffusely localized throughout axons (Ohtsuka et al. 2002); and (3) exogenously expressed CAST, lacking C-terminal IWA motif, is correctly localized with Bassoon in axons (Ohtsuka et al. 2002). These suggest that CAST functions as an anchoring protein for RIM1 at the active zone, while CAST may not require RIM1 for its correct localization to the active zones in cultured neurons. In addition, perturbation of interaction between CAST and Bassoon in the SCGNs significantly reduces EPSC in the SCGNs (Fig. 3.2f, g; Takao-Rikitsu et al. 2004). But, since exogenously expressed CAST, lacking the Bassoon-binding domain, and Bassoon, lacking the CAST-binding domain, are both correctly localized to the

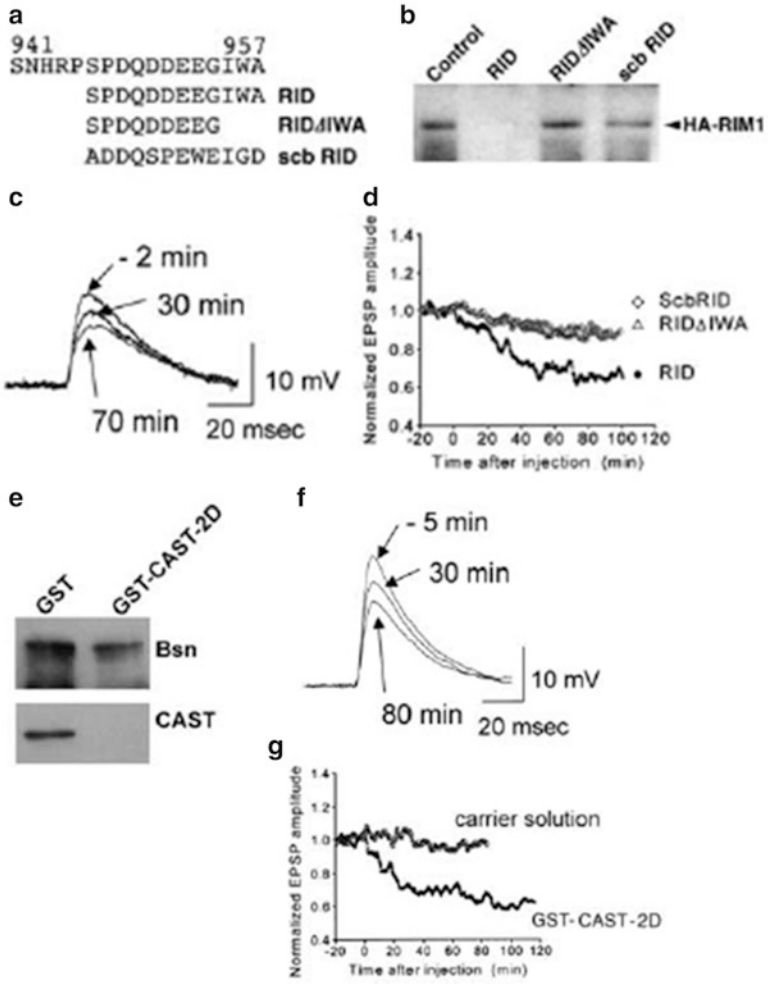

Fig. 3.2 Physiological significance of the protein-protein interaction. (**a–d**) Effects of the COOH-terminal regions of CAST on synaptic transmission. (**a**) Sequences of the CAST peptides. RID, RIM1-interacting domain; scb RID, scrambled RID. (**b**) Effects of the peptides (5 μM each) on the binding of HA-RIM1 to immobilized GST-CAST C-terminal region. The binding was inhibited by RID, but not by RIDΔIWA or scb RID. (**c**, **d**) Effects of the CAST peptides (1 mM each in the injection pipette) on synaptic transmission. Presynaptic neurons were stimulated every 20 s. CAST peptides were introduced into presynaptic neurons at $t = 0$. EPSPs from representative experiments with the injection are illustrated in **c**. Normalized and averaged EPSP amplitudes are plotted from five experiments with RID, RIDΔIWA, or scb RID peptide in **d**. (**e–g**) Effect of the Bassoon-binding domain (BsnBD) of CAST on synaptic transmission. (**e**) Effect of GST-CAST-2D (BsnBD) on the binding of CAST and Bassoon. Immunoprecipitation assay of Myc-CAST and EGFP-Bassoon was performed in the presence of GST alone or GST-CAST-2D (5 μM each), followed by Western blotting using the anti-Myc and anti-GFP Abs. GST-CAST-2D inhibited the binding, but GST alone did not. (**f**, **g**) Effect of GST-CAST-2D (150 μM) on synaptic transmission. The presynaptic neuron was stimulated every 20 s. The recombinant CAST protein was introduced into the presynaptic neuron at $t = 0$. EPSPs from the representative experiments with the injection are illustrated in (**f**). Normalized and averaged EPSP amplitudes are plotted from seven experiments with GST-CAST2D or the carrier solution alone in (**g**) (Source: Adapted from Takao-Rikitsu et al. 2004)

active zones in primary cultured neurons, these bindings are not required for each protein's localizations to the active zones. Bassoon has an N-terminal palmitoylation motif, which may partly contribute to its localization in the nerve terminals (Sanmarti-Vila et al. 2000; Dresbach et al. 2003). In contrast, the CAST-binding domain of Piccolo has no effect on the amplitude of EPSC under the conditions where that of Bassoon affects the amplitude as described above (Takao-Rikitsu et al. 2004). It is currently unknown for this, but it may be due to the instability of microinjected GST-fusion protein of Piccolo (CAST-binding region) or that the binding of CAST and Piccolo is less contributed in vivo than that of CAST and Bassoon.

3.4 Insights from Genetic Studies in Drosophila and Mice

Drosophila has the ELKS/CAST homologue, named Bruchpilot which is thought to play important roles in excitatory neurotransmission (Fig. 3.3; Kittel et al. 2006). Bruchpilot consists of coiled-coil regions over the entire sequence like CAST and ELKS, but it shows higher molecular weight ~190 kDa with a distinct C-terminal region, which also lacks the C-terminal IWA sequence characteristic for CAST and ELKS. Bruchpilot decorates around *Drosophila* active zone, so-called T-bars

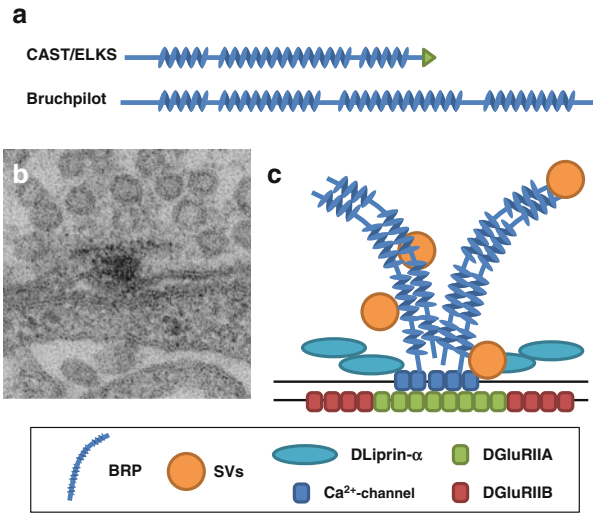

Fig. 3.3 T-bar, *Drosophila* active zone. (**a**) Comparison between molecular structures of CAST/ELKS and the *Drosophila* homologue Bruchpilot. Bruchpilot contains the distinct C-terminal region which lacks the IWA sequence. (**b**) Electron micrograph for T-bar in *Drosophila* neuromuscular junction. Courtesy from Drs. Stephan Sigrid and Christine Quentin. (**c**) Possible model of T-bar. The N-terminal region of Bruchpilot is closely associated with presynaptic plasma membranes where it directly binds to and recruits cacophony, Drosophila Ca^{2+} channel (Fouquet et al. 2009)

(Fig. 3.3b), and there directly interacts with Cacophony, a *Drosophila* Ca^{2+} channel, controlling its clustering at the presynaptic active zone (Kittel et al. 2006; Fouquet et al. 2009). Pulldown and yeast two-hybrid assay reveal that the N-terminal region of Bruchpilot physically interacts with the C-terminal region of cacophony (Fig. 3.3a; Fouquet et al. 2009). Indeed, in the Bruchpilot mutants, T-bar is lost and the Ca^{2+} channel is diffusely clustered, resulting in impairment of evoked synaptic vesicle release and short-term plasticity such as paired-pulse facilitation (Kittel et al. 2006). At least in *Drosophila*, Bruchpilot may be involved in the generation of slots for Ca^{2+} channel insertion into the presynaptic active zone, while Ca^{2+} channel itself is not involved in targeting of Bruchpilot to the active zone (Kawasaki et al. 2002; Fouquet et al. 2009).

Another line of evidence that CAST has physiological function for active zone structure comes from the studies of CAST KO retina (tom Dieck et al. 2012). Immunofluorescence and electron microscopy reveal that in the CAST KO retina, the size of the rod presynaptic active zone is reduced and Ca^{2+} channel complement is perturbed which results in significantly diminished amplitudes of the b-wave in scotopic electroretinogram. On the other hand, in the mice mutants, clustering of Ca^{2+} channels is intact although CAST directly interacts with the channels as described above (Fig. 3.1; Kiyonaka et al. 2012). It is thus currently an open question if CAST is involved in targeting of Ca^{2+} channels in mammals. Outside of the retina, CAST/ERC2 deletion was used to study hippocampal synapses where the overall structure of synapses is almost intact, whereas inhibitory synaptic transmission is significantly impaired, but excitatory transmission is not affected (Kaeser et al. 2009). This result seems to contradict with the previous observations in the mouse retina (tom Dieck et al. 2012), primary cultured SCG neurons (Fig. 3.2; Takao-Rikitsu et al. 2004), and *Drosophila* (Kittel et al. 2006) that CAST controls at least excitatory neurotransmitter release.

3.5 Perspective and Conclusion

Accumulating evidence suggests that the presynaptic active zone proteins play pivotal roles in neurotransmitter release and molecular organization of the active zone (Fig. 3.4), consequently affecting short- and long-term plasticity and animal behavior. As described in this chapter, several active zone-specific proteins have been identified and characterized, but exact molecular composition and their protein-protein network of interaction are still obscure and complex. One important issue is then the relationship between active zone proteins and Ca^{2+} channels of which elucidation will lead to more precise understating of spatially and temporally regulated Ca^{2+}-dependent transmitter release. It is not known for N-type Ca^{2+} channel-specific scaffold proteins. In addition, another crucial issue is how specific targeting of active zone proteins to the destination beneath the presynaptic plasma membranes is controlled. So far, in spite of numerous efforts, there have been no transmembrane proteins tightly and specifically associated with the presynaptic

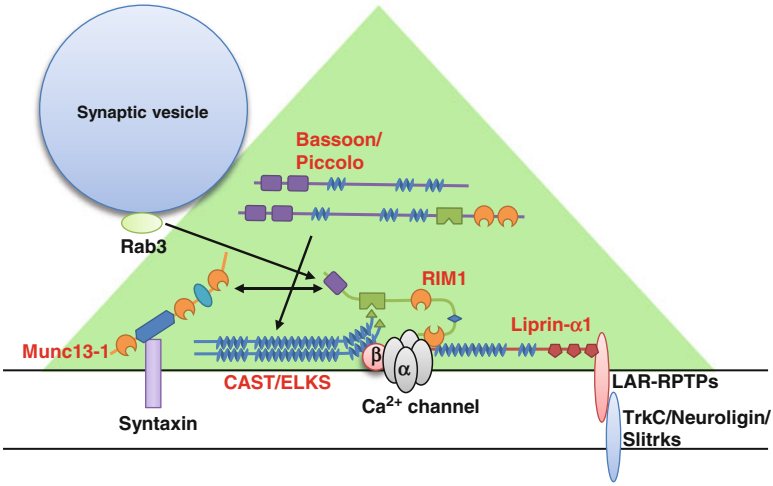

Fig. 3.4 Mode of protein-protein interactions among active zone proteins

active zone. Once such transmembrane proteins are identified, more mechanistic approaches could be available to examine the structure and function of the presynaptic active zone, which should open up a new avenue in this exciting field and shed new light on our understanding of higher brain functions.

Acknowledgments I thank Y. Hida for preparing the figures in this manuscript.

References

Augustin I, Rosenmund C, Südhof TC, Brose N (1999) Munc13-1 is essential for fusion competence of glutamatergic synaptic vesicles. Nature 400:457–461

Betz A, Okamoto M, Benseler F, Brose N (1997) Direct interaction of the rat unc-13 homologue Munc13-1 with the N terminus of syntaxin. J Biol Chem 272:2520–2526

Betz A, Thakur P, Junge HJ, Ashery U, Rhee JS, Scheuss V, Rosenmund C, Rettig J, Brose N (2001) Functional interaction of the active zone proteins Munc13-1 and RIM1 in synaptic vesicle priming. Neuron 30:183–196

Brenner S (1974) The genetics of Caenorhabditis elegans. Genetics 77:71–94

Brose N, Hofmann K, Hata Y, Südhof TC (1995) Mammalian homologues of Caenorhabditis elegans unc-13 gene define novel family of C2-domain proteins. J Biol Chem 270:25273–25280

Burns ME, Augustine GJ (1995) Synaptic structure and function: dynamic organization yields architectural precision. Cell 83:187–194

Cases-Langhoff C, Voss B, Garner AM, Appeltauer U, Takei K, Kindler S, Veh RW, De Camilli P, Gundelfinger ED, Garner CC (1996) Piccolo, a novel 420 kDa protein associated with the presynaptic cytomatrix. Eur J Cell Biol 69:214–223

Catterall WA (2005) International Union of Pharmacology. XLVIII. Nomenclature and structure-function relationships of voltage-gated calcium channels. Pharmacol Rev 57:411–425

Couteaux R, Pecot-Dechavassine M (1970) Synaptic vesicles and pouches at the level of "active zones" of the neuromuscular junction. C R Acad Sci Hebd Seances Acad Sci D 271:2346–2349

Dai Y, Taru H, Deken SL, Grill B, Ackley B, Nonet ML, Jin Y (2006) SYD-2 Liprin-alpha organizes presynaptic active zone formation through ELKS. Nat Neurosci 9:1479–1487

Davydova D, Marini C, King C, Klueva J, Bischof F, Romorini S, Montenegro-Venegas C, Heine M, Schneider R, Schröder MS, Altrock WD, Henneberger C, Rusakov DA, Gundelfinger ED, Fejtova A (2014) Bassoon specifically controls presynaptic P/Q-type Ca2+ channels via RIM-binding protein. Neuron 82:181–194

Deguchi-Tawarada M, Inoue E, Takao-Rikitsu E, Inoue M, Ohtsuka T, Takai Y (2004) CAST2: identification and characterization of a protein structurally related to the presynaptic cytomatrix protein CAST. Genes Cells 9:15–23

Deken SL, Vincent R, Hadwiger G, Liu Q, Wang ZW, Nonet ML (2005) Redundant localization mechanisms of RIM and ELKS in Caenorhabditis elegans. J Neurosci 25:5975–5983

Deng L, Kaeser PS, Xu W, Südhof TC (2011) RIM proteins activate vesicle priming by reversing autoinhibitory homodimerization of Munc13. Neuron 69:317–331

Dresbach T, Hempelmann A, Spilker C, tom Dieck S, Altrock WD, Zuschratter W, Garner CC, Gundelfinger ED (2003) Functional regions of the presynaptic cytomatrix protein bassoon: significance for synaptic targeting and cytomatrix anchoring. Mol Cell Neurosci 23:279–291

Dulubova I, Lou X, Lu J, Huryeva I, Alam A, Schneggenburger R, Südhof TC, Rizo J (2005) A Munc13/RIM/Rab3 tripartite complex: from priming to plasticity? EMBO J 24:2839–2850

Fenster SD, Chung WJ, Zhai R, Cases-Langhoff C, Voss B, Garner AM, Kaempf U, Kindler S, Gundelfinger ED, Garner CC (2000) Piccolo, a presynaptic zinc finger protein structurally related to bassoon. Neuron 25:203–214

Fouquet W, Owald D, Wichmann C, Mertel S, Depner H, Dyba M, Hallermann S, Kittel RJ, Eimer S, Sigrist SJ (2009) Maturation of active zone assembly by Drosophila Bruchpilot. J Cell Biol 186:129–145

Gerber SH, Garcia J, Rizo J, Südhof TC (2001) An unusual C(2)-domain in the active-zone protein piccolo: implications for Ca(2+) regulation of neurotransmitter release. EMBO J 20:1605–1619

Gray EG (1963) Electron microscopy of presynaptic organelles of the spinal cord. J Anat 97:101–106

Hibino H, Pironkova R, Onwumere O, Vologodskaia M, Hudspeth AJ, Lesage F (2002) RIM binding proteins (RBPs) couple Rab3-interacting molecules (RIMs) to voltage-gated Ca(2+) channels. Neuron 34:411–423

Hida Y, Ohtsuka T (2010) CAST and ELKS proteins: structural and functional determinants of the presynaptic active zone. J Biochem 148:131–137

Iezzi M, Regazzi R, Wollheim CB (2000) The Rab3-interacting molecule RIM is expressed in pancreatic beta-cells and is implicated in insulin exocytosis. FEBS Lett 474:66–70

Junge HJ, Rhee JS, Jahn O, Varoqueaux F, Spiess J, Waxham MN, Rosenmund C, Brose N (2004) Calmodulin and Munc13 form a Ca2+ sensor/effector complex that controls short-term synaptic plasticity. Cell 118:389–401

Kaeser PS, Kwon HB, Blundell J, Chevaleyre V, Morishita W, Malenka RC, Powell CM, Castillo PE, Südhof TC (2008) RIM1alpha phosphorylation at serine-413 by protein kinase A is not required for presynaptic long-term plasticity or learning. Proc Natl Acad Sci U S A 105:14680–14685

Kaeser PS, Deng L, Chavez AE, Liu X, Castillo PE, Südhof TC (2009) ELKS2alpha/CAST deletion selectively increases neurotransmitter release at inhibitory synapses. Neuron 64:227–239

Kaeser PS, Deng L, Wang Y, Dulubova I, Liu X, Rizo J, Südhof TC (2011) RIM proteins tether Ca (2+) channels to presynaptic active zones via a direct PDZ-domain interaction. Cell 144:282–295

Kawasaki F, Collins SC, Ordway RW (2002) Synaptic calcium-channel function in Drosophila: analysis and transformation rescue of temperature-sensitive paralytic and lethal mutations of cacophony. J Neurosci 22:5856–5864

Kittel RJ, Wichmann C, Rasse TM, Fouquet W, Schmidt M, Schmid A, Wagh DA, Pawlu C, Kellner RR, Willig KI, Hell SW, Buchner E, Heckmann M, Sigrist SJ (2006) Bruchpilot promotes active zone assembly, Ca2+ channel clustering, and vesicle release. Science 312:1051–1054

Kiyonaka S, Wakamori M, Miki T, Uriu Y, Nonaka M, Bito H, Beedle AM, Mori E, Hara Y, De Waard M, Kanagawa M, Itakura M, Takahashi M, Campbell KP, Mori Y (2007) RIM1 confers sustained activity and neurotransmitter vesicle anchoring to presynaptic Ca2+ channels. Nat Neurosci 10:691–701

Kiyonaka S, Nakajima H, Takada Y, Hida Y, Yoshioka T, Hagiwara A, Kitajima I, Mori Y, Ohtsuka T (2012) Physical and functional interaction of the active zone protein CAST/ERC2 and the beta-subunit of the voltage-dependent Ca2+ channel. J Biochem 152:149–159

Ko J, Kim S, Valtschanoff JG, Shin H, Lee JR, Sheng M, Premont RT, Weinberg RJ, Kim E (2003a) Interaction between liprin-alpha and GIT1 is required for AMPA receptor targeting. J Neurosci 23:1667–1677

Ko J, Na M, Kim S, Lee JR, Kim E (2003b) Interaction of the ERC family of RIM-binding proteins with the liprin-alpha family of multidomain proteins. J Biol Chem 278:42377–42385

Landis DM, Hall AK, Weinstein LA, Reese TS (1988) The organization of cytoplasm at the presynaptic active zone of a central nervous system synapse. Neuron 1:201–209

Lonart G, Schoch S, Kaeser PS, Larkin CJ, Südhof TC, Linden DJ (2003) Phosphorylation of RIM1alpha by PKA triggers presynaptic long-term potentiation at cerebellar parallel fiber synapses. Cell 115:49–60

Lu J, Li H, Wang Y, Südhof TC, Rizo J (2005) Solution structure of the RIM1α PDZ domain in complex with an ELKS1b C-terminal peptide. J Mol Biol 352:455–466

Lu J, Machius M, Dulubova I, Dai H, Südhof TC, Tomchick DR, Rizo J (2006) Structural basis for a Munc13-1 homodimer to Munc13-1/RIM heterodimer switch. PLoS Biol 4:e192

Mackintosh C (2004) Dynamic interactions between 14-3-3 proteins and phosphoproteins regulate diverse cellular processes. Biochem J 381:329–342

Monier S, Jollivet F, Janoueix-Lerosey I, Johannes L, Goud B (2002) Characterization of novel Rab6-interacting proteins involved in endosome-to-TGN transport. Traffic 3:289–297

Nakata T, Kitamura Y, Shimizu K, Tanaka S, Fujimori M, Yokoyama S, Ito K, Emi M (1999) Fusion of a novel gene, ELKS, to RET due to translocation t(10;12)(q11;p13) in a papillary thyroid carcinoma. Genes Chromosomes Cancer 25:97–103

Neeb A, Koch H, Schürmann A, Brose N (1999) Direct interaction between the ARF-specific guanine nucleotide exchange factor msec7-1 and presynaptic Munc13-1. Eur J Cell Biol 78:533–538

Ohtsuka T, Takao-Rikitsu E, Inoue E, Inoue M, Takeuchi M, Matsubara K, Deguchi-Tawarada M, Satoh K, Morimoto K, Nakanishi H, Takai Y (2002) CAST: a novel protein of the cytomatrix at the active zone of synapses that forms a ternary complex with RIM1 and Munc13-1. J Cell Biol 158:577–590

Olsen O, Moore KA, Fukata M, Kazuta T, Trinidad JC, Kauer FW, Streuli M, Misawa H, Burlingame AL, Nicoll RA, Bredt DS (2005) Neurotransmitter release regulated by a MALS-liprin-alpha presynaptic complex. J Cell Biol 170:1127–1134

Orita S, Naito A, Sakaguchi G, Maeda M, Igarashi H, Sasaki T, Takai Y (1997) Physical and functional interactions of Doc2 and Munc13 in Ca2+-dependent exocytotic machinery. J Biol Chem 272:16081–16084

Richmond JE, Weimer RM, Jorgensen EM (2001) An open form of syntaxin bypasses the requirement for UNC-13 in vesicle priming. Nature 412:338–341

Sanmarti-Vila L, tom Dieck S, Richter K, Altrock W, Zhang L, Volknandt W, Zimmermann H, Garner CC, Gundelfinger ED, Dresbach T (2000) Membrane association of presynaptic cytomatrix protein bassoon. Biochem Biophys Res Commun 275:43–46

Schoch S, Gundelfinger ED (2006) Molecular organization of the presynaptic active zone. Cell Tissue Res 326:379–391

Schoch S, Castillo PE, Jo T, Mukherjee K, Geppert M, Wang Y, Schmitz F, Malenka RC, Südhof TC (2002) RIM1alpha forms a protein scaffold for regulating neurotransmitter release at the active zone. Nature 415:321–326

Schröder MS, Stellmacher A, Romorini S, Marini C, Montenegro-Venegas C, Altrock WD, Gundelfinger ED, Fejtova A (2013) Regulation of presynaptic anchoring of the scaffold protein Bassoon by phosphorylation-dependent interaction with 14-3-3 adaptor proteins. PLoS One 8: e58814

Serra-Pages C, Kedersha NL, Fazikas L, Medley Q, Debant A, Streuli M (1995) The LAR transmembrane protein tyrosine phosphatase and a coiled-coil LAR-interacting protein co-localize at focal adhesions. EMBO J 14:2827–2838

Serra-Pages C, Medley QG, Tang M, Hart A, Streuli M (1998) Liprins, a family of LAR transmembrane protein-tyrosine phosphatase-interacting proteins. J Biol Chem 273:15611–15620

Sheng ZH (2014) Mitochondrial trafficking and anchoring in neurons: new insight and implications. J Cell Biol 204:1087–1098

Stryker E, Johnson KG (2007) LAR, liprin and the regulation of active zone morphogenesis. J Cell Sci 120:3723–3728

Südhof TC (1995) The synaptic vesicle cycle: a cascade of protein-protein interactions. Nature 375:645–653

Südhof TC (2012) The presynaptic active zone. Neuron 75:11–25

Takao-Rikitsu E, Mochida S, Inoue E, Deguchi-Tawarada M, Inoue M, Ohtsuka T, Takai Y (2004) Physical and functional interaction of the active zone proteins, CAST, RIM1, and Bassoon, in neurotransmitter release. J Cell Biol 164:301–311

tom Dieck S, Sanmartí-Vila L, Langnaese K, Richter K, Kindler S, Soyke A, Wex H, Smalla KH, Kämpf U, Fränzer JT, Stumm M, Garner CC, Gundelfinger ED (1998) Bassoon, a novel zinc-finger CAG/glutamine-repeat protein selectively localized at the active zone of presynaptic nerve terminals. J Cell Biol 142:499–509

tom Dieck S, Specht D, Strenzke N, Hida Y, Krishnamoorthy V, Schmidt KF, Inoue E, Ishizaki H, Tanaka-Okamoto M, Miyoshi J, Hagiwara A, Brandstatter JH, Lowel S, Gollisch T, Ohtsuka T, Moser T (2012) Deletion of the presynaptic scaffold CAST reduces active zone size in rod photoreceptors and impairs visual processing. J Neurosci 32:12192–12203

Wagh DA, Rasse TM, Asan E, Hofbauer A, Schwenkert I, Dürrbeck H, Buchner S, Dabauvalle MC, Schmidt M, Qin G, Wichmann C, Kittel R, Sigrist SJ, Buchner E (2006) Bruchpilot, a protein with homology to ELKS/CAST, is required for structural integrity and function of synaptic active zones in Drosophila. Neuron 49:833–844

Wang Y, Okamoto M, Schmitz F, Hofmann K, Südhof TC (1997) Rim is a putative Rab3 effector in regulating synaptic-vesicle fusion. Nature 388:593–598

Wang Y, Sugita S, Südhof TC (2000) The RIM/NIM family of neuronal C2 domain proteins. Interactions with Rab3 and a new class of Src homology 3 domain proteins. J Biol Chem 275:20033–20044

Wang Y, Liu X, Biederer T, Südhof TC (2002) A family of RIM-binding proteins regulated by alternative splicing: implications for the genesis of synaptic active zones. Proc Natl Acad Sci U S A 99:14464–14469

Wang X, Hu B, Zieba A, Neumann NG, Kasper-Sonnenberg M, Honsbein A, Hultqvist G, Conze T, Witt W, Limbach C, Geitmann M, Danielson H, Kolarow R, Niemann G, Lessmann V, Kilimann MW (2009) A protein interaction node at the neurotransmitter release site: domains of Aczonin/Piccolo, Bassoon, CAST, and rim converge on the N-terminal domain of Munc13-1. J Neurosci 29:12584–12596

Wu LG, Westenbroek RE, Borst JG, Catterall WA, Sakmann B (1999) Calcium channel types with distinct presynaptic localization couple differentially to transmitter release in single calyx-type synapses. J Neurosci 19:726–736

Zhen M, Jin Y (1999) The liprin protein SYD-2 regulates the differentiation of presynaptic termini in C. elegans. Nature 401:371–375

Part III
Synaptic Vesicle Fusion

Chapter 4
Fusion Machinery: SNARE Protein Complex

Ira Milosevic and Jakob B. Sørensen

Abstract SNARE proteins constitute the minimal machinery needed for membrane fusion. SNAREs operate by forming a complex, which pulls the lipid bilayers into close contact and provides the mechanical force needed for lipid bilayer fusion. At the chemical synapse, SNARE-complex formation between the vesicular SNARE VAMP2/synaptobrevin-2 and the target (plasma membrane) SNAREs SNAP25 and syntaxin-1 results in fusion and release of neurotransmitter, synchronized to the electrical activity of the cell by calcium influx and binding to synaptotagmin. Formation of the SNARE complex is tightly regulated and appears to start with syntaxin-1 bound to an SM (Sec1/Munc18-like) protein. Proteins of the Munc13-family are responsible for opening up syntaxin and allowing sequential binding of SNAP-25 and VAMP2/synaptobrevin-2. N- to C-terminal "zippering" of the SNARE domains leads to membrane fusion. An intermediate, half-zippered, state represents the "primed" vesicle, which is ready for release when C-terminal SNARE assembly is triggered by synaptotagmin. Following fusion, the SNAREs are recycled by the action of the AAA-ATPase NSF (N-ethylmaleimide-sensitive factor). In recent years, the lipid requirements for the SNARE mechanism have been scrutinized, and roles for the "noncanonical" SNAREs in the synapse are emerging, yet much remains to be learned about the spatial and temporal regulation of fusion.

Keywords Exocytosis • Membrane fusion • SNARE protein • Synapse • Neuron • Synaptic vesicle

I. Milosevic
European Neuroscience Institute (ENI), Grisebachstr. 5, D-37077 Göttingen, Germany
e-mail: imilose@gwdg.de

J.B. Sørensen (✉)
Department of Neuroscience and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen, Blegdamsvej 3B, DK-2200 Copenhagen N, Denmark
e-mail: jakobbs@sund.ku.dk

S. Mochida (ed.), *Presynaptic Terminals*, DOI 10.1007/978-4-431-55166-9_4

4.1 SNARE Complex and Membrane Fusion: Introduction

Many fundamental cellular functions rely on the transport processes through membrane fusion because cells are subdivided into subcellular compartments by lipid bilayer membranes. Membrane fusion is a cooperative and synchronized process characterized by three central steps, although each of these steps represents a complex sequence of events on its own (Risselada and Grubmuller 2012). In the first step, two bilayers come into very close contact, sometimes as close as 1 nm. During the second step, an initial lipid structure develops that connects two bilayers. In the third step, this structure transforms into a funnel-like structure, the fusion pore, which connects the two membranes and allows flux of interior content (e.g., neurotransmitters). The exact mechanisms underlying this complex sequence of events are not fully understood, in spite of the fact that some aforementioned intermediates have been directly observed (Risselada and Grubmuller 2012).

One of the most important, as well as best-studied, examples of membrane fusion is neurotransmitter release at the neuronal synapse, a process which requires fusion of synaptic vesicles with the presynaptic plasma membrane. The current prevailing view is that regulated neurotransmitter release at the neuronal synapse relies on the same basic proteinaceous machinery as other membrane trafficking events (Jahn and Fasshauer 2012; Sudhof 2013). Soluble NSF attachment protein (SNAP) receptors (SNAREs) are presently considered the core constituents of the protein machinery responsible for membrane fusion. Most members of the SNARE protein family were identified by their possession of a conserved homologous stretch of 60–70 amino acids, referred to as the SNARE motif (Kloepper et al. 2007; Terrian and White 1997; Weimbs et al. 1998). Four SNARE motifs assemble spontaneously into a thermostable, sodium dodecyl sulfate- and protease-resistant coiled-coil bundle, called the SNARE core complex (Antonin et al. 2000a; Fasshauer et al. 1998a; Sollner et al. 1993b; Sutton et al. 1998). Heptad repeats in components of the core complex form 16 conserved layers of interacting amino acid side chains, which are arranged perpendicularly to the axis of the complex. All layers except one contain hydrophobic amino acids. The unique central layer, termed the "0-layer," is hydrophilic and consists of three glutamines (Q_s) and one arginine (R) stabilized by ionic interactions (Sutton et al. 1998). Based on this characteristic, SNARE proteins are classified into four subfamilies: Q_a-, Q_b-, Q_c-SNAREs (Q-SNAREs contribute a glutamine residue to the 0-layer), and R-SNAREs (R-SNAREs contribute an arginine residue to the 0-layer) (Fig. 4.1a, b) (Bock et al. 2001; Fasshauer et al. 1998b). All analyzed SNARE complexes to date have a $Q_aQ_bQ_cR$ composition and consist of four SNARE proteins, with the exception of the neuronal synaptic SNARE complex, which is comprised of three SNARE proteins: synaptosome-associated protein of 25 kDa (SNAP-25; (Oyler et al. 1989)), syntaxin-1 (Bennett et al. 1992), and synaptobrevin-2/vesicle-associated membrane protein (VAMP) 2 (Baumert et al. 1989; Trimble et al. 1988).

The fusogenicity of the SNAREs relies on their differential localization: synaptobrevin/VAMP is a vesicular protein, whereas syntaxin-1 and SNAP-25

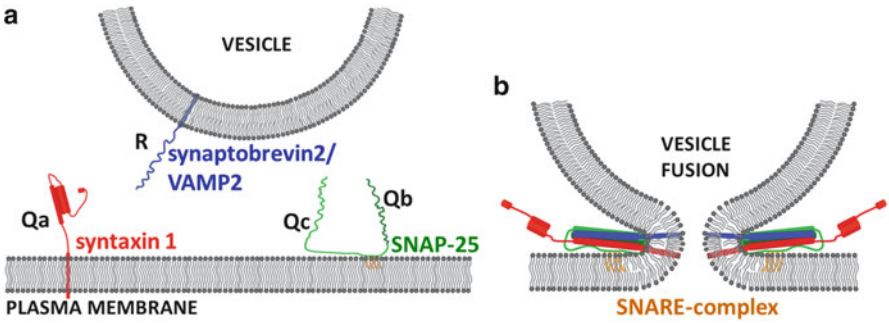

Fig. 4.1 Schematic of the three neuronal SNARE proteins that mediate synaptic vesicle fusion by forming a SNARE complex: syntaxin-1 (*red*), SNAP-25 (*green*) and synaptobrevin-2/VAMP2 (*blue*). The color coding for these proteins is used in all other figures. (**a**) Syntaxin-1 (Qa) and SNAP-25 (Qbc) are present in the plasma membrane, while synaptobrevin-2/VAMP2 (R) is in the synaptic vesicle. (**b**) Formation of SNARE complex composed of syntaxin-1, SNAP-25, and synaptobrevin-2/VAMP2 drives fusion of the vesicle with the plasma membrane

are attached to the plasma membrane. Assembly of a SNARE complex between the membranes (in *trans*) leads to fusion, driven by the energy liberated during SNARE-complex formation. During fusion, the membranes merge and the SNARE proteins find themselves in the same membrane, forming what is referred to as a *cis*-SNARE complex. SNARE-complex disassembly by the ATPase NSF is needed to recycle SNAREs for another round of fusion (see below). The central role of the SNARE complex in exocytosis at the neuronal synapse is well documented. The first demonstration came from studies with neurotoxins that selectively cleave SNAREs and potently inhibit exocytosis (Montecucco and Schiavo 1995; Niemann et al. 1994). Tetanus toxin cleaves synaptobrevin/VAMP, while botulinum neurotoxins (BoNT) A, B, and C cleave SNAP-25, synaptobrevin/VAMP, and syntaxin, respectively. Reconstitution studies of purified proteins in liposomes indicated that SNAP-25, syntaxin-1, and synaptobrevin-2/VAMP2 are sufficient to fuse membranes and can be viewed as representing a "minimal fusion machinery" (Weber et al. 1998). Another study expressed the same SNARE proteins on the cellular surface and detected spontaneous cell-to-cell fusion, showing that SNAREs are sufficient to fuse biological membranes (Hu et al. 2003).

SNAP-25 (Table 4.1) is a Q_{bc}-SNARE and contributes two of the four α-helices to the neuronal SNARE complex (Fasshauer et al. 1998b; Sutton et al. 1998). This arrangement is unique to the SNAP-25 family, which is involved in fusion of vesicles with the plasma membrane, whereas in intracellular fusion reactions, the Q_b- and Q_c-SNARE motifs are supplied by separate proteins (Fukuda et al. 2000). The SNARE motifs are present at the N- and C-termini of SNAP-25 and are separated by a central cysteine-rich membrane targeting domain (also called a linker domain; (Sutton et al. 1998)). SNAP-25 is highly conserved among species, with little variation in length. The two alternatively spliced variants, SNAP-25a and SNAP-25b, have high homology, differing only by nine amino acids (Bark and Wilson 1994). They are efficiently targeted to the plasma membrane due to the

palmitoylation of the four cysteine residues in the linker domain (Gonzalo et al. 1999; Hess et al. 1992). SNAP-25 is most abundant in brain, where an interesting developmental shift in the expression of the isoforms has been described: SNAP-25a is more abundant in embryonic brain, whereas the expression of SNAP-25b increases robustly after birth to become the predominant isoform in most, but not all, adult brain areas (Bark et al. 1995; Boschert et al. 1996). Impairment of this switch towards the SNAP-25b isoform in mice leads to premature mortality and a change in short-term plasticity in hippocampal synapses (Bark et al. 2004). Ablation of the SNAP-25 gene in mice results in embryonic lethality (Washbourne et al. 2002), and Ca^{2+}-triggered secretion from neuroendocrine cells without SNAP-25 is nearly abolished (Sorensen et al. 2003). Both SNAP-25 isoforms can rescue secretion when expressed in SNAP-25 knock-out cells, but the SNAP-25b isoform is more efficient in driving vesicle priming than the SNAP-25a isoform (Sorensen et al. 2003), which has been attributed to a more efficient binding of synaptotagmin-1, the calcium sensor for exocytosis, by SNAP-25b (Mohrmann et al. 2013).

Syntaxin-1 (Table 4.1) is a prototypic Q_a-SNARE and it contributes one of the four α-helices forming the neuronal SNARE complex (Fasshauer et al. 1998b; Sutton et al. 1998). This 35 kDa plasma membrane protein consists of a transmembrane domain, a SNARE motif, and an N-terminal H_{abc}-domain (Bennett

Table 4.1 Major proteins involved in synaptic vesicle exocytosis at the neuronal synapse

Exocytic protein	Function
Syntaxin	Plasma membrane transmembrane protein and central component of SNARE complex; involved in docking, priming, and fusion
SNAP25	Plasma membrane-anchored protein and central component of SNARE complex; involved in docking, priming, and fusion
VAMP/ synaptobrevin	Synaptic vesicle transmembrane protein and central component of SNARE complex; involved in priming and fusion
Synaptotagmin	Ca^{2+} sensor, binds $PI(4,5)P_2$, lipids, and the SNARE complex and regulates fusion through docking, priming, and fusion triggering
Complexin	Binds and regulates SNARE complex
Munc13	Involved in synaptic vesicle priming; opens syntaxin within Munc18 and interacts with RIM
Munc18	Involved in synaptic vesicle docking and priming; binds syntaxin in the closed configuration
NSF	Disassembles SNARE complex
α- and β-SNAP	Cofactor to NSF
Rab3	Regulates synaptic vesicle cycle; interacts with RIM and rabphilin
RIM1	Involved in synaptic vesicle priming; interacts with synaptotagmin, Munc13, and RimBPs (Rim-binding proteins)
N-, P-, Q-type Ca^{2+} channels	Regulate calcium influx to the presynaptic terminus and subsequently trigger exocytosis
PIPKIγ	Key $PI(4,5)P_2$-synthesizing enzyme at the presynaptic plasma membrane

et al. 1992). Two paralogs, syntaxin-1A and syntaxin-1B, share high homology and localization to the plasma membrane (Bennett et al. 1992). While their expression pattern largely overlaps, with only subtle changes in distribution, it is essentially restricted to neuronal and neuroendocrine cells (Ruiz-Montasell et al. 1996). The N-terminal H_{abc}-domain of syntaxin-1 is implicated in the regulation of protein accessibility. This region reversibly binds to the SNARE motif and can maintain two distinct syntaxin-1 conformations. In the "open" conformation (H_{abc}-domain not bound to the SNARE motif), the protein is able to form a functional SNARE complex, whereas in the "closed" conformation (H_{abc}-domain bound to the SNARE motif) it is not (Dulubova et al. 1999). In *Drosophila*, deletion of the syntaxin-1A homologue completely blocks neurotransmitter release (Schulze et al. 1995). In mouse, genetic ablation of syntaxin-1A does not lead to dramatic phenotypes, indicating that syntaxin-1B is sufficient to maintain vital functions (Fujiwara et al. 2006; Gerber et al. 2008), but long-term potentiation was affected due to a defect in the catecholamine systems (Fujiwara et al. 2006; Mishima et al. 2012).

Synaptobrevin/VAMP (Table 4.1) is a prototypic R-SNARE and contributes one of the four α-helices that compose the neuronal SNARE complex (Fasshauer et al. 1998b; Sutton et al. 1998). It is an abundant 13 kDa synaptic vesicle protein with a central SNARE motif, a C-terminal transmembrane region, and a proline-rich N-terminus. Two of the best-studied isoforms, synaptobrevin-1/VAMP1 and synaptobrevin-2/VAMP2, differ mainly in the hydrophobic C-terminus and in the poorly conserved N-terminus (Elferink et al. 1989; Trimble et al. 1988). Although a partial overlap in their expression pattern is apparent, synaptobrevin-1 and synaptobrevin-2 are differentially distributed in the brain, suggesting specialized functions for each isoform. Synaptobrevin-2/VAMP2 is, in general, more evenly distributed, while synaptobrevin-1/VAMP1 expression is located predominantly in neurons with somatomotor function (Trimble et al. 1990). Ablation of the synaptobrevin-2/VAMP2 gene is postnatally lethal (Schoch et al. 2001). Evoked synaptic exocytosis from hippocampal neurons without synaptobrevin-2/VAMP2 is severely decreased, but fusion is not completely abolished (Schoch et al. 2001). The vesicles that fuse in the absence of synaptobrevin-2/VAMP2 are unable to endocytose and recycle quickly, implying that synaptobrevin-2/VAMP2 is also necessary for rapid synaptic vesicle endocytosis (Deak et al. 2004). Synaptobrevin-2 is by far the most abundant synaptic vesicle protein, with 60–70 copies per vesicle (Takamori et al. 2006).

Fusion mediated by SNAREs only is relatively slow, due to uncoordinated fusion events. Given that neurons require high levels of spatially and temporally coordinated activity, SNARE proteins in neurons are tightly regulated at different stages of their generation and action: transcriptional regulation of gene expression, targeting to the correct compartment membranes, functionality in targeted membranes, posttranslational modification (e.g., phosphorylation), assembly and disassembly of the SNARE complex, and fusion triggering by calcium. Consequently, many accessory factors that modulate multiple SNARE complexes are needed to ensure the sophisticated control that characterizes membrane fusion in neurons (Sudhof 2013).

4.2 SNARE-Assembly Mechanism and Fusion

4.2.1 SNARE-Complex Assembly I: Role of Closed Syntaxin and Munc18-1

Syntaxin-1 is found in dense clusters with diameters of 50–70 nm on the plasma membrane (Barg et al. 2010; Lang et al. 2001; Rickman et al. 2010; Sieber et al. 2007). The lipids phosphatidylinositol-4,5-bisphosphate (PIP_2) and cholesterol both participate in clustering syntaxin-1 (Honigmann et al. 2013; Lang et al. 2001; van den Bogaart et al. 2011a) – but see Murray and Tamm (2009). The syntaxin-1 clusters are so dense that they are thought to preclude the formation of the SNAP-25/syntaxin acceptor complex for synaptobrevin/VAMP and possibly even the closed conformation of syntaxin-1 (Sieber et al. 2007) (Fig. 4.2a). SNAP-25 also forms clusters, which are distinct or partly overlapping with the syntaxin clusters (Knowles et al. 2010; Lang et al. 2001). The first event leading to SNARE-complex assembly and fusion is therefore arguably the recruitment of syntaxin-1 and SNAP-25 from these clusters. This might happen spontaneously by lateral diffusion of SNAREs into the membrane from the rim of clusters, resulting in a certain abundance of reactive syntaxin-1 in the membrane, in equilibrium with clustered and thus unavailable syntaxin-1 or SNAP-25 (Bar-On et al. 2009, 2012; Lang et al. 2002), or conversely the vesicles might themselves induce recruit SNAREs to the underlying membrane (Barg et al. 2010; Knowles et al. 2010).

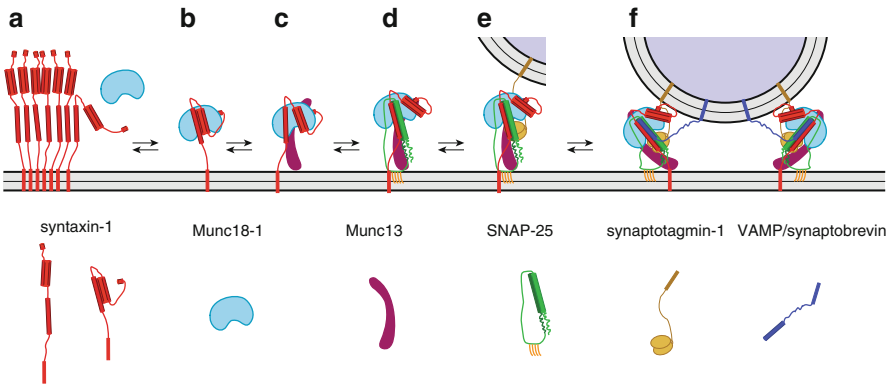

Fig. 4.2 Overview of events leading up to formation of a fusion complex between vesicle and plasma membrane. (**a**) Syntaxin-1 (and SNAP-25) exists in the plasma membrane as clusters of ~70 nm diameter. (**b**) Munc18-1 binds to syntaxin-1 in its closed configuration, where the H_{abc}-domain is folded back on the SNARE domain. (**c**) Munc13 proteins bind to and open syntaxin-1 within syntaxin/Munc18 dimers. (**d**) Open syntaxin can now bind to SNAP-25. (**e**) Vesicle docking in adrenal chromaffin cells involves binding of synaptotagmin to syntaxin/SNAP-25 dimer. (**f**) Synaptobrevin-2/VAMP2 binds to the acceptor complex, creating the fusogenic complex. Note that it is unknown whether Munc13 and Munc18 are still bound to the complex at this time. Also note that complexin, which probably binds to the assembling SNARE-complex, has been left out

Once outside the clusters, syntaxin-1 can associate with SNAP-25 in various configurations (An and Almers 2004; Freedman et al. 2003; Halemani et al. 2010; Laage et al. 2000; Margittai et al. 2001; Misura et al. 2001a; Rickman et al. 2010).

Munc18-1/nsec-1 (Table 4.1) is a soluble kidney-shaped protein, which stabilizes closed syntaxin by binding the H_{abc}-H3 helical bundle through its central cavity (Misura et al. 2000) (Fig. 4.2a, b). This naturally leads to the expectation that Munc18-1 is a negative regulator of secretion, but knockout experiments revealed that Munc18-1 and its homologues are required for fusion in the cell (Harrison et al. 1994; Hosono et al. 1992; Verhage et al. 2000). Further experiments showed that membrane-anchored syntaxin can form SNARE complexes even when bound to Munc18-1 (Zilly et al. 2006). The issue of the essential positive function of Munc18-1 in exocytosis has still not been resolved and remains one of the most interesting open questions in the exocytosis field (for a recent review on SNAREs and Munc18, see (Rizo and Sudhof 2012)). One important function for Munc18 is its binding to syntaxin-1 in the closed configuration during trafficking of both proteins to the cell membrane (Medine et al. 2007; Rowe et al. 1999). In this configuration, Munc18-1 protects the cell from the formation of ectopic SNARE complexes when syntaxin-1 traffics through the Golgi-TGN area (Rowe et al. 1999). Accordingly, in the Munc18-1 knockout mouse, the level of syntaxin-1 is reduced (by about 70 %), although part of the syntaxin-1 population of syntaxin-1 is still properly localized at synapses (Toonen et al. 2005). Thus, the function of Munc18-1 as a chaperone for syntaxin is important, but not sufficient to explain the complete arrest of secretion in Munc18-1 knockout mice (Verhage et al. 2000). It appears likely that initial recruitment of syntaxin from the dense clusters on the plasma membrane might be regulated by Munc18-1 binding to syntaxin-1 adopting the closed configuration at the edge of syntaxin clusters (Fig. 4.2a).

Munc18-1 exerts several actions along the exocytotic pathway. In the absence of Munc18 (or Unc18), vesicle docking to the plasma membrane is absent (Voets et al. 2001; Weimer et al. 2003). This might be expected from the chaperone function of Munc18, because docking also depends on syntaxin-1 expression (de Wit et al. 2006; Hammarlund et al. 2007). However, expression of a Munc18-1 mutant in chromaffin cells, which rescued syntaxin abundance, was unable to rescue docking or secretion, and, conversely, expression of Munc18-2 rescued syntaxin abundance and docking, but not secretion (Gulyas-Kovacs et al. 2007). Expression of a mutant defective in syntaxin binding stimulated release in PC12-cells (Schutz et al. 2005). Therefore, Munc18-1 must play a positive role downstream from docking and distinct from binding to closed syntaxin. Further experiments showed that overexpression of SNAP-25 in Munc18-1 null cells, which presumably increases the amount of syntaxin/SNAP-25 dimers, fully rescued vesicle docking, but secretion remained fully depressed (de Wit et al. 2009). The vesicular docking factor in chromaffin cells was identified as synaptotagmin-1 (de Wit et al. 2009), whereas there is conflicting evidence as to whether VAMP-2/synaptobrevin-2 is involved in this step (Borisovska et al. 2005; Wu et al. 2012). Synaptotagmin-1 seems to be necessary for docking in chromaffin cells by binding

to SNAP-25, presumably within SNAP-25/syntaxin dimers (Mohrmann et al. 2013), but Munc18-1 carries out an essential role in fusion downstream of this step.

The different functions of Munc18-1 along the exocytotic pathway might correlate with different binding configurations with the SNAREs. One configuration is the aforementioned binding to closed syntaxin; another is the binding to the assembled (or assembling) SNARE complex (Carr et al. 1999; Dulubova et al. 2007; Khvotchev et al. 2007; Shen et al. 2007). Both configurations include an interaction with an N-terminal peptide of syntaxin-1 (Burkhardt et al. 2008; Dulubova et al. 2007; Shen et al. 2007). Interfering with the N-terminal peptide reduces Munc18 stimulation of lipid mixing in vitro (Schollmeier et al. 2011; Shen et al. 2007) and exocytosis in vivo (Deak et al. 2009; Khvotchev et al. 2007; Zhou et al. 2013a). Mutations in Munc18 (and its homologue in *Caenorhabditis elegans*), which abolish binding to the N-terminal syntaxin, also strongly reduce synaptic transmission in some (Deak et al. 2009; Johnson et al. 2009), but not all (Meijer et al. 2012) investigations. In in vitro fusion assays, it was surprisingly found that only the N-terminal part of syntaxin, and not the Habc-domain, is essential for Munc18-1 dependent stimulation of vesicle fusion (Rathore et al. 2010; Shen et al. 2010), and furthermore, in *C. elegans*, the N-terminal domain of syntaxin could be transferred onto SNAP-25 without loss of synaptic transmission (Rathore et al. 2010). This indicates that the N-terminal domain of syntaxin acts to recruit Munc18-1 to the assembled (or assembling) SNARE complex to perform its essential stimulating function. However, it is still unclear what this function is.

One possibility is that Munc18-1 assists in nucleating or finalizing SNARE-complex assembly (Shen et al. 2007). This is supported by the observation that the stimulatory role of Munc18-1 in in vitro fusion assays depends on the exact nature of the R-SNARE and is abolished upon mutation of synaptobrevin-2 or its replacement by other isoforms (Schollmeier et al. 2011; Shen et al. 2007). Biochemical experiments have shown that the four-helix SNARE bundle binds to the same cavity in Munc18, which interacts with the H_{abc}-domain of closed syntaxin (Xu et al. 2010), but binding to the SNARE complex has a lower affinity than binding to closed syntaxin (Burkhardt et al. 2008; Xu et al. 2010). Binding was also found to occur directly between the 3a-domain of Munc18-1 and the C-terminal end of the synaptobrevin/VAMP SNARE domain (Xu et al. 2010), which would localize this part of Munc18-1 right at the fusion pore. Notably, the 3a-domain has been shown by random and targeted mutagenesis to be essential for fusion (Boyd et al. 2008; Martin et al. 2013). The basic conceptual problem with the hypothesis that Munc18-1 stimulates SNARE-complex assembly is that SNAREs assemble readily to drive membrane fusion in vitro without the need for Munc18-1 (Schuette et al. 2004; Weber et al. 1998). Consequently, in in vitro fusion assays, Munc18-1 only has a relatively mild effect on fusion and it only becomes stimulatory after preincubation of liposomes under conditions that probably result in initial SNARE-complex formation. Thus, it is currently hard to understand from these findings why Munc18-1 should be absolutely required for SNARE-complex formation in the cell.

It has been suggested that Munc18 might directly catalyze lipid mixing itself, once it is brought into contact with the fusion site by binding to the SNAREs (Rizo and Sudhof 2012). In this model, assembly of the SNAREs would bring the membranes together by exerting force on them, leading to tight apposition and dehydration of the two membranes. Munc18 might then act to stimulate lipid splaying, which might be the rate-limiting step for fusion (see below), and would result in the formation of a lipid stalk. Possibly, Munc18 could provide a surface over which the lipids might bend, catalyzing fusion. While there is no direct evidence to support this model – and it does not account for the difference between in vitro and in vivo findings – it is in agreement with the notion that all SNARE-driven fusion events in the cell require a SM-protein.

4.2.2 SNARE-Complex Assembly II: Opening Syntaxin and Roles of Munc13/CAPS

Since the closed conformation of syntaxin blocks SNARE-complex assembly, opening syntaxin is a key step towards membrane fusion. This opening step must be correlated with a change in interaction mode of Munc18-1. Replacement of wild-type syntaxin with a mutated version – the so-called LE-mutant (Dulubova et al. 1999), which tends to adopt the open conformation more readily (Dulubova et al. 1999) – promoted spontaneous release and increased vesicular release probability (Gerber et al. 2008), presumably because of facilitated formation of SNARE complexes between vesicles and plasma membrane.

In the cell, the opening of syntaxin must be followed or accompanied by binding to SNAP-25, which yields the acceptor complex for synaptobrevin-2/VAMP2 (Fig. 4.2c, d). A role for Munc18-1 in catalyzing this step by keeping syntaxin in a half-open configuration has been proposed based on biochemical studies (Burkhardt et al. 2008). Structural studies have led to the suggestion that a conformational change in Munc18-1 might drive the formation of the acceptor complex while syntaxin remains bound to Munc18-1 (Christie et al. 2012; Colbert et al. 2013; Hu et al. 2011), although these studies did not agree on whether the N-terminal part of syntaxin would engage Munc18-1 to open or close syntaxin.

Proteins of the Munc13/CAPS family (Table 4.1) act by mediating or stimulating the opening of syntaxin, probably within Munc18/syntaxin dimers (Fig. 4.2c). These proteins contain a catalytic so-called Mun domain, which mediates vesicle priming (Basu et al. 2005; Stevens et al. 2005). In the absence of Munc13 proteins, neurosecretion is completely abolished in neurons (Augustin et al. 1999; Richmond et al. 1999; Varoqueaux et al. 2002), whereas overexpression strongly stimulates priming in adrenal chromaffin cells (Ashery et al. 2000). Munc13 interacts directly with syntaxin, with the SNARE complex and with Munc18 (Ma et al. 2011) . These rather weak interactions might play key roles in opening syntaxin within syntaxin/Munc18 dimers and allowing SNARE-complex assembly (Ma et al. 2011). The

important role of Munc13 proteins in opening syntaxin was demonstrated in *Drosophila*, where expression of the LE-mutated open syntaxin partly overcomes the secretion defect in Munc13-deficient neurons (McEwen et al. 2006; Richmond et al. 2001). However, in mouse open syntaxin-1B did not overcome the lethal phenotype of the Munc13-1 knockout (Gerber et al. 2008).

CAPS-1 and CAPS-2 also contain a Mun domain, in addition to a PIP_2-binding pleckstrin homology domain, which are both essential for their function (Grishanin et al. 2002; Khodthong et al. 2011). Deletion of CAPS-1 and CAPS-2 leads to a subtle phenotype in neurons, where vesicles can be primed only transiently, by increases in the basal calcium concentration, but soon fall back to the non-primed state (Jockusch et al. 2007). Therefore, there might be two different priming pathways, one governed by Munc13 proteins and one by CAPS proteins. In fusion of dense-core vesicles, CAPS acts at the priming step (Elhamdani et al. 1999; Liu et al. 2008; Speidel et al. 2008), and it has been further shown that CAPS binds to the individual SNAREs and orchestrates the formation of the SNARE complex (Daily et al. 2010; James et al. 2009). Thus, CAPS proteins might play similar roles as Munc13 proteins, but the presence of a PH domain in CAPS and C1- and C2-domains in Munc13 might confer the proteins with different regulatory properties. In fusion of dense-core vesicles, the absence of CAPS proteins can be partly overcome by expression of open syntaxin (Hammarlund et al. 2008; Liu et al. 2010), indicating the similarity in function between Munc13 and CAPS in opening syntaxin. However, Munc13 overexpression could not compensate for CAPS deficiency, or vice versa, (Jockusch et al. 2007; Liu et al. 2010); therefore, CAPS and Munc13 proteins must perform other, distinct, functions in the exocytotic cascade.

Deletion of the H_{abc}-domain of syntaxin – while maintaining the N-terminal peptide intact – supported fast evoked release in syntaxin-1-deficient neurons, but the RRP and spontaneous release were depressed (Zhou et al. 2013b). This finding, together with the only partial rescue of Munc13 or CAPS deficiencies by LE-mutated open syntaxin, indicates that although the closed conformation of syntaxin-1 is inhibitory for SNARE-complex formation per se, the ability to open syntaxin up – presumably catalyzed by Munc13 or CAPS proteins – plays a specific positive role in vesicle priming, which cannot be overcome by constitutively open syntaxin. This seems to play the largest role during sustained calcium elevations, where priming has to be fast to keep up with fusion. This correlates well with the fact that Munc13 proteins are stimulated by calcium and diacylglycerol (Lipstein et al. 2013; Rhee et al. 2002; Shin et al. 2010). Thus, the opening of syntaxin by Munc13 might be a pivotal regulation point of the synaptic cycle, and the regulatory domains of Munc13 (and CAPS) might have developed to link the activity of G-protein-regulated receptors and the calcium concentration to the synaptic priming speed.

The above establish the involvement of the major players in (calcium-independent) exocytosis and neurotransmitter release: the SNARE proteins, Munc18, and Munc13. In a striking experiment, it was shown that the essential functions of SNAREs, Munc18, and Munc13 can be reconstituted in vitro in a

fusion assay (Ma et al. 2013). In these experiments, Munc18 displaced SNAP-25 from syntaxin, and fusion then became Munc13 dependent. Thus, it was concluded that syntaxin being bound to Munc18 in the closed conformation is the starting point for fusion (Fig. 4.2a–c), not "free" syntaxin/SNAP-25 dimers, which would be susceptible to spontaneous disassembly by the activity of α-SNAP/NSF (see below). These syntaxin/Munc18 dimers then bind SNAP-25 and synaptobrevin-2/VAMP2 through the action of Munc13 (Fig. 4.2d), in a pathway which is resistant to α-SNAP/NSF action (Ma et al. 2013). It should be noted that this conclusion does not contradict the observation that the LE-mutated "open syntaxin" can partly bypass the need for Munc13 in vivo, because recent studies have shown that the LE-mutated syntaxin in fact binds to Munc18 in a closed configuration with only slightly lower affinity than wild-type syntaxin (Burkhardt et al. 2008; Colbert et al. 2013). But when bound to Munc18, LE-mutated syntaxin allows formation of the SNARE complex without further cofactors (Burkhardt et al. 2008). Therefore, LE-mutated syntaxin might be called "open" in the sense that it allows spontaneous formation of the SNARE complex within syntaxin/Munc18 dimers.

4.2.3 SNARE-Complex Assembly III: Zippering the SNARE Bundle

Productive formation of the syntaxin/SNAP-25 dimer probably takes place while syntaxin remains bound to Munc18-1 (see above). Biochemical experiments and experiments in adrenal chromaffin cells are consistent with the idea that this dimer binds to synaptotagmin, which docks vesicles to the SNARE acceptor complex (de Wit et al. 2009; Mohrmann et al. 2013; Rickman et al. 2004, 2006) (Fig. 4.2e). A specific step corresponding to synaptotagmin binding to SNAP-25/syntaxin has not been detected in neurons, where morphological docking as detected by the electron microscope is driven by another complex consisting of RIM (Rab3-interacting molecule), RIM-BP (RIM-binding protein), and Rab3 or Rab27 (Sudhof 2013). Nevertheless, it is likely that the same sequence of events takes place in neurons, even though it does not correspond to a visible phenotype in EM micrographs. The next event on the path to vesicle fusion is most likely synaptobrevin/VAMP binding to the acceptor complex to form the ternary SNARE complex, which drives membrane fusion itself (Fig. 4.2f). This is probably a heavily regulated step towards fusion. The protein tomosyn, which carries a SNARE motif and is classified as an R-SNARE, binds to the SNAP-25/syntaxin dimer to block or limit vesicle priming, which then requires synaptobrevin-2/VAMP2 to replace tomosyn in a poorly characterized fashion (for a review, see (Ashery et al. 2009)).

The main framework for our thinking about the SNARE-assembly step is the "zipper hypothesis" (Hanson et al. 1997a; Lin and Scheller 1997), which was suggested following the realization that SNARE-complex formation aligns the SNARE domains in parallel (Hanson et al. 1997b; Sutton et al. 1998). This placed

the C-terminal membrane anchors of synaptobrevin-2/VAMP2 and syntaxin in the same end of the complex, suggestive of a mechanism where N- to C-terminal assembly ("zippering") brings the membranes closer and closer together, until fusion results. In the simplest possible version of this model, the complex would be linked to the membrane via stiff linker between the SNARE domains and transmembrane anchors, linking complex formation directly to deformation of the membranes (see below).

Experiments relying on deletion studies, peptide interference in cells, and in vitro fusion assays uniformly support the idea that ternary SNARE-complex formation starts in the N-terminal end and progresses towards the C-terminus (Chen et al. 2001; Fasshauer and Margittai 2004; Matos et al. 2003; Melia et al. 2002; Pobbati et al. 2006; Xu et al. 1999b). It is the formation of the SNARE complex, which leads to structuring of the SNARE domains of SNAP-25 and synaptobrevin-2/VAMP2, which are unstructured when free in solution (Fasshauer et al. 1997). However, the α-helical SNARE motifs are fairly promiscuous, which in vitro can lead to formation of several other products, such as a 2:1 complex, where the binding site for synaptobrevin-2/VAMP2 is occupied by a second syntaxin (Fasshauer and Margittai 2004; Xiao et al. 2001). This structure – as well as others (Misura et al. 2001a, b) – represents off-pathway products (kinetic traps), which are unproductive for fusion and therefore exacerbate the very slow kinetics of SNARE-driven fusion in vitro. Blocking formation of these alternative complexes results in markedly sped up in vitro fusion (Pobbati et al. 2006). In the cell, it is likely that the formation of the SNAP-25/syntaxin acceptor complex while bound to Munc18 protects against the formation of off-pathway complexes, which might be yet another function for Munc18 (Rizo and Sudhof 2012).

Experiments performed in cells support the idea of N- to C-terminal assembly of the SNARE complex. Infusion of an antibody, which blocked SNARE-complex assembly, into chromaffin cells led to the suggestion that the SNARE complex might coexist in two different states, a "loose" and a "tight" state, before exocytosis (Xu et al. 1999b). Differences in the activity dependence of neurotransmission block caused by tetanus toxin (TeNT) and botulinum neurotoxin D (BoNT/D), which bind to the N- or the C-terminal end of the synaptobrevin-2/VAMP2 SNARE domain, respectively, led to the suggestion that the N-terminal, but not the C-terminal, half of the SNARE domain is shielded before fusion (Hua and Charlton 1999).

Mutagenesis studies have shown that mutating the hydrophobic layers in the middle of the SNARE bundle to cause a local destabilization has very different consequences depending on where along the bundle the mutation is placed. Mutations in the middle of the bundle – or towards the N-terminal end – caused a decrease in forward vesicle priming rate, whereas mutations in the most C-terminal layers – layers +7 and +8 – cause a depression in fusion speed (Sorensen et al. 2006; Walter et al. 2010; Weber et al. 2010; Wei et al. 2000). The depression in speed was graded when several destabilizing mutations were compared, and – importantly – temperature unfolding experiments showed that C-terminal mutations caused the C-terminal end of the complex to disassemble at lower temperatures, while the

N-terminal end of the complex remained unaffected, indicating that the two ends of the SNARE complex fold independently (Sorensen et al. 2006). Conversely, a mutation in syntaxin layer +7, which tightens the C-terminal end, caused increased spontaneous and evoked release in *Drosophila* (Lagow et al. 2007). Thus, fusion rate or, equivalently, fusion probability might correlate directly with the stability of the very C-terminal end of the SNARE bundle.

Since N-terminal assembly correlates with vesicle priming, whereas C-terminal assembly causes vesicle fusion, some mechanism must arrest further SNARE complex zippering after initial N-terminal assembly until arrival of the calcium signal (but see below for a different view). This mechanism could in principle be (1) intrinsic to the SNARE complex, such that two sub-domains fold independently, separated by an energy barrier; (2) the repulsion between the membranes, which will put up an energy barrier for C-terminal assembly; or (3) an accessory protein, which arrests the SNARE complex in the half-zippered state. In fact, it is likely that all of these mechanisms contribute to the partial assembly of SNARE complexes. First, elegant experiments using forced unzippering and rezippering of single SNARE complexes (without accessory proteins) by optical or magnetic tweezers have revealed that the N-terminal and C-terminal halves of the SNARE bundle fold/unfold in discrete steps (Gao et al. 2012; Min et al. 2013). This is followed by zippering of the linker and transmembrane domains to yield the fully zippered complex (Stein et al. 2009). Interestingly, the N- and C-terminal halves seem to assemble as binary switches, so that further intermediate assembly steps are not discernible. The assembly of the N-terminal half of the SNARE complex arranges the C-terminal domains of the acceptor complex into the same structure as the *cis*-complex (Li et al. 2014), indicating that N-terminal assembly sets the stage for later C-terminal assembly. Second, the same experiments showed that, with a repulsion force on the C-terminal end of the SNAREs between 12pN and 20pN (Gao et al. 2012), or between 11 pN and 34 pN (Min et al. 2013), the SNARE complex would rest in a state where only the N-terminal end was assembled. Above this range, the N-terminal end would disassemble. Thus, it is likely that repulsion between membranes would keep the SNARE complex in a partially assembled state. Third, recent in vitro and in vivo experiments have found that the accessory protein complexin binds to the SNARE complex and inserts its so-called accessory helix into the C-terminal end of the partially assembled complex, where it blocks further assembly until calcium binds to synaptotagmin and relieves the complexin block [e.g., (Giraudo et al. 2006; Kummel et al. 2011; Malsam et al. 2012; Tang et al. 2006; Xue et al. 2007)] (See chapter on complexins; Chap. 6). Because the binding site for complexin is created during formation of the SNARE complex (Chen et al. 2002; Pabst et al. 2000), the two former mechanisms might be required to arrest the SNARE-complex formation long enough for complexin to bind.

Not all data support the view that assembly of the C-terminal half of the SNARE complex leads to membrane fusion. Fully assembled SNARE complexes – or SNARE complexes only slightly frayed at the very C-terminal end – have been identified between non-fused – or hemifused – membranes (Hernandez et al. 2012; Shin et al. 2014). Thus, full assembly of the SNARE domains into a complex might

not be sufficient to fuse the membranes. According to this view, it might be the zippering of the linker domains and transmembrane anchors that triggers fusion, assisted by calcium binding to synaptotagmin. This scenario is consistent with mutagenesis conducted in cells, because the only mutations that were found to selectively compromise secretion rate were located in layers +7 and +8, and indeed it was suggested that the "partially assembled" complex might only need to assemble layer +8 (Walter et al. 2010). Thus, whether SNARE-complex assembly is arrested at the zero layer in the cell is unclear.

The physical movements in the SNAREs associated with membrane fusion in living cells have been detected using inter- and intramolecular FRET (fluorescence resonance energy transfer) (An and Almers 2004; Degtyar et al. 2013; Wang et al. 2008; Zhao et al. 2013). These studies have detected changes in FRET reflecting SNARE-complex formation, or other conformational changes, before and after fusion. However, because of the long exposures required to detect the small FRET signals, it has been hard to establish unequivocally which signals are derived from changes before or after membrane fusion. Recently, this problem was overcome using a four-electrode electrochemical detector array on a glass coverslip to detect and localize fusing vesicles in chromaffin cells in TIRF microscopy independently of the FRET signal (Zhao et al. 2013). Averaging the FRET signal around many fusing vesicles led to the detection of a FRET signal, which preceded release by ~90 ms. The FRET probe used [SCORE for SNARE complex reporter (An and Almers 2004)] was an intramolecular SNAP-25 probe constructed to display FRET upon SNARE-complex formation. Since 90 ms is probably too fast to be caused by vesicle priming in chromaffin cells, this experiment detected a structural change in SNAP-25 linked to membrane fusion itself. This might be interpreted as evidence that the second SNARE motif of SNAP-25 only joins the SNARE complex at the time of fusion (An and Almers 2004). Alternatively, the FRET signal might reflect a more subtle movement, for instance, a rotation, associated with final SNARE-complex zippering.

Recently, it was suggested that synaptotagmin might act as a distance regulator, which would prevent SNARE-complex assembly entirely until calcium influx (Jahn and Fasshauer 2012; van den Bogaart et al. 2011b). In this model, the SNARE complex acts as a "one-shot" device, which assembles uninterrupted to fuse the membranes as soon as the N-terminal ends engage. Thus, in this model there is no function for a partially assembled SNARE complex. While this model is attractive in that it can explain the FRET signal detected immediately before fusion (Zhao et al. 2013), it is hard to reconcile with the known effects of complexin on vesicle priming and fusion, since complexin cannot bind until the SNARE complex has formed. It further appears inconsistent with the finding that mutations in the N-terminal end of the SNARE complex selectively affect vesicle priming, a reaction that takes part upstream of calcium influx.

4.2.4 SNARE-Complex Assembly IV: Fusing the Membranes

Whereas a consistent picture is emerging regarding the steps leading up to membrane fusion, membrane merger itself remains incompletely understood. This is due, at least in part, to the fact that the membrane merger process cannot be studied directly in living cells, whereas in vitro systems might not reproduce essential features of the cellular fusion process. Nevertheless, recent years have seen substantial progress using indirect methods and mathematical simulations that allow us to paint a preliminary picture, which will undoubtedly become more complete as cellular methods for the measurement of lipid mixture with high temporal resolution are developed.

It is commonly assumed that SNARE-dependent membrane fusion follows the same basal steps as protein-less membrane fusion (Chernomordik and Kozlov 2003) (Fig. 4.3). Initially, the proximal leaflets approach each other (Fig. 4.3a), which results in dehydration of the lipid head groups, splaying of lipids (Fig. 4.3b) and lipid stalk formation, where the proximal leaflets are fused. This lipid stalk can now either expand, yielding a hemifused state and then a fusion pore, or the lipid stalk can directly yield a fusion pore. There is extensive evidence that SNARE-mediated fusion can pass through the hemifused state (Abdulreda et al. 2008; Giraudo et al. 2005; Lu et al. 2005; Reese et al. 2005; Wong et al. 2007) (Fig. 4.3c). The next step is fusion pore generation by breaking the hemifused membrane to yield the fully fused state (Fig. 4.3d). In synaptic transmission, neurotransmitter release starts as soon as the fusion pore is generated, and for small synaptic vesicles, it is expected that even the formation of a transient fusion pore might be enough to empty the vesicle for neurotransmitter (Bruns and Jahn 1995). In contrast, for dense-core vesicles, transmitter might be released both

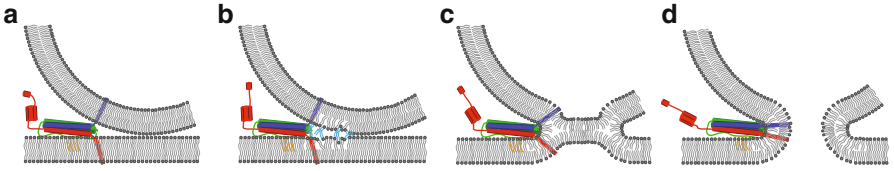

Fig. 4.3 Final SNARE-mediated fusion of membranes. Note that only the SNARE complex has been drawn, even though complexin and synaptotagmin also are expected to participate at this stage (**a**) Formation of the SNARE complex leads to close apposition between the membranes, leading to dehydration of the leaflets and approach of lipid head groups from the two membranes. (**b**) The next step is probably lipid splaying, i.e., some lipids will flip out of the leaflet and form bridges with lipids from the opposing leaflet. Splayed lipids have been drawn in cyan. (**c**) After formation of a lipid stalk (not shown), the fusion pathway might transit through the hemifused state, where distal leaflets meet to form a bilayer. (**d**) The C-terminal ends of syntaxin and synaptobrevin-2/VAMP2 cause destabilization of the hemifused state and lead to full membrane fusion, which relaxes bending stress in the SNAREs and leads to a fully zippered complex. See Risselada and Grubmuller (2012) for a more detailed account of the last events leading to membrane fusion

during fusion pore formation and as the fusion pore expands (Albillos et al. 1997; Zhou et al. 1996).

How is SNARE-complex assembly linked to the fusion of the lipid membranes? One view sees the conclusion of the SNARE-catalyzed membrane fusion process as a highly structured and reproducible superstructure, often consisting of a large number (5–15) of SNARE complexes arranged in a radial and symmetrical fashion around the nascent fusion pore (Megighian et al. 2013; Montecucco et al. 2005). Whereas most investigators assume that the fusion pore would be lined by lipids, in some models this lipid pore would be preceded by a transient proteinaceous pore lined by the transmembrane domains of syntaxin and synaptobrevin-2/VAMP2 (Han et al. 2004). Recent findings make such a fixed high-order structure unlikely. In vitro experiments have shown that a single SNARE complex suffices to fuse membranes (Shi et al. 2012; van den Bogaart et al. 2010), whereas three complexes are necessary for keeping the fusion pore open (Shi et al. 2012). This fits very well with titration experiments of all three SNAREs in living cells, which is consistent with two to three SNARE complexes being enough to cause fast fusion (Arancillo et al. 2013; Mohrmann et al. 2010; Sinha et al. 2011). Similar results had been obtained previously in cracked-open PC12 cells (Hua and Scheller 2001). Even though more experiments are needed to understand whether the number of engaging SNARE complexes is fixed or variable, and whether it is stochastic or under regulatory control, these experiments are most easily reconciled with a model wherein SNAREs fuse membranes essentially as independent devices resulting in variable overall stoichiometry, which might be reflected in the multiple kinetic components of exocytosis. This is further in agreement with the fact that no mechanism that would organize ring-formed higher-order SNARE structures in the cell has so far been established. One such possible mechanism would be domain sharing by SNAP-25, i.e., the two SNARE domains of SNAP-25 might join different but neighboring complexes, which might organize multiple SNARE complexes in a ring-formed structure (Tokumaru et al. 2001). However, this arrangement was not supported by experiments in chromaffin cells (Mohrmann et al. 2010).

The assumption that the energy of formation of the SNARE complex drives membrane fusion has prompted considerations about whether the energy of a single SNARE complex would suffice to overcome the energy barrier for fusion. Theoretical calculations using continuum models have placed the energy barrier for membrane fusion at ~40 k_BT (k_B is the Boltzmann constant; T is the absolute temperature) (Cohen and Melikyan 2004; Kuzmin et al. 2001). This estimate has been compared to the energy released by forming a single SNARE complex as measured in a surface force apparatus (~35 k_BT) (Li et al. 2007) or as measured using optical tweezer manipulation of single complexes (~65 k_BT) (Gao et al. 2012). However, isothermal calorimetry resulted in markedly lower estimates of the energy release by a SNARE complex, around ~19 k_BT (Wiederhold and Fasshauer 2009). The large discrepancy between those three studies (note that ~2.3 k_BT corresponds to a factor 10 difference in equilibrium constant at physiological temperatures) remains unexplained but is likely related to the difficulty in

obtaining reversible measurements within an experimentally accessible time scale, combined with the fact that assembly and disassembly displays hysteresis (Fasshauer et al. 2002). In any case, it is unlikely that all of the energy liberated by the formation of a SNARE complex can be harnessed for membrane fusion. Assembly of the C-terminal half of the SNARE complex liberates less energy than assembly of the N-terminal end (Wiederhold and Fasshauer 2009), even though, in the optical tweezers experiment, the C-terminal end still released an impressive ~28 k_BT (Gao et al. 2012). It is formation of the C-terminal end that couples to membrane fusion, whereas assembly of the N-terminal end drives vesicle priming (see above). Therefore, it is important to understand whether assembly of the N-terminal end results in a tense structure that stores energy eventually used for C-terminal assembly. However, destabilizing mutations in the N-terminal end did not compromise fusion speeds in chromaffin cells (Sorensen et al. 2006; Walter et al. 2010; Wiederhold et al. 2010), whereas in neurons such mutations actually slightly increased release probability (Weber et al. 2010). This is most easily reconciled with the assembly of the N-terminal end of the SNARE complex resulting in a stable structure (i.e., a minimum in the energy landscape), which might result in an increase in the effective size of the energy barrier for fusion. Thus, partial SNARE-complex assembly might actually increase the energy barrier for fusion, in order to set the state for fast calcium-triggered fusion. This is exactly what is expected in the "complexin as a clamp" model (see above), where tight assembly of the N-terminal end of the SNARE complex leads to complexin binding and clamping of C-terminal SNARE-complex assembly. Another possibility is that a similar feature is encoded in the SNARE complex itself and then exacerbated by interaction with complexin/synaptotagmin.

The energy barrier for membrane fusion is lowered by ~10 k_BT during the arrival of an action potential (Rhee et al. 2005). Thus, a low number of SNARE complexes (down to a single one) might release enough energy to fuse the membranes with a rate consistent with neurotransmission. Simulations using coarse-grain models have resulted in lower estimates for the overall fusion barrier [reviewed in (Risselada and Grubmuller 2012)]. Notably, several energy barriers could be distinguished. The first consists of close approach and dehydration of the membranes. The next is the occurrence of splayed lipids, i.e., lipids with exposed hydrophobic tails reaching over towards the opposite membrane. This state might constitute the main energy barrier, while the stalk is at a local energy minimum (Markvoort and Marrink 2011; Risselada and Grubmuller 2012). Expansion of the fusion pore might constitute another downstream energy barrier (Katsov et al. 2004). SNARE assembly will likely be able to overcome the first energy barrier and cause close membrane apposition. It has been suggested that Munc18 might directly act to induce lipid mixing (Rizo and Sudhof 2012), which would help overcome the second energy barrier. Similarly, it has been found that synaptotagmins help expand the fusion pore (Bai et al. 2004b; Wang et al. 2001, 2006). Thus, it is likely that we will come to see the SNAREs as part of a larger fusion machine, which has to overcome several distinct energy barriers.

Whatever the exact nature of the energy barrier(s), if the energy of formation of the SNARE complex is harvested to fuse the membranes, then force must be transduced to the membranes via the linkers between the SNARE domains and the transmembrane domains (TMD) of syntaxin-1 and synaptobrevin-2/VAMP2. Force transduction might take place by the formation of continuous α-helices throughout the linker and TMD, as found in a crystal structure that included those regions (Stein et al. 2009). Because α-helices are stiff structures, progressive formation of a continuous α-helix from the N- to the C-terminal would link the conformation of synaptobrevin-2/VAMP2 to membrane deformation. However, mutagenesis experiments have shown that α-helical continuity per se is not absolutely required for membrane fusion. In in vitro fusion assays, inserting helix breakers (five amino acids, including two prolines) in synaptobrevin-2/VAMP2 did not inhibit lipid mixing, whereas insertion in syntaxin reduced fusion by a factor of two (McNew et al. 1999). Insertion of flexible domains into the linkers progressively reduced lipid mixing, and again syntaxin was more susceptible than synaptobrevin-2/VAMP2, indicating that the SNARE complex acts asymmetrically on the two membranes. Simulations have shown that the syntaxin linker has considerable stiffness, making the molecule adopt an upright posture in the membrane (Knecht and Grubmuller 2003), while the synaptobrevin-2/VAMP2 linker is more flexible and might be partly inserted into the membrane (Ellena et al. 2009; Kweon et al. 2003).

Experiments in chromaffin cells and cultured neurons have shown that insertion of additional flexible sequences of 2–8 amino acids in the synaptobrevin-2/VAMP2 linker compromises vesicle priming, induces a longer delay before secretion starts, and slows down release from individual vesicles (Bretou et al. 2008; Guzman et al. 2010; Kesavan et al. 2007). Surprisingly, in chromaffin cells expressing a synaptobrevin-2/VAMP2 with a 6-amino acid addition in linker, secretion – when it got under way after a longer delay – was as fast as in the wild-type situation (Kesavan et al. 2007). With a linker encompassing 22 additional amino acids, secretion was indistinguishable from synaptobrevin-2/VAMP2 knockout cells (Kesavan et al. 2007). In neurons, insertion of three or seven amino acids in the linker of syntaxin-1 eliminated the ability to support evoked release (Zhou et al. 2013a). Further studies have identified two tryptophans in the synaptobrevin-2/VAMP2 linker as essential in imposing a fusion clamp, which favors evoked rather than spontaneous fusion (Fang et al. 2013; Maximov et al. 2009). Overall, there is little doubt that the linkers of syntaxin and synaptobrevin-2/VAMP2 are important for transducing force to the membranes as part of the evoked release mechanism (see discussion of spontaneous release below).

In a very recent and fascinating study, it was demonstrated that replacing the TMDs of both syntaxin and synaptobrevin-2/VAMP2 with lipid anchors, which inserted into only one membrane leaflet, still allowed a substantial amount of fast evoked release from central neurons (Zhou et al. 2013a). This was unexpected, because a previous study performed in vitro was unable to reconstitute fusion upon anchoring the SNARE domains to lipids (McNew et al. 2000). One important

difference between the studies – apart from the obvious difference between in vitro studies and studies carried out in cells – is that in the 2000 study, SNARE domains were linked to single lipids, whereas in the 2013 study, the SNAREs were linked to longer palmitoylation stretches, which presumably conferred a much more solid lipid anchor, consisting of several lipid moieties, onto the SNAREs. These considerations are thus in line with the idea that the main function of the TMD regions of the SNAREs is to transfer force to the membranes, whereas the details of how this is achieved appear to be of secondary importance.

Likewise, the finding that fast release was still present, although depressed in magnitude, after insertion of 4–5 amino acids in the linker of synaptobrevin-2/VAMP2 (Kesavan et al. 2007), indicates that the exact properties of the synaptobrevin-2/VAMP2 linker – while obviously important to optimize release of neurotransmitter – are not crucial to obtain membrane fusion per se. It is interesting to compare linker mutations with mutations in the C-terminal layers of the SNARE complex, where even single-point mutations, or deletions of single amino acids, lead to severe phenotypes in vivo (Criado et al. 1999; Gil et al. 2002; Sorensen et al. 2006; Walter et al. 2010) and in vitro (Hernandez et al. 2012; Siddiqui et al. 2007). Thus, it appears that although the stability and detailed topology of the SNARE bundle are very important, linker domains and transmembrane domains are less restricted. Nevertheless, analysis of single-vesicle fusion events has shown that the SNAREs add force to drive membrane fusion not only during fusion pore formation but also during the subsequent expansion of the fusion pore (Bretou et al. 2008; Guzman et al. 2010; Kesavan et al. 2007).

Thus, within the wild-type proteins, the SNARE TMDs are likely to play an important role in fusion pore formation and expansion. Deletion of the C-terminal half of the synaptobrevin-2/VAMP2 TMD was found to suppress secretion in PC12 cells (Fdez et al. 2010), possibly due to arrest in the hemifused state. Dimerization of the TMD of synaptobrevin-2/VAMP2 has also been demonstrated (Laage and Langosch 1997; Laage et al. 2000), but the mutation that eliminated dimerization did not inhibit fusion (Fdez et al. 2010). Addition of one or two amino acid residues to the very C-terminal (i.e., the intravascular) end of synaptobrevin-2/VAMP2 inhibited fusion in chromaffin cells, according to the transfer energy of the residues from water to the membrane (Ngatchou et al. 2010). This led to the suggestion that the C-terminal end of the TMD is pulled into the membrane – driven by formation of the SNARE complex – leading to disruption of the hemifused stalk or membrane, and fusion pore formation.

Simulations have provided important insights into the final events leading up to membrane fusion [reviewed in (Markvoort and Marrink 2011; Risselada and Grubmuller 2012)]. Molecular dynamics simulations confirmed experimental findings that a single or a few SNARE complexes can drive fusion and identified important roles for the SNARE linkers and TMRs in inducing lipid disordering that eventually leads to stalk formation (Risselada et al. 2011). Following formation of the hemifused state, the C-terminal ends of the TMRs help formation of the fusion pore, which releases bending stress in the SNAREs by placing both charged C-terminal ends into the hydrophilic pore (Lindau et al. 2012; Risselada

et al. 2011), consistent with the crystal structure (Stein et al. 2009). One simulation also identified important roles for dimerization (Risselada et al. 2011). These findings all contrast to a certain degree with the experimental evidence mentioned above that dimerization is not necessary and that the TMD and linkers can be manipulated without losing fusogenicity. However, it is important to remember that until molecular dynamics simulations have been carried out on these mutants, it is unclear to what extent they might have furnished the system with alternative pathways of fusion that might substitute for specific endogenous properties.

In conclusion, there is compelling – even overwhelming – evidence to conclude that SNARE-complex formation liberates energy, which is transferred to the membrane through the linkers and TMD as a corresponding force to drive membrane fusion. This force participates in closely aligning the membranes, leading to dehydration of the lipid head groups, at which point the fusion process becomes governed by poorly characterized lipid-protein and lipid-lipid interactions. It appears most likely that the subsequent events are lipid splaying, stalk formation, and the formation of a hemifusion intermediate, which gives way to the formation of a fusion pore and release of the vesicular content [for a model incorporating a transient proteinaceous pore, see (Jackson 2010)]. The SNAREs continue to drive fusion throughout these stages, but it seems likely that the last part of the process can proceed in several different ways.

4.3 Disassembly and Recycling of the SNARE Proteins

After synaptic vesicle fusion, the tight *cis*-SNARE complexes need to be disassembled, thereby recycling the SNAREs for subsequent fusion reactions (Fig. 4.4). The SNARE-complex disassembly process is achieved by the action of a complex consisting of the ATPase NSF and its cofactors α-/β-/γ-SNAP (Sollner et al. 1993a) (Table 4.1). α-SNAP is known to bind to the *cis*-SNARE complexes and, in turn, to recruit and activate NSF, which liberates individual SNAREs from the complex. α-SNAP and its two homologues, β- and γ-SNAP, were originally discovered as factors required for recruiting NSF to membranes in cell-free transport assays (Clary and Rothman 1990; Whiteheart et al. 2001). Both NSF and α-SNAP have been shown to actively participate in all intracellular processes involving membrane fusion.

In spite of the fact that SNARE proteins are in vast excess, they still may become limiting during the vesicle cycle if they are not rapidly recycled. The best example is given by the analysis of a temperature-sensitive mutation in the *Drosophila* homologue of NSF, *comatose* or dNSF-1, which revealed pronounced synaptic depression during repetitive stimulation (Littleton et al. 1998, 2001). These and other data (Banerjee et al. 1996; Xu et al. 1999a) led to the realization that NSF is not necessary for fusion per se but required to recycle the SNAREs. Likewise, it was recently reported that mammalian neurons without α-SNAP and with a hypomorphic β-SNAP level displayed additional rundown during phases of high

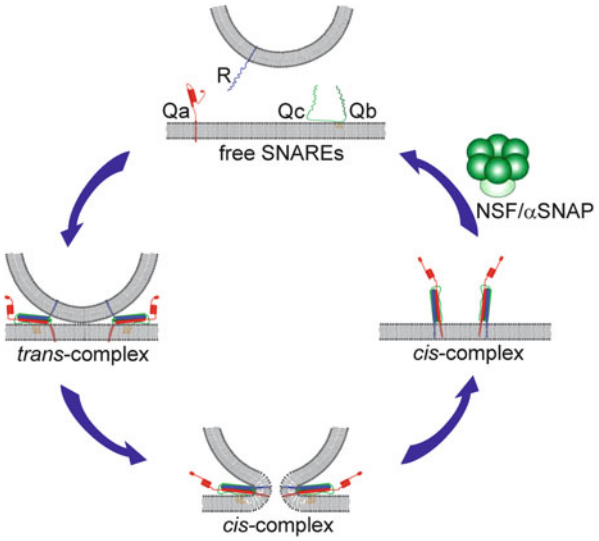

Fig. 4.4 Schematic of the canonical SNARE protein cycle. SNARE components: synatxin-1 (*red*), SNAP-25 (*green*), and synaptobrevin-2/VAMP2 (*blue*). Membrane fusion through formation of a *trans*-SNARE complex proceeds from a loose state (in which only the N-terminal portion of the SNARE motifs is "zipped up") to a tight state (in which the zippering process is mostly completed), and this is followed by the opening of the fusion pore. During fusion, the *trans*-complex relaxes into a *cis*-configuration. Dissociation of the *cis*-complex and repriming requires the energy input of ATP hydrolysis and is achieved through the binding of the ATPase protein NSF (N-ethylmaleimide-sensitive factor) together with SNAPs (soluble NSF attachment proteins) that function as cofactors

synaptic activity (Burgalossi et al. 2010), demonstrating the importance of NSF's cofactors in priming new vesicles for release.

While *cis*-SNARE-complexes should act as substrates for α-/β-/γ-SNAP and NSF in order to be disassembled for another round of fusion, *trans*-SNARE complexes should be resistant, lest their disassembly would block fusion. Different mechanisms have been suggested to protect trans-SNARE complexes from disassembly, including steric hindrance by the membrane or resistance of partially assembled complexes to SNAP binding (Weber et al. 2000). In a recent study, it was found that formation of the trans-SNARE complex within the confined environment of Munc18 and Munc13 protects trans-SNARE complexes from NSF-mediated disassembly (Ma et al. 2013).

Generally, it is believed that the activity of NSF is so high that it acts soon after fusion to disassemble the resultant *cis*-SNARE complexes and to liberate the SNAREs that drove the merger of membranes. Consequently, in the resting state most SNAREs in the membrane of neurosecretory cells are uncomplexed (Lang et al. 2002), and when blocking the action of NSF, secretion runs down only slowly, whereas blocking the function of syntaxin leads to a much faster block of secretion

(Littleton et al. 1998). However, there is some evidence that NSF and α-/β-SNAP could have an acute function immediately prior to vesicle fusion (Burgalossi et al. 2010; Kuner et al. 2008). This was taken to indicate that under some conditions, α-SNAP/NSF-dependent priming occurs immediately prior to the fusion step and is needed to free the SNAREs from the inactive *cis*-complex state (Kuner et al. 2008). However, it is also possible that NSF and α-/β-SNAP interact with the *trans*-SNARE complex in a way which has yet to determined.

4.4 SNAREs, Lipids, and Membrane Fusion

Apart from the SNAREs and associated proteins, other cellular factors also regulate the kinetics, the extent of fusion and the preparation of vesicle for release. Among those factors, membrane lipids are especially noteworthy [for review see (Chasserot-Golaz et al. 2010; Darios et al. 2007)]. Since lipids are the main constituents of the fusing membranes, modifying lipids can directly change the intrinsic fusogenic properties of membranes. In addition, lipids act to recruit and/or activate a large number of different proteins to create a local environment in which exocytosis takes place.

Although it has been known since the 1950s that stimulation of pancreatic cell secretion leads to increased phosphorylation of phosphatidylinositides (PIs) (Hokin and Hokin 1953), it is still an ongoing work to characterize all the PI molecules that are needed for the exocytic process. This is partially due to the PI versatility given that the inositol head group of PI can be reversibly phosphorylated at various positions, resulting in seven naturally occurring PIs. All PIs show distributions restricted to well-characterized membrane territories and can be rapidly interconverted by specialized lipid kinases and phosphatases, which add or remove specific phosphate groups; some forms are also broken down by phospholipases (Cremona and De Camilli 2001; Di Paolo and De Camilli 2006).

$PI(4,5)P_2$ is a key PI player in regulated exocytosis as well as endocytosis in neurons and neuroendocrine cells [reviewed by (Cremona and De Camilli 2001; Martin 2001; Saheki and De Camilli 2012)]. The canonical pathway of PI metabolism places PI(4)P as the precursor of $PI(4,5)P_2$. In this pathway, PI4-kinases (PI4Ks) phosphorylate PI to produce PI(4)P, which then serves as a substrate for PIP5-kinases (PI5Ks). Two types of PIP5Ks are responsible for the $PI(4,5)P_2$ synthesis and each exists as several isoforms. For example, phosphatidylinositol 4-phosphate 5-kinase Iγ (PI4P5K-Iγ) is the major isoform that produces $PI(4,5)P_2$ at the neuronal active zone (Wenk et al. 2001). To account for the complex demands for $PI(4,5)P_2$ at the active zone, PI4P5K-Iγ is tightly regulated by Ca^{2+}, Arf6, phosphorylation, and phosphatidic acid, the product of phospholipase D activity [see also below; (Aikawa and Martin 2003; Fruman et al. 1998)].

The first clue suggesting a direct role of $PI(4,5)P_2$ in regulated exocytosis came from studies on permeabilized chromaffin and PC12 cells which showed that $PI(4,5)P_2$ was required for an ATP-dependent priming step preceding Ca^{2+}-triggered

fusion (Eberhard et al. 1990; Hay et al. 1995). A search for the cytosolic factors required for this energy requiring priming step in permeabilized neuroendocrine cells led to the identification of two enzymes involved in the PI metabolism: a phosphatidylinositol transfer protein (PITP; (Hay and Martin 1993)) and a PI4P5K (Hay et al. 1995). A model was suggested in which PI was delivered to the vesicle membrane via PITP, phosphorylated to PI(4)P by vesicular protein PI4K-II, and finally converted to PI(4,5)P_2 by PI4P5K recruited from the cytoplasm (Hay et al. 1995). In subsequent work it was observed that the PI(4,5)P_2-binding PH domain from PLCδ_1 became localized to the plasma membrane and inhibited Ca^{2+}-dependent exocytosis in chromaffin cells (Holz et al. 2000). Further, overexpression of the PI4P5K-Iγ caused an increase in the plasmalemmal PI(4,5)P_2 level and the primed vesicle pool, whereas overexpression of a membrane-tagged PI(4,5)P_2 phosphatase eliminated plasmalemmal PI(4,5)P_2 and inhibited secretion, showing that the balance between the generation and degradation rates of the plasmalemmal PI(4,5)P_2 directly regulates vesicle priming (Milosevic et al. 2005). Dual roles of PI(4,5)P_2 in both exocytosis and endocytosis suggest that this lipid may control the plasma membrane trafficking and a model in which a PI cycle is nested within the secretory vesicle cycle was proposed (Cremona and De Camilli 2001).

Many proteins involved in regulated exocytosis have been shown to interact with PI(4,5)P_2 in vitro, and based on these interactions, it can be postulated that PI(4,5)P_2 has a function in vesicle docking, in priming, and in particular the fusion reaction. Most notably, PI(4,5)P_2 binds to synaptotagmin family members and CAPS proteins (Bai et al. 2004a; Loyet et al. 1998; Schiavo et al. 1996; van den Bogaart et al. 2012). Other relevant interactions include binding of PI(4,5)P_2 to Mints, which bind Munc18s and are implicated in docking (Okamoto and Sudhof 1997), and rabphilin 3, an effector of Rab3 proteins, which might control SNARE-complex formation (Chung et al. 1998). Molecular details of how PI(4,5)P_2 forms a platform for vesicle recruitment have recently been proposed (Honigmann et al. 2013). Specifically, synaptotagmin-1 was shown to interact independently of calcium with the polybasic linker region of syntaxin-1 already associated with PI(4,5)P_2 at the plasma membrane. This interaction might cause vesicle docking at least in vitro (Kim et al. 2012).

Besides PI(4,5)P_2, PI hydrolysis products or other PIs may act as recruitment or signaling factors to prime secretory vesicles for fusion. Diacylglycerol (DAG) production through hydrolysis of PI(4,5)P_2 by phospholipase C is now considered to be needed for the priming process, owing to the activation of protein kinase C and Munc13, which then modulate the function of syntaxin-1 (Bauer et al. 2007). DAG is further hydrolyzed by DAG lipases to liberate fatty acids and monoacylglycerols. PI(3)P is located on a subpopulation of neurosecretory vesicles and positively regulates secretion (Meunier et al. 2005). In addition, PIKfyve kinase that can produce PI(3,5)P_2 from PI(3)P on secretory vesicles has been proposed to negatively affect exocytosis (Osborne et al. 2008), yielding an opposite effect and revealing how fine-tuning of membrane fusion by PIs can potentially control the number of vesicles undergoing priming. Finally, synaptic PI(3,4,5)P3 has recently been shown to contribute to syntaxin clustering and exocytosis (Khuong et al. 2013).

Phosphatidylserine (PS) and cholesterol are involved in the spatial definition of exocytotic sites [recently reviewed by (Ammar et al. 2013)]. In the plasma membrane, PS is mainly present in the inner membrane leaflet and it contributes substantially to its negative charge. PS is necessary for synaptotagmin binding and thus for fusion triggering (Zhang et al. 2009; Zhang and Jackson 2010). It was recently found that exocytosis is associated with outward translocation of PS, which in turn is required for compensatory endocytosis (Ory et al. 2013). Cholesterol depletion provided a clue for a role of cholesterol in neurosecretory cell exocytosis (Chamberlain et al. 2001), which was supported by additional biochemical and imaging experiments implying that SNARE proteins concentrate in cholesterol-dependent clusters (Lang et al. 2001).

Growing evidence also supports a role for phosphatidic acid (PA) during exocytosis: the local formation of PA by phospholipase D1 underneath the vesicle regulates the fusion competency of secretory vesicles docked at the plasma membrane of neurosecretory cells, suggesting a direct role in membrane fusion (Vitale et al. 2001; Zeniou-Meyer et al. 2007). Because PA is a cone-shaped lipid, it will promote the formation of bend lipid structures displaying negative curvature, which is required during formation of the hemifusion state. Several constituents and regulators of the fusion machinery have also been shown to bind to PA, including NSF, small GTPases, and syntaxin-1 (Jang et al. 2012). As mentioned above, PA is an essential cofactor of PI4P5K-Iγ, which produces $PI(4,5)P_2$, which in turn recruits and activates phospholipase D, suggesting a positive feedback loop in the synthesis of $PI(4,5)P_2$ and PA (Jang et al. 2012).

Finally, fatty acids have been proposed to play an important function in membrane fusion. Arachidonic acid, omega-3, and omega-6 unsaturated fatty acids were found to directly promote SNAP-25/syntaxin-3 assembly and the formation of the ternary SNARE complex leading to dendrite expansion (Darios and Davletov 2006). In chromaffin cells, arachidonic acid promoted vesicle docking and increased quantal size (Garcia-Martinez et al. 2013). It remains to be seen whether endogenous levels of free fatty acids suffice to stimulate the SNARE mechanism.

In summary, lipids play multiple roles in membrane fusion, acting either individually, sequentially, or simultaneously with other lipids. The rapid enzymatic production and degradation of lipids has the potential to modify the physiological function at the synapse within seconds or minutes without the need for protein synthesis/degradation. Further studies will be needed to understand the interplay between lipids and SNAREs in regulation membrane fusion.

4.5 Noncanonical SNAREs in Synaptic Transmission

Spontaneous miniature release is the release of single neurotransmitter quanta in the absence of an action potential. Even though most spontaneous release events are triggered by calcium – similar to evoked release – there is much evidence to show that spontaneous events are subject to separate regulation and that at least some of

the vesicle fusion events follow a different mechanistic route [for a recent review, see (Ramirez and Kavalali 2011)]. Although spontaneous release cannot transfer time-locked information from one neuron to another, it can nevertheless be important for controlling firing in the postsynaptic cell (Carter and Regehr 2002). Whether the fusion machinery and the vesicles themselves are different from those that support evoked release is, however, controversial.

Knockout of SNAP-25 or synaptobrevin-2/VAMP2 almost eliminates evoked release, whereas spontaneous release is affected much less (Bronk et al. 2007; Deitcher et al. 1998; Delgado-Martinez et al. 2007; Schoch et al. 2001; Schulze et al. 1995; Washbourne et al. 2002). Indeed, normalizing spontaneous release in SNAP-25 and synaptobrevin-2/VAMP2 knockouts to the primed vesicle pool would lead to frequencies at least as high as in wild-type neurons (Bronk et al. 2007; Delgado-Martinez et al. 2007; Schoch et al. 2001). The most likely explanation for this is substitution by non-cognate SNAREs, which form complexes with syntaxin-1 and support spontaneous release. For instance, exogenously expressed SNAP-23 can fully restore spontaneous release in SNAP-25 KO neurons, whereas evoked release is strongly asynchronous (Delgado-Martinez et al. 2007). As another example, endogenous or exogenous cellubrevin effectively substitutes for synaptobrevin-2/VAMP2 in its absence, although it seems to play no role in the presence of synaptobrevin-2/VAMP2 (Borisovska et al. 2005; Deak et al. 2006). These findings imply that SNAREs are partly interchangeable in the cell as long as the 3Q:R-rule is observed (Fasshauer et al. 1998b), but only a few of them – the neuronal SNAREs – are able to effectively link to complexin and synaptotagmin to support evoked release. In contrast, knockout or reduction of the syntaxin-1 level appears to reduce both spontaneous and evoked release in parallel (Arancillo et al. 2013; Stewart et al. 2000; Zhou et al. 2013a). Thus, syntaxin-1 might be special and not amenable to substitution. The most likely explanation is the structure of this SNARE, combined with the interaction with Munc18-1 and Munc13 proteins, which are necessary to open up syntaxin-1 as an obligatory part of the vesicular priming machinery.

Likewise, mutation of SNAP-25 and synaptobrevin-2/VAMP2 often leads to milder phenotypes for spontaneous release than for evoked release. This is true for insertions in the synaptobrevin-2/VAMP2 linker domain (Deak et al. 2006; Guzman et al. 2010) and for deletion of two phenylalanines within the linker (Fang et al. 2013; Maximov et al. 2009). Furthermore, mutations inside the SNARE bundle of SNAP-25 around the middle of the complex increased spontaneous release rates (Weber et al. 2010). These and other findings indicate that the structural requirements for spontaneous release are easier to fulfill than those for evoked release. In contrast, upon destabilizing mutation in the C-terminal end of the SNAP-25 SNARE motif, spontaneous release suffered even more than evoked release (Weber et al. 2010). Conversely, a mutation in syntaxin that tightened the C-terminal end of the SNARE complex led to an increase of both evoked and spontaneous release (Lagow et al. 2007). These findings indicate that an obligatory prerequisite for spontaneous release is the firm assembly of the C-terminal end of the SNARE bundle, just as for evoked release. Since this is the process that initiates

lipid splaying and stalk formation (see above), the membrane fusion pathway itself might be conserved between spontaneous and evoked release.

What is then the difference between evoked and spontaneous release in terms of SNARE-complex assembly? As explained above, the prerequisite for evoked release is the presence of a fusion clamp, which is most likely engaged in the partly zippered SNARE complex, preventing C-terminal assembly. This clamp might include complexin and synaptotagmin, or the repulsion between the membranes, but it is likely that it also depends on the details of the SNARE-complex assembly pathway. Firm N-terminal assembly might stabilize the vesicle in a trough in the energy landscape, which sets up an additional energy barrier for fusion compared to the unprimed vesicle. Conversely, a looser assembly of the N-terminal end might allow the complex to "skip over" the clamped state, progressing directly to C-terminal assembly and release (Weber et al. 2010). This might explain why mutations will often disinhibit spontaneous release, whereas evoked release is much more susceptible to mutation and substitution by other SNAREs.

Synaptic vesicles contain several other SNAREs in addition to synaptobrevin-2/VAMP2, including vti1a and VAMP4 (Antonin et al. 2000b; Raingo et al. 2012; Ramirez et al. 2012; Takamori et al. 2006). In recent years, it has been found that these SNAREs might participate in synaptic vesicle fusion, which results in either spontaneous or asynchronous release. VAMP-4 expression is able to restore evoked release in synaptobrevin-2/VAMP2 knockout neurons, but the resulting release is asynchronous release, which is more susceptible to the calcium buffer EGTA (Raingo et al. 2012). Conversely, knockdown of VAMP-4 under some circumstances attenuated asynchronous release, and using pHluorin assays, it was shown that VAMP-4 traffics independently of synaptobrevin-2/VAMP2 (Raingo et al. 2012). Finally, it was shown that VAMP-4 – as a R-SNARE – is able to substitute for synaptobrevin-2/VAMP2 and form a SNARE complex with syntaxin-1 and SNAP-25; however, this SNARE complex did not bind complexin or synaptotagmin-1, which accounts for the asynchronicity of release.

Vti1a is a Q_b-SNARE and is therefore expected to take up the same position in a SNARE complex as the N-terminal domain of SNAP-25. It has been mainly associated with intracellular fusion events, such as endosome fusion and endosome-to-Golgi fusion (Brandhorst et al. 2006; Mallard et al. 2002). Using pHluorin fusion proteins, one group concluded that vti1a traffics on spontaneously fusing synaptic vesicles, and knockdown of vti1a reduced the frequency of spontaneous release (Ramirez et al. 2012). However, vti1a-pHluorin-carrying vesicles fused during prolonged stimulation trains, although this was less prominent than for synaptobrevin-pHluorin (Hoopmann et al. 2010; Ramirez et al. 2012), showing that vti1a-carrying vesicles are not entirely resistant to calcium. It was concluded that vti1a is resident on vesicles that do not contain synaptobrevin-2/VAMP2 (Ramirez et al. 2012). It remains to be understood what the vti1a SNARE partners for this fusion pathway would be, since synaptobrevin-2/VAMP2 as an R-SNARE is not expected to be replaceable by a Q_b-SNARE. VAMP7/TI-VAMP is an R-SNARE belonging to the longin family of R-SNAREs with extended N-terminal domains (Filippini et al. 2001). VAMP7 was identified as another

marker for the resting vesicle pool, vesicles of which cycle only spontaneously or reluctantly (Hua et al. 2011). However, differences in the cycling between VAMP-7 and vti1a make it unlikely that they reside in the same vesicle population or participate in the same SNARE complex (Ramirez and Kavalali 2012). Indeed, it was recently shown that Reelin, a secreted glycoprotein, stimulates spontaneous release by specifically recruiting VAMP-7 – but not vti1a containing – vesicles (Bal et al. 2013).

An open question is how dominant a role the noncanonical SNAREs play in asynchronous and spontaneous release under physiological conditions, given that the neuronal SNAREs – syntaxin-1, synaptobrevin-2/VAMP2, and SNAP-25 – are very abundant and also participate in those events, and knockout or expression of mutants of the neuronal SNAREs can eliminate most spontaneous events and asynchronous release in cultured neurons. Most likely, the relative expression levels and/or separation into different vesicle pools will determine the relative participation of the different SNAREs in fusion. In this scenario, the noncanonical SNAREs might play their largest role in minority neuron populations displaying stronger expression; for instance, VAMP-4 was found to be strongly expressed in CCK-interneurons (Raingo et al. 2012), which are known to display strongly asynchronous release (Hefft and Jonas 2005). Much more research is needed before the importance of the noncanonical SNAREs in synaptic transmission can be assessed.

4.6 Membrane Fusion and SNARE Complex: Beyond 2014

Most of the research work described in this review has been designed to explore the properties and mechanistic action of one or a few specific components of the membrane fusion machinery. These types of studies have been essential to understand how the SNARE machinery works and are leading the field towards a stage of maturity in which the inner workings of the fusion machinery in the living cell can be described concisely and reconstituted in vitro. A few key questions that will still have to be answered are:

- What is the minimal essential function of Munc18 in membrane fusion?
- Is the stoichiometry of SNARE complexes participating in fusion regulated, and is it predetermined or stochastic?
- What is the nature of the fusion pathway(s) downstream of SNARE-complex assembly?

While the basic discoveries in membrane fusion and SNAREs emerged from biochemical and electrophysiological studies, continuous progress in this research field will be driven by new technologies and methodologies. In recent years, new genomic, biophysical (computational and molecular modelling), and imaging (super-resolution microscopy) approaches have pointed to new properties of membrane fusion and SNARE-complex action. In the future, expression from

endogenous loci of SNARE proteins bearing genetically encoded fluorescent tags will likely provide new ways to study SNAREs and mechanisms of membrane fusion in space and time in living cells and organisms. Some of the key questions for this new phase of discovery are:

- How, and at which stage, is the SNARE mechanism regulated – and how are different levels of regulation (for instance, expression level, phosphorylation, Munc13-activation, and inhibition by tomosyn) integrated?
- How does regulation of the SNARE mechanism result in tissue- and cell-specific release properties?
- What role do the basal biophysical properties of the SNARE mechanism (and its regulation) play in the context of neuronal networks, and how does the SNARE mechanism influence the working of the brain in health and disease?

Acknowledgments We would like to apologize to the authors of many important papers that we could not cite because of space constraints. Work in the author's laboratories is supported by the Lundbeck Foundation, the Novo Nordisk Foundation, the EU FP7 program ("SYNSYS," grant agreement no. HEALTH-F2-2009-242167), the Danish Medical Research Council, the University of Copenhagen 2016 funds, and the Emmy Noether Young Investigator Award from German Research Foundation (DFG).

References

Abdulreda MH, Bhalla A, Chapman ER, Moy VT (2008) Atomic force microscope spectroscopy reveals a hemifusion intermediate during soluble N-ethylmaleimide-sensitive factor-attachment protein receptors-mediated membrane fusion. Biophys J 94:648–655

Aikawa Y, Martin TF (2003) ARF6 regulates a plasma membrane pool of phosphatidylinositol (4,5)bisphosphate required for regulated exocytosis. J Cell Biol 162:647–659

Albillos A, Dernick G, Horstmann H, Almers W, Alvarez de Toledo G, Lindau M (1997) The exocytotic event in chromaffin cells revealed by patch amperometry. Nature 389:509–512

Ammar MR, Kassas N, Chasserot-Golaz S, Bader MF, Vitale N (2013) Lipids in regulated exocytosis: what are they doing? Front Endocrinol 4:125

An SJ, Almers W (2004) Tracking SNARE complex formation in live endocrine cells. Science 306:1042–1046

Antonin W, Holroyd C, Fasshauer D, Pabst S, Von Mollard GF, Jahn R (2000a) A SNARE complex mediating fusion of late endosomes defines conserved properties of SNARE structure and function. EMBO J 19:6453–6464

Antonin W, Riedel D, von Mollard GF (2000b) The SNARE Vti1a-beta is localized to small synaptic vesicles and participates in a novel SNARE complex. J Neurosci 20:5724–5732

Arancillo M, Min SW, Gerber S, Munster-Wandowski A, Wu YJ, Herman M, Trimbuch T, Rah JC, Ahnert-Hilger G, Riedel D, Sudhof TC, Rosenmund C (2013) Titration of Syntaxin1 in mammalian synapses reveals multiple roles in vesicle docking, priming, and release probability. J Neurosci 33:16698–16714

Ashery U, Varoqueaux F, Voets T, Betz A, Thakur P, Koch H, Neher E, Brose N, Rettig J (2000) Munc13-1 acts as a priming factor for large dense-core vesicles in bovine chromaffin cells. EMBO J 19:3586–3596

Ashery U, Bielopolski N, Barak B, Yizhar O (2009) Friends and foes in synaptic transmission: the role of tomosyn in vesicle priming. Trends Neurosci 32:275–282

Augustin I, Rosenmund C, Sudhof TC, Brose N (1999) Munc13-1 is essential for fusion competence of glutamatergic synaptic vesicles. Nature 400:457–461

Bai J, Tucker WC, Chapman ER (2004a) PIP2 increases the speed of response of synaptotagmin and steers its membrane-penetration activity toward the plasma membrane. Nat Struct Mol Biol 11:36–44

Bai J, Wang CT, Richards DA, Jackson MB, Chapman ER (2004b) Fusion pore dynamics are regulated by synaptotagmin*t-SNARE interactions. Neuron 41:929–942

Bal M, Leitz J, Reese AL, Ramirez DM, Durakoglugil M, Herz J, Monteggia LM, Kavalali ET (2013) Reelin mobilizes a VAMP7-dependent synaptic vesicle pool and selectively augments spontaneous neurotransmission. Neuron 80:934–946

Banerjee A, Barry VA, DasGupta BR, Martin TF (1996) N-Ethylmaleimide-sensitive factor acts at a prefusion ATP-dependent step in Ca2+-activated exocytosis. J Biol Chem 271:20223–20226

Bar-On D, Gutman M, Mezer A, Ashery U, Lang T, Nachliel E (2009) Evaluation of the heterogeneous reactivity of the syntaxin molecules on the inner leaflet of the plasma membrane. J Neurosci 29:12292–12301

Bar-On D, Wolter S, van de Linde S, Heilemann M, Nudelman G, Nachliel E, Gutman M, Sauer M, Ashery U (2012) Super-resolution imaging reveals the internal architecture of nano-sized syntaxin clusters. J Biol Chem 287:27158–27167

Barg S, Knowles MK, Chen X, Midorikawa M, Almers W (2010) Syntaxin clusters assemble reversibly at sites of secretory granules in live cells. Proc Natl Acad Sci USA 107:20804–20809

Bark IC, Wilson MC (1994) Human cDNA clones encoding two different isoforms of the nerve terminal protein SNAP-25. Gene 139:291–292

Bark IC, Hahn KM, Ryabinin AE, Wilson MC (1995) Differential expression of SNAP-25 protein isoforms during divergent vesicle fusion events of neural development. Proc Natl Acad Sci USA 92:1510–1514

Bark C, Bellinger FP, Kaushal A, Mathews JR, Partridge LD, Wilson MC (2004) Developmentally regulated switch in alternatively spliced SNAP-25 isoforms alters facilitation of synaptic transmission. J Neurosci 24:8796–8805

Basu J, Shen N, Dulubova I, Lu J, Guan R, Guryev O, Grishin NV, Rosenmund C, Rizo J (2005) A minimal domain responsible for Munc13 activity. Nat Struct Mol Biol 12:1017–1018

Bauer CS, Woolley RJ, Teschemacher AG, Seward EP (2007) Potentiation of exocytosis by phospholipase C-coupled G-protein-coupled receptors requires the priming protein Munc13-1. J Neurosci 27:212–219

Baumert M, Maycox PR, Navone F, De Camilli P, Jahn R (1989) Synaptobrevin: an integral membrane protein of 18,000 daltons present in small synaptic vesicles of rat brain. EMBO J 8:379–384

Bennett MK, Calakos N, Scheller RH (1992) Syntaxin: a synaptic protein implicated in docking of synaptic vesicles at presynaptic active zones. Science 257:255–259

Bock JB, Matern HT, Peden AA, Scheller RH (2001) A genomic perspective on membrane compartment organization. Nature 409:839–841

Borisovska M, Zhao Y, Tsytsyura Y, Glyvuk N, Takamori S, Matti U, Rettig J, Sudhof T, Bruns D (2005) v-SNAREs control exocytosis of vesicles from priming to fusion. EMBO J 24:2114–2126

Boschert U, O'Shaughnessy C, Dickinson R, Tessari M, Bendotti C, Catsicas S, Pich EM (1996) Developmental and plasticity-related differential expression of two SNAP-25 isoforms in the rat brain. J Comp Neurol 367:177–193

Boyd A, Ciufo LF, Barclay JW, Graham ME, Haynes LP, Doherty MK, Riesen M, Burgoyne RD, Morgan A (2008) A random mutagenesis approach to isolate dominant-negative yeast sec1 mutants reveals a functional role for domain 3a in yeast and mammalian Sec1/Munc18 proteins. Genetics 180:165–178

Brandhorst D, Zwilling D, Rizzoli SO, Lippert U, Lang T, Jahn R (2006) Homotypic fusion of early endosomes: SNAREs do not determine fusion specificity. Proc Natl Acad Sci USA 103:2701–2706

Bretou M, Anne C, Darchen F (2008) A fast mode of membrane fusion dependent on tight SNARE zippering. J Neurosci 28:8470–8476

Bronk P, Deak F, Wilson MC, Liu X, Sudhof TC, Kavalali ET (2007) Differential effects of SNAP-25 deletion on Ca2+ -dependent and Ca2+ -independent neurotransmission. J Neurophysiol 98:794–806

Bruns D, Jahn R (1995) Real-time measurement of transmitter release from single synaptic vesicles. Nature 377:62–65

Burgalossi A, Jung S, Meyer G, Jockusch WJ, Jahn O, Taschenberger H, O'Connor VM, Nishiki T, Takahashi M, Brose N, Rhee JS (2010) SNARE protein recycling by alphaSNAP and betaSNAP supports synaptic vesicle priming. Neuron 68:473–487

Burkhardt P, Hattendorf DA, Weis WI, Fasshauer D (2008) Munc18a controls SNARE assembly through its interaction with the syntaxin N-peptide. EMBO J 27:923–933

Carr CM, Grote E, Munson M, Hughson FM, Novick PJ (1999) Sec1p binds to SNARE complexes and concentrates at sites of secretion. J Cell Biol 146:333–344

Carter AG, Regehr WG (2002) Quantal events shape cerebellar interneuron firing. Nat Neurosci 5:1309–1318

Chamberlain LH, Burgoyne RD, Gould GW (2001) SNARE proteins are highly enriched in lipid rafts in PC12 cells: implications for the spatial control of exocytosis. Proc Natl Acad Sci USA 98:5619–5624

Chasserot-Golaz S, Coorssen JR, Meunier FA, Vitale N (2010) Lipid dynamics in exocytosis. Cell Mol Neurobiol 30:1335–1342

Chen YA, Scales SJ, Scheller RH (2001) Sequential SNARE assembly underlies priming and triggering of exocytosis. Neuron 30:161–170

Chen X, Tomchick DR, Kovrigin E, Arac D, Machius M, Sudhof TC, Rizo J (2002) Three-dimensional structure of the complexin/SNARE complex. Neuron 33:397–409

Chernomordik LV, Kozlov MM (2003) Protein-lipid interplay in fusion and fission of biological membranes. Annu Rev Biochem 72:175–207

Christie MP, Whitten AE, King GJ, Hu SH, Jarrott RJ, Chen KE, Duff AP, Callow P, Collins BM, James DE, Martin JL (2012) Low-resolution solution structures of Munc18:Syntaxin protein complexes indicate an open binding mode driven by the Syntaxin N-peptide. Proc Natl Acad Sci USA 109:9816–9821

Chung SH, Song WJ, Kim K, Bednarski JJ, Chen J, Prestwich GD, Holz RW (1998) The C2 domains of Rabphilin3A specifically bind phosphatidylinositol 4,5-bisphosphate containing vesicles in a Ca2+-dependent manner. In vitro characteristics and possible significance. J Biol Chem 273:10240–10248

Clary DO, Rothman JE (1990) Purification of three related peripheral membrane proteins needed for vesicular transport. J Biol Chem 265:10109–10117

Cohen FS, Melikyan GB (2004) The energetics of membrane fusion from binding, through hemifusion, pore formation, and pore enlargement. J Membr Biol 199:1–14

Colbert KN, Hattendorf DA, Weiss TM, Burkhardt P, Fasshauer D, Weis WI (2013) Syntaxin1a variants lacking an N-peptide or bearing the LE mutation bind to Munc18a in a closed conformation. Proc Natl Acad Sci USA 110:12637–12642

Cremona O, De Camilli P (2001) Phosphoinositides in membrane traffic at the synapse. J Cell Sci 114:1041–1052

Criado M, Gil A, Viniegra S, Gutierrez LM (1999) A single amino acid near the C terminus of the synaptosome associated protein of 25 kDa (SNAP-25) is essential for exocytosis in chromaffin cells. Proc Natl Acad Sci USA 96:7256–7261

Daily NJ, Boswell KL, James DJ, Martin TF (2010) Novel interactions of CAPS (Ca2+-dependent activator protein for secretion) with the three neuronal SNARE proteins required for vesicle fusion. J Biol Chem 285:35320–35329

Darios F, Davletov B (2006) Omega-3 and omega-6 fatty acids stimulate cell membrane expansion by acting on syntaxin 3. Nature 440:813–817

Darios F, Connell E, Davletov B (2007) Phospholipases and fatty acid signalling in exocytosis. J Physiol 585:699–704

de Wit H, Cornelisse LN, Toonen RF, Verhage M (2006) Docking of secretory vesicles is syntaxin dependent. PLoS One 1:e126

de Wit H, Walter AM, Milosevic I, Gulyas-Kovacs A, Riedel D, Sorensen JB, Verhage M (2009) Synaptotagmin-1 docks secretory vesicles to syntaxin-1/SNAP-25 acceptor complexes. Cell 138:935–946

Deak F, Schoch S, Liu X, Sudhof TC, Kavalali ET (2004) Synaptobrevin is essential for fast synaptic-vesicle endocytosis. Nat Cell Biol 6:1102–1108

Deak F, Shin OH, Kavalali ET, Sudhof TC (2006) Structural determinants of synaptobrevin 2 function in synaptic vesicle fusion. J Neurosci 26:6668–6676

Deak F, Xu Y, Chang WP, Dulubova I, Khvotchev M, Liu X, Sudhof TC, Rizo J (2009) Munc18-1 binding to the neuronal SNARE complex controls synaptic vesicle priming. J Cell Biol 184:751–764

Degtyar V, Hafez IM, Bray C, Zucker RS (2013) Dance of the SNAREs: assembly and rearrangements detected with FRET at neuronal synapses. J Neurosci 33:5507–5523

Deitcher DL, Ueda A, Stewart BA, Burgess RW, Kidokoro Y, Schwarz TL (1998) Distinct requirements for evoked and spontaneous release of neurotransmitter are revealed by mutations in the Drosophila gene neuronal-synaptobrevin. J Neurosci 18:2028–2039

Delgado-Martinez I, Nehring RB, Sorensen JB (2007) Differential abilities of SNAP-25 homologs to support neuronal function. J Neurosci 27:9380–9391

Di Paolo G, De Camilli P (2006) Phosphoinositides in cell regulation and membrane dynamics. Nature 443:651–657

Dulubova I, Sugita S, Hill S, Hosaka M, Fernandez I, Sudhof TC, Rizo J (1999) A conformational switch in syntaxin during exocytosis: role of munc18. EMBO J 18:4372–4382

Dulubova I, Khvotchev M, Liu S, Huryeva I, Sudhof TC, Rizo J (2007) Munc18-1 binds directly to the neuronal SNARE complex. Proc Natl Acad Sci USA 104:2697–2702

Eberhard DA, Cooper CL, Low MG, Holz RW (1990) Evidence that the inositol phospholipids are necessary for exocytosis. Loss of inositol phospholipids and inhibition of secretion in permeabilized cells caused by a bacterial phospholipase C and removal of ATP. Biochem J 268:15–25

Elferink LA, Trimble WS, Scheller RH (1989) Two vesicle-associated membrane protein genes are differentially expressed in the rat central nervous system. J Biol Chem 264:11061–11064

Elhamdani A, Martin TF, Kowalchyk JA, Artalejo CR (1999) Ca(2+)-dependent activator protein for secretion is critical for the fusion of dense-core vesicles with the membrane in calf adrenal chromaffin cells. J Neurosci 19:7375–7383

Ellena JF, Liang B, Wiktor M, Stein A, Cafiso DS, Jahn R, Tamm LK (2009) Dynamic structure of lipid-bound synaptobrevin suggests a nucleation-propagation mechanism for trans-SNARE complex formation. Proc Natl Acad Sci USA 106:20306–20311

Fang Q, Zhao Y, Lindau M (2013) Juxtamembrane tryptophans of synaptobrevin 2 control the process of membrane fusion. FEBS Lett 587:67–72

Fasshauer D, Margittai M (2004) A transient N-terminal interaction of SNAP-25 and syntaxin nucleates SNARE assembly. J Biol Chem 279:7613–7621

Fasshauer D, Otto H, Eliason WK, Jahn R, Brunger AT (1997) Structural changes are associated with soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor complex formation. J Biol Chem 272:28036–28041

Fasshauer D, Eliason WK, Brunger AT, Jahn R (1998a) Identification of a minimal core of the synaptic SNARE complex sufficient for reversible assembly and disassembly. Biochemistry 37:10354–10362

Fasshauer D, Sutton RB, Brunger AT, Jahn R (1998b) Conserved structural features of the synaptic fusion complex: SNARE proteins reclassified as Q- and R-SNAREs. Proc Natl Acad Sci USA 95:15781–15786

Fasshauer D, Antonin W, Subramaniam V, Jahn R (2002) SNARE assembly and disassembly exhibit a pronounced hysteresis. Nat Struct Biol 9:144–151

Fdez E, Martinez-Salvador M, Beard M, Woodman P, Hilfiker S (2010) Transmembrane-domain determinants for SNARE-mediated membrane fusion. J Cell Sci 123:2473–2480

Filippini F, Rossi V, Galli T, Budillon A, D'Urso M, D'Esposito M (2001) Longins: a new evolutionary conserved VAMP family sharing a novel SNARE domain. Trends Biochem Sci 26:407–409

Freedman SJ, Song HK, Xu Y, Sun ZY, Eck MJ (2003) Homotetrameric structure of the SNAP-23 N-terminal coiled-coil domain. J Biol Chem 278:13462–13467

Fruman DA, Meyers RE, Cantley LC (1998) Phosphoinositide kinases. Annu Rev Biochem 67:481–507

Fujiwara T, Mishima T, Kofuji T, Chiba T, Tanaka K, Yamamoto A, Akagawa K (2006) Analysis of knock-out mice to determine the role of HPC-1/syntaxin 1A in expressing synaptic plasticity. J Neurosci 26:5767–5776

Fukuda R, McNew JA, Weber T, Parlati F, Engel T, Nickel W, Rothman JE, Sollner TH (2000) Functional architecture of an intracellular membrane t-SNARE. Nature 407:198–202

Gao Y, Zorman S, Gundersen G, Xi Z, Ma L, Sirinakis G, Rothman JE, Zhang Y (2012) Single reconstituted neuronal SNARE complexes zipper in three distinct stages. Science 337:1340–1343

Garcia-Martinez V, Villanueva J, Torregrosa-Hetland CJ, Bittman R, Higdon A, Darley-Usmar VM, Davletov B, Gutierrez LM (2013) Lipid metabolites enhance secretion acting on SNARE microdomains and altering the extent and kinetics of single release events in bovine adrenal chromaffin cells. PLoS One 8:e75845

Gerber SH, Rah JC, Min SW, Liu X, de Wit H, Dulubova I, Meyer AC, Rizo J, Arancillo M, Hammer RE, Verhage M, Rosenmund C, Sudhof TC (2008) Conformational switch of syntaxin-1 controls synaptic vesicle fusion. Science 321:1507–1510

Gil A, Gutierrez LM, Carrasco-Serrano C, Alonso MT, Viniegra S, Criado M (2002) Modifications in the C terminus of the synaptosome-associated protein of 25 kDa (SNAP-25) and in the complementary region of synaptobrevin affect the final steps of exocytosis. J Biol Chem 277:9904–9910

Giraudo CG, Hu C, You D, Slovic AM, Mosharov EV, Sulzer D, Melia TJ, Rothman JE (2005) SNAREs can promote complete fusion and hemifusion as alternative outcomes. J Cell Biol 170:249–260

Giraudo CG, Eng WS, Melia TJ, Rothman JE (2006) A clamping mechanism involved in SNARE-dependent exocytosis. Science 313:676–680

Gonzalo S, Greentree WK, Linder ME (1999) SNAP-25 is targeted to the plasma membrane through a novel membrane-binding domain. J Biol Chem 274:21313–21318

Grishanin RN, Klenchin VA, Loyet KM, Kowalchyk JA, Ann K, Martin TF (2002) Membrane association domains in Ca2+-dependent activator protein for secretion mediate plasma membrane and dense-core vesicle binding required for Ca2+-dependent exocytosis. J Biol Chem 277:22025–22034

Gulyas-Kovacs A, de Wit H, Milosevic I, Kochubey O, Toonen R, Klingauf J, Verhage M, Sorensen JB (2007) Munc18-1: sequential interactions with the fusion machinery stimulate vesicle docking and priming. J Neurosci 27:8676–8686

Guzman RE, Schwarz YN, Rettig J, Bruns D (2010) SNARE force synchronizes synaptic vesicle fusion and controls the kinetics of quantal synaptic transmission. J Neurosci 30:10272–10281

Halemani ND, Bethani I, Rizzoli SO, Lang T (2010) Structure and dynamics of a two-helix SNARE complex in live cells. Traffic 11:394–404

Hammarlund M, Palfreyman MT, Watanabe S, Olsen S, Jorgensen EM (2007) Open syntaxin docks synaptic vesicles. PLoS Biol 5:e198

Hammarlund M, Watanabe S, Schuske K, Jorgensen EM (2008) CAPS and syntaxin dock dense core vesicles to the plasma membrane in neurons. J Cell Biol 180:483–491

Han X, Wang CT, Bai J, Chapman ER, Jackson MB (2004) Transmembrane segments of syntaxin line the fusion pore of Ca2+-triggered exocytosis. Science 304:289–292

Hanson PI, Heuser JE, Jahn R (1997a) Neurotransmitter release – four years of SNARE complexes. Curr Opin Neurobiol 7:310–315

Hanson PI, Roth R, Morisaki H, Jahn R, Heuser JE (1997b) Structure and conformational changes in NSF and its membrane receptor complexes visualized by quick-freeze/deep-etch electron microscopy. Cell 90:523–535

Harrison SD, Broadie K, van de Goor J, Rubin GM (1994) Mutations in the Drosophila Rop gene suggest a function in general secretion and synaptic transmission. Neuron 13:555–566

Hay JC, Martin TF (1993) Phosphatidylinositol transfer protein required for ATP-dependent priming of Ca(2+)-activated secretion. Nature 366:572–575

Hay JC, Fisette PL, Jenkins GH, Fukami K, Takenawa T, Anderson RA, Martin TF (1995) ATP-dependent inositide phosphorylation required for Ca(2+)-activated secretion. Nature 374:173–177

Hefft S, Jonas P (2005) Asynchronous GABA release generates long-lasting inhibition at a hippocampal interneuron-principal neuron synapse. Nat Neurosci 8:1319–1328

Hernandez JM, Stein A, Behrmann E, Riedel D, Cypionka A, Farsi Z, Walla PJ, Raunser S, Jahn R (2012) Membrane fusion intermediates via directional and full assembly of the SNARE complex. Science 336:1581–1584

Hess DT, Slater TM, Wilson MC, Skene JH (1992) The 25 kDa synaptosomal-associated protein SNAP-25 is the major methionine-rich polypeptide in rapid axonal transport and a major substrate for palmitoylation in adult CNS. J Neurosci 12:4634–4641

Hokin MR, Hokin LE (1953) Enzyme secretion and the incorporation of P32 into phospholipides of pancreas slices. J Biol Chem 203:967–977

Holz RW, Hlubek MD, Sorensen SD, Fisher SK, Balla T, Ozaki S, Prestwich GD, Stuenkel EL, Bittner MA (2000) A pleckstrin homology domain specific for phosphatidylinositol 4, 5-bisphosphate (PtdIns-4,5-P2) and fused to green fluorescent protein identifies plasma membrane PtdIns-4,5-P2 as being important in exocytosis. J Biol Chem 275:17878–17885

Honigmann A, van den Bogaart G, Iraheta E, Risselada HJ, Milovanovic D, Mueller V, Mullar S, Diederichsen U, Fasshauer D, Grubmuller H, Hell SW, Eggeling C, Kuhnel K, Jahn R (2013) Phosphatidylinositol 4,5-bisphosphate clusters act as molecular beacons for vesicle recruitment. Nat Struct Mol Biol 20:679–686

Hoopmann P, Punge A, Barysch SV, Westphal V, Buckers J, Opazo F, Bethani I, Lauterbach MA, Hell SW, Rizzoli SO (2010) Endosomal sorting of readily releasable synaptic vesicles. Proc Natl Acad Sci USA 107:19055–19060

Hosono R, Hekimi S, Kamiya Y, Sassa T, Murakami S, Nishiwaki K, Miwa J, Taketo A, Kodaira KI (1992) The unc-18 gene encodes a novel protein affecting the kinetics of acetylcholine metabolism in the nematode Caenorhabditis elegans. J Neurochem 58:1517–1525

Hu C, Ahmed M, Melia TJ, Sollner TH, Mayer T, Rothman JE (2003) Fusion of cells by flipped SNAREs. Science 300:1745–1749

Hu SH, Christie MP, Saez NJ, Latham CF, Jarrott R, Lua LH, Collins BM, Martin JL (2011) Possible roles for Munc18-1 domain 3a and Syntaxin1 N-peptide and C-terminal anchor in SNARE complex formation. Proc Natl Acad Sci USA 108:1040–1045

Hua SY, Charlton MP (1999) Activity-dependent changes in partial VAMP complexes during neurotransmitter release. Nat Neurosci 2:1078–1083

Hua Y, Scheller RH (2001) Three SNARE complexes cooperate to mediate membrane fusion. Proc Natl Acad Sci USA 98:8065–8070

Hua Z, Leal-Ortiz S, Foss SM, Waites CL, Garner CC, Voglmaier SM, Edwards RH (2011) v-SNARE composition distinguishes synaptic vesicle pools. Neuron 71:474–487

Jackson MB (2010) SNARE complex zipping as a driving force in the dilation of proteinaceous fusion pores. J Membr Biol 235:89–100

Jahn R, Fasshauer D (2012) Molecular machines governing exocytosis of synaptic vesicles. Nature 490:201–207

James DJ, Kowalchyk J, Daily N, Petrie M, Martin TF (2009) CAPS drives trans-SNARE complex formation and membrane fusion through syntaxin interactions. Proc Natl Acad Sci USA 106:17308–17313

Jang JH, Lee CS, Hwang D, Ryu SH (2012) Understanding of the roles of phospholipase D and phosphatidic acid through their binding partners. Prog Lipid Res 51:71–81

Jockusch WJ, Speidel D, Sigler A, Sorensen JB, Varoqueaux F, Rhee JS, Brose N (2007) CAPS-1 and CAPS-2 are essential synaptic vesicle priming proteins. Cell 131:796–808

Johnson JR, Ferdek P, Lian LY, Barclay JW, Burgoyne RD, Morgan A (2009) Binding of UNC-18 to the N-terminus of syntaxin is essential for neurotransmission in Caenorhabditis elegans. Biochem J 418:73–80

Katsov K, Muller M, Schick M (2004) Field theoretic study of bilayer membrane fusion. I. Hemifusion mechanism. Biophys J 87:3277–3290

Kesavan J, Borisovska M, Bruns D (2007) v-SNARE actions during Ca(2+)-triggered exocytosis. Cell 131:351–363

Khodthong C, Kabachinski G, James DJ, Martin TF (2011) Munc13 homology domain-1 in CAPS/UNC31 mediates SNARE binding required for priming vesicle exocytosis. Cell Metab 14:254–263

Khuong TM, Habets RL, Kuenen S, Witkowska A, Kasprowicz J, Swerts J, Jahn R, van den Bogaart G, Verstreken P (2013) Synaptic PI(3,4,5)P3 is required for Syntaxin1A clustering and neurotransmitter release. Neuron 77:1097–1108

Khvotchev M, Dulubova I, Sun J, Dai H, Rizo J, Sudhof TC (2007) Dual modes of Munc18-1/SNARE interactions are coupled by functionally critical binding to syntaxin-1 N terminus. J Neurosci 27:12147–12155

Kim JY, Choi BK, Choi MG, Kim SA, Lai Y, Shin YK, Lee NK (2012) Solution single-vesicle assay reveals PIP2-mediated sequential actions of synaptotagmin-1 on SNAREs. EMBO J 31:2144–2155

Kloepper TH, Kienle CN, Fasshauer D (2007) An elaborate classification of SNARE proteins sheds light on the conservation of the eukaryotic endomembrane system. Mol Biol Cell 18:3463–3471

Knecht V, Grubmuller H (2003) Mechanical coupling via the membrane fusion SNARE protein syntaxin 1A: a molecular dynamics study. Biophys J 84:1527–1547

Knowles MK, Barg S, Wan L, Midorikawa M, Chen X, Almers W (2010) Single secretory granules of live cells recruit syntaxin-1 and synaptosomal associated protein 25 (SNAP-25) in large copy numbers. Proc Natl Acad Sci USA 107:20810–20815

Kummel D, Krishnakumar SS, Radoff DT, Li F, Giraudo CG, Pincet F, Rothman JE, Reinisch KM (2011) Complexin cross-links prefusion SNAREs into a zigzag array. Nat Struct Mol Biol 18:927–933

Kuner T, Li Y, Gee KR, Bonewald LF, Augustine GJ (2008) Photolysis of a caged peptide reveals rapid action of N-ethylmaleimide sensitive factor before neurotransmitter release. Proc Natl Acad Sci USA 105:347–352

Kuzmin PI, Zimmerberg J, Chizmadzhev YA, Cohen FS (2001) A quantitative model for membrane fusion based on low-energy intermediates. Proc Natl Acad Sci USA 98:7235–7240

Kweon DH, Kim CS, Shin YK (2003) Insertion of the membrane-proximal region of the neuronal SNARE coiled coil into the membrane. J Biol Chem 278:12367–12373

Laage R, Langosch D (1997) Dimerization of the synaptic vesicle protein synaptobrevin (vesicle-associated membrane protein) II depends on specific residues within the transmembrane segment. Eur J Biochem/FEBS 249:540–546

Laage R, Rohde J, Brosig B, Langosch D (2000) A conserved membrane-spanning amino acid motif drives homomeric and supports heteromeric assembly of presynaptic SNARE proteins. J Biol Chem 275:17481–17487

Lagow RD, Bao H, Cohen EN, Daniels RW, Zuzek A, Williams WH, Macleod GT, Sutton RB, Zhang B (2007) Modification of a hydrophobic layer by a point mutation in syntaxin 1A regulates the rate of synaptic vesicle fusion. PLoS Biol 5:e72

Lang T, Bruns D, Wenzel D, Riedel D, Holroyd P, Thiele C, Jahn R (2001) SNAREs are concentrated in cholesterol-dependent clusters that define docking and fusion sites for exocytosis. EMBO J 20:2202–2213

Lang T, Margittai M, Holzler H, Jahn R (2002) SNAREs in native plasma membranes are active and readily form core complexes with endogenous and exogenous SNAREs. J Cell Biol 158:751–760

Li F, Pincet F, Perez E, Eng WS, Melia TJ, Rothman JE, Tareste D (2007) Energetics and dynamics of SNAREpin folding across lipid bilayers. Nat Struct Mol Biol 14:890–896

Li F, Kummel D, Coleman J, Reinisch KM, Rothman JE, Pincet F (2014) A half-zippered SNARE complex represents a functional intermediate in membrane fusion. J Am Chem Soc 136:3456–3464

Lin RC, Scheller RH (1997) Structural organization of the synaptic exocytosis core complex. Neuron 19:1087–1094

Lindau M, Hall BA, Chetwynd A, Beckstein O, Sansom MS (2012) Coarse-grain simulations reveal movement of the synaptobrevin C-terminus in response to piconewton forces. Biophys J 103:959–969

Lipstein N, Sakaba T, Cooper BH, Lin KH, Strenzke N, Ashery U, Rhee JS, Taschenberger H, Neher E, Brose N (2013) Dynamic control of synaptic vesicle replenishment and short-term plasticity by Ca(2+)-calmodulin-Munc13-1 signaling. Neuron 79:82–96

Littleton JT, Chapman ER, Kreber R, Garment MB, Carlson SD, Ganetzky B (1998) Temperature-sensitive paralytic mutations demonstrate that synaptic exocytosis requires SNARE complex assembly and disassembly. Neuron 21:401–413

Littleton JT, Barnard RJ, Titus SA, Slind J, Chapman ER, Ganetzky B (2001) SNARE-complex disassembly by NSF follows synaptic-vesicle fusion. Proc Natl Acad Sci USA 98:12233–12238

Liu Y, Schirra C, Stevens DR, Matti U, Speidel D, Hof D, Bruns D, Brose N, Rettig J (2008) CAPS facilitates filling of the rapidly releasable pool of large dense-core vesicles. J Neurosci 28:5594–5601

Liu Y, Schirra C, Edelmann L, Matti U, Rhee J, Hof D, Bruns D, Brose N, Rieger H, Stevens DR, Rettig J (2010) Two distinct secretory vesicle-priming steps in adrenal chromaffin cells. J Cell Biol 190:1067–1077

Loyet KM, Kowalchyk JA, Chaudhary A, Chen J, Prestwich GD, Martin TF (1998) Specific binding of phosphatidylinositol 4,5-bisphosphate to calcium-dependent activator protein for secretion (CAPS), a potential phosphoinositide effector protein for regulated exocytosis. J Biol Chem 273:8337–8343

Lu X, Zhang F, McNew JA, Shin YK (2005) Membrane fusion induced by neuronal SNAREs transits through hemifusion. J Biol Chem 280:30538–30541

Ma C, Li W, Xu Y, Rizo J (2011) Munc13 mediates the transition from the closed syntaxin-Munc18 complex to the SNARE complex. Nat Struct Mol Biol 18:542–549

Ma C, Su L, Seven AB, Xu Y, Rizo J (2013) Reconstitution of the vital functions of Munc18 and Munc13 in neurotransmitter release. Science 339:421–425

Mallard F, Tang BL, Galli T, Tenza D, Saint-Pol A, Yue X, Antony C, Hong W, Goud B, Johannes L (2002) Early/recycling endosomes-to-TGN transport involves two SNARE complexes and a Rab6 isoform. J Cell Biol 156:653–664

Malsam J, Parisotto D, Bharat TA, Scheutzow A, Krause JM, Briggs JA, Sollner TH (2012) Complexin arrests a pool of docked vesicles for fast Ca2+-dependent release. EMBO J 31:3270–3281

Margittai M, Fasshauer D, Pabst S, Jahn R, Langen R (2001) Homo- and heterooligomeric SNARE complexes studied by site-directed spin labeling. J Biol Chem 276:13169–13177

Markvoort AJ, Marrink SJ (2011) Lipid acrobatics in the membrane fusion arena. Curr Top Membr 68:259–294

Martin TF (2001) PI(4,5)P(2) regulation of surface membrane traffic. Curr Opin Cell Biol 13:493–499

Martin S, Tomatis VM, Papadopulos A, Christie MP, Malintan NT, Gormal RS, Sugita S, Martin JL, Collins BM, Meunier FA (2013) The Munc18-1 domain 3a loop is essential for neuroexocytosis but not for syntaxin-1A transport to the plasma membrane. J Cell Sci 126:2353–2360

Matos MF, Mukherjee K, Chen X, Rizo J, Sudhof TC (2003) Evidence for SNARE zippering during Ca2+-triggered exocytosis in PC12 cells. Neuropharmacology 45:777–786

Maximov A, Tang J, Yang X, Pang ZP, Sudhof TC (2009) Complexin controls the force transfer from SNARE complexes to membranes in fusion. Science 323:516–521

McEwen JM, Madison JM, Dybbs M, Kaplan JM (2006) Antagonistic regulation of synaptic vesicle priming by Tomosyn and UNC-13. Neuron 51:303–315

McNew JA, Weber T, Engelman DM, Sollner TH, Rothman JE (1999) The length of the flexible SNAREpin juxtamembrane region is a critical determinant of SNARE-dependent fusion. Mol Cell 4:415–421

McNew JA, Weber T, Parlati F, Johnston RJ, Melia TJ, Sollner TH, Rothman JE (2000) Close is not enough: SNARE-dependent membrane fusion requires an active mechanism that transduces force to membrane anchors. J Cell Biol 150:105–117

Medine CN, Rickman C, Chamberlain LH, Duncan RR (2007) Munc18-1 prevents the formation of ectopic SNARE complexes in living cells. J Cell Sci 120:4407–4415

Megighian A, Zordan M, Pantano S, Scorzeto M, Rigoni M, Zanini D, Rossetto O, Montecucco C (2013) Evidence for a radial SNARE super-complex mediating neurotransmitter release at the Drosophila neuromuscular junction. J Cell Sci 126:3134–3140

Meijer M, Burkhardt P, de Wit H, Toonen RF, Fasshauer D, Verhage M (2012) Munc18-1 mutations that strongly impair SNARE-complex binding support normal synaptic transmission. EMBO J 31:2156–2168

Melia TJ, Weber T, McNew JA, Fisher LE, Johnston RJ, Parlati F, Mahal LK, Sollner TH, Rothman JE (2002) Regulation of membrane fusion by the membrane-proximal coil of the t-SNARE during zippering of SNAREpins. J Cell Biol 158:929–940

Meunier FA, Osborne SL, Hammond GR, Cooke FT, Parker PJ, Domin J, Schiavo G (2005) Phosphatidylinositol 3-kinase C2alpha is essential for ATP-dependent priming of neurosecretory granule exocytosis. Mol Biol Cell 16:4841–4851

Milosevic I, Sorensen JB, Lang T, Krauss M, Nagy G, Haucke V, Jahn R, Neher E (2005) Plasmalemmal phosphatidylinositol-4,5-bisphosphate level regulates the releasable vesicle pool size in chromaffin cells. J Neurosci 25:2557–2565

Min D, Kim K, Hyeon C, Cho YH, Shin YK, Yoon TY (2013) Mechanical unzipping and rezipping of a single SNARE complex reveals hysteresis as a force-generating mechanism. Nat Commun 4:1705

Mishima T, Fujiwara T, Kofuji T, Akagawa K (2012) Impairment of catecholamine systems during induction of long-term potentiation at hippocampal CA1 synapses in HPC-1/syntaxin 1A knock-out mice. J Neurosci 32:381–389

Misura KM, Scheller RH, Weis WI (2000) Three-dimensional structure of the neuronal-Sec1-syntaxin 1a complex. Nature 404:355–362

Misura KM, Gonzalez LC Jr, May AP, Scheller RH, Weis WI (2001a) Crystal structure and biophysical properties of a complex between the N-terminal SNARE region of SNAP25 and syntaxin 1a. J Biol Chem 276:41301–41309

Misura KM, Scheller RH, Weis WI (2001b) Self-association of the H3 region of syntaxin 1A. Implications for intermediates in SNARE complex assembly. J Biol Chem 276:13273–13282

Mohrmann R, de Wit H, Verhage M, Neher E, Sorensen JB (2010) Fast vesicle fusion in living cells requires at least three SNARE complexes. Science 330:502–505

Mohrmann R, de Wit H, Connell E, Pinheiro PS, Leese C, Bruns D, Davletov B, Verhage M, Sorensen JB (2013) Synaptotagmin interaction with SNAP-25 governs vesicle docking, priming, and fusion triggering. J Neurosci 33:14417–14430

Montecucco C, Schiavo G (1995) Structure and function of tetanus and botulinum neurotoxins. Q Rev Biophys 28:423–472

Montecucco C, Schiavo G, Pantano S (2005) SNARE complexes and neuroexocytosis: how many, how close? Trends Biochem Sci 30:367–372

Murray DH, Tamm LK (2009) Clustering of syntaxin-1A in model membranes is modulated by phosphatidylinositol 4,5-bisphosphate and cholesterol. Biochemistry 48:4617–4625

Ngatchou AN, Kisler K, Fang Q, Walter AM, Zhao Y, Bruns D, Sorensen JB, Lindau M (2010) Role of the synaptobrevin C terminus in fusion pore formation. Proc Natl Acad Sci USA 107:18463–18468

Niemann H, Blasi J, Jahn R (1994) Clostridial neurotoxins: new tools for dissecting exocytosis. Trends Cell Biol 4:179–185

Okamoto M, Sudhof TC (1997) Mints, Munc18-interacting proteins in synaptic vesicle exocytosis. J Biol Chem 272:31459–31464

Ory S, Ceridono M, Momboisse F, Houy S, Chasserot-Golaz S, Heintz D, Calco V, Haeberle AM, Espinoza FA, Sims PJ, Bailly Y, Bader MF, Gasman S (2013) Phospholipid scramblase-1-induced lipid reorganization regulates compensatory endocytosis in neuroendocrine cells. J Neurosci 33:3545–3556

Osborne SL, Wen PJ, Boucheron C, Nguyen HN, Hayakawa M, Kaizawa H, Parker PJ, Vitale N, Meunier FA (2008) PIKfyve negatively regulates exocytosis in neurosecretory cells. J Biol Chem 283:2804–2813

Oyler GA, Higgins GA, Hart RA, Battenberg E, Billingsley M, Bloom FE, Wilson MC (1989) The identification of a novel synaptosomal-associated protein, SNAP-25, differentially expressed by neuronal subpopulations. J Cell Biol 109:3039–3052

Pabst S, Hazzard JW, Antonin W, Sudhof TC, Jahn R, Rizo J, Fasshauer D (2000) Selective interaction of complexin with the neuronal SNARE complex. Determination of the binding regions. J Biol Chem 275:19808–19818

Pobbati AV, Stein A, Fasshauer D (2006) N- to C-terminal SNARE complex assembly promotes rapid membrane fusion. Science 313:673–676

Raingo J, Khvotchev M, Liu P, Darios F, Li YC, Ramirez DM, Adachi M, Lemieux P, Toth K, Davletov B, Kavalali ET (2012) VAMP4 directs synaptic vesicles to a pool that selectively maintains asynchronous neurotransmission. Nat Neurosci 15:738–745

Ramirez DM, Kavalali ET (2011) Differential regulation of spontaneous and evoked neurotransmitter release at central synapses. Curr Opin Neurobiol 21:275–282

Ramirez DM, Kavalali ET (2012) The role of non-canonical SNAREs in synaptic vesicle recycling. Cell Logist 2:20–27

Ramirez DM, Khvotchev M, Trauterman B, Kavalali ET (2012) Vti1a identifies a vesicle pool that preferentially recycles at rest and maintains spontaneous neurotransmission. Neuron 73:121–134

Rathore SS, Bend EG, Yu H, Hammarlund M, Jorgensen EM, Shen J (2010) Syntaxin N-terminal peptide motif is an initiation factor for the assembly of the SNARE-Sec1/Munc18 membrane fusion complex. Proc Natl Acad Sci USA 107:22399–22406

Reese C, Heise F, Mayer A (2005) Trans-SNARE pairing can precede a hemifusion intermediate in intracellular membrane fusion. Nature 436:410–414

Rhee JS, Betz A, Pyott S, Reim K, Varoqueaux F, Augustin I, Hesse D, Sudhof TC, Takahashi M, Rosenmund C, Brose N (2002) Beta phorbol ester- and diacylglycerol-induced augmentation of transmitter release is mediated by Munc13s and not by PKCs. Cell 108:121–133

Rhee JS, Li LY, Shin OH, Rah JC, Rizo J, Sudhof TC, Rosenmund C (2005) Augmenting neurotransmitter release by enhancing the apparent Ca2+ affinity of synaptotagmin 1. Proc Natl Acad Sci USA 102:18664–18669

Richmond JE, Davis WS, Jorgensen EM (1999) UNC-13 is required for synaptic vesicle fusion in C. elegans. Nat Neurosci 2:959–964

Richmond JE, Weimer RM, Jorgensen EM (2001) An open form of syntaxin bypasses the requirement for UNC-13 in vesicle priming. Nature 412:338–341

Rickman C, Archer DA, Meunier FA, Craxton M, Fukuda M, Burgoyne RD, Davletov B (2004) Synaptotagmin interaction with the syntaxin/SNAP-25 dimer is mediated by an evolutionarily conserved motif and is sensitive to inositol hexakisphosphate. J Biol Chem 279:12574–12579

Rickman C, Jimenez JL, Graham ME, Archer DA, Soloviev M, Burgoyne RD, Davletov B (2006) Conserved prefusion protein assembly in regulated exocytosis. Mol Biol Cell 17:283–294

Rickman C, Medine CN, Dun AR, Moulton DJ, Mandula O, Halemani ND, Rizzoli SO, Chamberlain LH, Duncan RR (2010) t-SNARE protein conformations patterned by the lipid microenvironment. J Biol Chem 285:13535–13541

Risselada HJ, Grubmuller H (2012) How SNARE molecules mediate membrane fusion: recent insights from molecular simulations. Curr Opin Struct Biol 22:187–196

Risselada HJ, Kutzner C, Grubmuller H (2011) Caught in the act: visualization of SNARE-mediated fusion events in molecular detail. Chembiochem 12:1049–1055

Rizo J, Sudhof TC (2012) The membrane fusion enigma: SNAREs, Sec1/Munc18 proteins, and their accomplices – guilty as charged? Annu Rev Cell Dev Biol 28:279–308

Rowe J, Corradi N, Malosio ML, Taverna E, Halban P, Meldolesi J, Rosa P (1999) Blockade of membrane transport and disassembly of the Golgi complex by expression of syntaxin 1A in neurosecretion-incompetent cells: prevention by rbSEC1. J Cell Sci 112(Pt 12):1865–1877

Ruiz-Montasell B, Aguado F, Majo G, Chapman ER, Canals JM, Marsal J, Blasi J (1996) Differential distribution of syntaxin isoforms 1A and 1B in the rat central nervous system. Eur J Neurosci 8:2544–2552

Saheki Y, De Camilli P (2012) Synaptic vesicle endocytosis. Cold Spring Harb Perspect Biol 4: a005645

Schiavo G, Gu QM, Prestwich GD, Sollner TH, Rothman JE (1996) Calcium-dependent switching of the specificity of phosphoinositide binding to synaptotagmin. Proc Natl Acad Sci USA 93:13327–13332

Schoch S, Deak F, Konigstorfer A, Mozhayeva M, Sara Y, Sudhof TC, Kavalali ET (2001) SNARE function analyzed in synaptobrevin/VAMP knockout mice. Science 294:1117–1122

Schollmeier Y, Krause JM, Kreye S, Malsam J, Sollner TH (2011) Resolving the function of distinct Munc18-1/SNARE protein interaction modes in a reconstituted membrane fusion assay. J Biol Chem 286:30582–30590

Schuette CG, Hatsuzawa K, Margittai M, Stein A, Riedel D, Kuster P, Konig M, Seidel C, Jahn R (2004) Determinants of liposome fusion mediated by synaptic SNARE proteins. Proc Natl Acad Sci USA 101:2858–2863

Schulze KL, Broadie K, Perin MS, Bellen HJ (1995) Genetic and electrophysiological studies of Drosophila syntaxin-1A demonstrate its role in nonneuronal secretion and neurotransmission. Cell 80:311–320

Schutz D, Zilly F, Lang T, Jahn R, Bruns D (2005) A dual function for Munc-18 in exocytosis of PC12 cells. Eur J Neurosci 21:2419–2432

Shen J, Tareste DC, Paumet F, Rothman JE, Melia TJ (2007) Selective activation of cognate SNAREpins by Sec1/Munc18 proteins. Cell 128:183–195

Shen J, Rathore SS, Khandan L, Rothman JE (2010) SNARE bundle and syntaxin N-peptide constitute a minimal complement for Munc18-1 activation of membrane fusion. J Cell Biol 190:55–63

Shi L, Shen QT, Kiel A, Wang J, Wang HW, Melia TJ, Rothman JE, Pincet F (2012) SNARE proteins: one to fuse and three to keep the nascent fusion pore open. Science 335:1355–1359

Shin OH, Lu J, Rhee JS, Tomchick DR, Pang ZP, Wojcik SM, Camacho-Perez M, Brose N, Machius M, Rizo J, Rosenmund C, Sudhof TC (2010) Munc13 C2B domain is an activity-dependent Ca2+ regulator of synaptic exocytosis. Nat Struct Mol Biol 17:280–288

Shin J, Lou X, Kweon DH, Shin YK (2014) Multiple conformations of a single SNAREpin between two nanodisc membranes reveal diverse pre-fusion states. Biochem J 459:95–102

Siddiqui TJ, Vites O, Stein A, Heintzmann R, Jahn R, Fasshauer D (2007) Determinants of synaptobrevin regulation in membranes. Mol Biol Cell 18:2037–2046

Sieber JJ, Willig KI, Kutzner C, Gerding-Reimers C, Harke B, Donnert G, Rammner B, Eggeling C, Hell SW, Grubmuller H, Lang T (2007) Anatomy and dynamics of a supramolecular membrane protein cluster. Science 317:1072–1076

Sinha R, Ahmed S, Jahn R, Klingauf J (2011) Two synaptobrevin molecules are sufficient for vesicle fusion in central nervous system synapses. Proc Natl Acad Sci USA 108:14318–14323

Sollner T, Bennett MK, Whiteheart SW, Scheller RH, Rothman JE (1993a) A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. Cell 75:409–418

Sollner T, Whiteheart SW, Brunner M, Erdjument-Bromage H, Geromanos S, Tempst P, Rothman JE (1993b) SNAP receptors implicated in vesicle targeting and fusion. Nature 362:318–324

Sorensen JB, Nagy G, Varoqueaux F, Nehring RB, Brose N, Wilson MC, Neher E (2003) Differential control of the releasable vesicle pools by SNAP-25 splice variants and SNAP-23. Cell 114:75–86

Sorensen JB, Wiederhold K, Muller EM, Milosevic I, Nagy G, de Groot BL, Grubmuller H, Fasshauer D (2006) Sequential N- to C-terminal SNARE complex assembly drives priming and fusion of secretory vesicles. EMBO J 25:955–966

Speidel D, Salehi A, Obermueller S, Lundquist I, Brose N, Renstrom E, Rorsman P (2008) CAPS1 and CAPS2 regulate stability and recruitment of insulin granules in mouse pancreatic beta cells. Cell Metab 7:57–67

Stein A, Weber G, Wahl MC, Jahn R (2009) Helical extension of the neuronal SNARE complex into the membrane. Nature 460:525–528

Stevens DR, Wu ZX, Matti U, Junge HJ, Schirra C, Becherer U, Wojcik SM, Brose N, Rettig J (2005) Identification of the minimal protein domain required for priming activity of Munc13-1. Curr Biol 15:2243–2248

Stewart BA, Mohtashami M, Trimble WS, Boulianne GL (2000) SNARE proteins contribute to calcium cooperativity of synaptic transmission. Proc Natl Acad Sci USA 97:13955–13960

Sudhof TC (2013) Neurotransmitter release: the last millisecond in the life of a synaptic vesicle. Neuron 80:675–690

Sutton RB, Fasshauer D, Jahn R, Brunger AT (1998) Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 A resolution. Nature 395:347–353

Takamori S, Holt M, Stenius K, Lemke EA, Gronborg M, Riedel D, Urlaub H, Schenck S, Brugger B, Ringler P, Muller SA, Rammner B, Grater F, Hub JS, De Groot BL, Mieskes G, Moriyama Y, Klingauf J, Grubmuller H, Heuser J, Wieland F, Jahn R (2006) Molecular anatomy of a trafficking organelle. Cell 127:831–846

Tang J, Maximov A, Shin OH, Dai H, Rizo J, Sudhof TC (2006) A complexin/synaptotagmin 1 switch controls fast synaptic vesicle exocytosis. Cell 126:1175–1187

Terrian DM, White MK (1997) Phylogenetic analysis of membrane trafficking proteins: a family reunion and secondary structure predictions. Eur J Cell Biol 73:198–204

Tokumaru H, Umayahara K, Pellegrini LL, Ishizuka T, Saisu H, Betz H, Augustine GJ, Abe T (2001) SNARE complex oligomerization by synaphin/complexin is essential for synaptic vesicle exocytosis. Cell 104:421–432

Toonen RF, de Vries KJ, Zalm R, Sudhof TC, Verhage M (2005) Munc18-1 stabilizes syntaxin 1, but is not essential for syntaxin 1 targeting and SNARE complex formation. J Neurochem 93:1393–1400

Trimble WS, Cowan DM, Scheller RH (1988) VAMP-1: a synaptic vesicle-associated integral membrane protein. Proc Natl Acad Sci USA 85:4538–4542

Trimble WS, Gray TS, Elferink LA, Wilson MC, Scheller RH (1990) Distinct patterns of expression of two VAMP genes within the rat brain. J Neurosci 10:1380–1387

van den Bogaart G, Holt MG, Bunt G, Riedel D, Wouters FS, Jahn R (2010) One SNARE complex is sufficient for membrane fusion. Nat Struct Mol Biol 17:358–364

van den Bogaart G, Meyenberg K, Risselada HJ, Amin H, Willig KI, Hubrich BE, Dier M, Hell SW, Grubmuller H, Diederichsen U, Jahn R (2011a) Membrane protein sequestering by ionic protein-lipid interactions. Nature 479:552–555

van den Bogaart G, Thutupalli S, Risselada JH, Meyenberg K, Holt M, Riedel D, Diederichsen U, Herminghaus S, Grubmuller H, Jahn R (2011b) Synaptotagmin-1 may be a distance regulator acting upstream of SNARE nucleation. Nat Struct Mol Biol 18:805–812

van den Bogaart G, Meyenberg K, Diederichsen U, Jahn R (2012) Phosphatidylinositol 4,5-bisphosphate increases Ca2+ affinity of synaptotagmin-1 by 40-fold. J Biol Chem 287:16447–16453

Varoqueaux F, Sigler A, Rhee JS, Brose N, Enk C, Reim K, Rosenmund C (2002) Total arrest of spontaneous and evoked synaptic transmission but normal synaptogenesis in the absence of Munc13-mediated vesicle priming. Proc Natl Acad Sci USA 99:9037–9042

Verhage M, Maia AS, Plomp JJ, Brussaard AB, Heeroma JH, Vermeer H, Toonen RF, Hammer RE, van den Berg TK, Missler M, Geuze HJ, Sudhof TC (2000) Synaptic assembly of the brain in the absence of neurotransmitter secretion. Science 287:864–869

Vitale N, Caumont AS, Chasserot-Golaz S, Du G, Wu S, Sciorra VA, Morris AJ, Frohman MA, Bader MF (2001) Phospholipase D1: a key factor for the exocytotic machinery in neuroendocrine cells. EMBO J 20:2424–2434

Voets T, Toonen RF, Brian EC, de Wit H, Moser T, Rettig J, Sudhof TC, Neher E, Verhage M (2001) Munc18-1 promotes large dense-core vesicle docking. Neuron 31:581–591

Walter AM, Wiederhold K, Bruns D, Fasshauer D, Sorensen JB (2010) Synaptobrevin N-terminally bound to syntaxin-SNAP-25 defines the primed vesicle state in regulated exocytosis. J Cell Biol 188:401–413

Wang CT, Grishanin R, Earles CA, Chang PY, Martin TF, Chapman ER, Jackson MB (2001) Synaptotagmin modulation of fusion pore kinetics in regulated exocytosis of dense-core vesicles. Science 294:1111–1115

Wang CT, Bai J, Chang PY, Chapman ER, Jackson MB (2006) Synaptotagmin-Ca2+ triggers two sequential steps in regulated exocytosis in rat PC12 cells: fusion pore opening and fusion pore dilation. J Physiol 570:295–307

Wang L, Bittner MA, Axelrod D, Holz RW (2008) The structural and functional implications of linked SNARE motifs in SNAP25. Mol Biol Cell 19:3944–3955

Washbourne P, Thompson PM, Carta M, Costa ET, Mathews JR, Lopez-Bendito G, Molnar Z, Becher MW, Valenzuela CF, Partridge LD, Wilson MC (2002) Genetic ablation of the t-SNARE SNAP-25 distinguishes mechanisms of neuroexocytosis. Nat Neurosci 5:19–26

Weber T, Zemelman BV, McNew JA, Westermann B, Gmachl M, Parlati F, Sollner TH, Rothman JE (1998) SNAREpins: minimal machinery for membrane fusion. Cell 92:759–772

Weber T, Parlati F, McNew JA, Johnston RJ, Westermann B, Sollner TH, Rothman JE (2000) SNAREpins are functionally resistant to disruption by NSF and alphaSNAP. J Cell Biol 149:1063–1072

Weber JP, Reim K, Sorensen JB (2010) Opposing functions of two sub-domains of the SNARE-complex in neurotransmission. EMBO J 29:2477–2490

Wei S, Xu T, Ashery U, Kollewe A, Matti U, Antonin W, Rettig J, Neher E (2000) Exocytotic mechanism studied by truncated and zero layer mutants of the C-terminus of SNAP-25. EMBO J 19:1279–1289

Weimbs T, Mostov K, Low SH, Hofmann K (1998) A model for structural similarity between different SNARE complexes based on sequence relationships. Trends Cell Biol 8:260–262

Weimer RM, Richmond JE, Davis WS, Hadwiger G, Nonet ML, Jorgensen EM (2003) Defects in synaptic vesicle docking in unc-18 mutants. Nat Neurosci 6:1023–1030

Wenk MR, Pellegrini L, Klenchin VA, Di Paolo G, Chang S, Daniell L, Arioka M, Martin TF, De Camilli P (2001) PIP kinase Igamma is the major PI(4,5)P(2) synthesizing enzyme at the synapse. Neuron 32:79–88

Whiteheart SW, Schraw T, Matveeva EA (2001) N-ethylmaleimide sensitive factor (NSF) structure and function. Int Rev Cytol 207:71–112

Wiederhold K, Fasshauer D (2009) Is assembly of the SNARE complex enough to fuel membrane fusion? J Biol Chem 284:13143–13152

Wiederhold K, Kloepper TH, Walter AM, Stein A, Kienle N, Sorensen JB, Fasshauer D (2010) A coiled coil trigger site is essential for rapid binding of synaptobrevin to the SNARE acceptor complex. J Biol Chem 285:21549–21559

Wong JL, Koppel DE, Cowan AE, Wessel GM (2007) Membrane hemifusion is a stable intermediate of exocytosis. Dev Cell 12:653–659

Wu Y, Gu Y, Morphew MK, Yao J, Yeh FL, Dong M, Chapman ER (2012) All three components of the neuronal SNARE complex contribute to secretory vesicle docking. J Cell Biol 198:323–330

Xiao W, Poirier MA, Bennett MK, Shin YK (2001) The neuronal t-SNARE complex is a parallel four-helix bundle. Nat Struct Biol 8:308–311

Xu T, Ashery U, Burgoyne RD, Neher E (1999a) Early requirement for alpha-SNAP and NSF in the secretory cascade in chromaffin cells. EMBO J 18:3293–3304

Xu T, Rammner B, Margittai M, Artalejo AR, Neher E, Jahn R (1999b) Inhibition of SNARE complex assembly differentially affects kinetic components of exocytosis. Cell 99:713–722

Xu Y, Su L, Rizo J (2010) Binding of Munc18-1 to synaptobrevin and to the SNARE four-helix bundle. Biochemistry 49:1568–1576

Xue M, Reim K, Chen X, Chao HT, Deng H, Rizo J, Brose N, Rosenmund C (2007) Distinct domains of complexin I differentially regulate neurotransmitter release. Nat Struct Mol Biol 14:949–958

Zeniou-Meyer M, Zabari N, Ashery U, Chasserot-Golaz S, Haeberle AM, Demais V, Bailly Y, Gottfried I, Nakanishi H, Neiman AM, Du G, Frohman MA, Bader MF, Vitale N (2007) Phospholipase D1 production of phosphatidic acid at the plasma membrane promotes exocytosis of large dense-core granules at a late stage. J Biol Chem 282:21746–21757

Zhang Z, Jackson MB (2010) Membrane bending energy and fusion pore kinetics in Ca(2+)-triggered exocytosis. Biophys J 98:2524–2534

Zhang Z, Hui E, Chapman ER, Jackson MB (2009) Phosphatidylserine regulation of Ca2+-triggered exocytosis and fusion pores in PC12 cells. Mol Biol Cell 20:5086–5095

Zhao Y, Fang Q, Herbst AD, Berberian KN, Almers W, Lindau M (2013) Rapid structural change in synaptosomal-associated protein 25 (SNAP25) precedes the fusion of single vesicles with the plasma membrane in live chromaffin cells. Proc Natl Acad Sci USA 110:14249–14254

Zhou Z, Misler S, Chow RH (1996) Rapid fluctuations in transmitter release from single vesicles in bovine adrenal chromaffin cells. Biophys J 70:1543–1552

Zhou P, Bacaj T, Yang X, Pang ZP, Sudhof TC (2013a) Lipid-anchored SNAREs lacking transmembrane regions fully support membrane fusion during neurotransmitter release. Neuron 80:470–483

Zhou P, Pang ZP, Yang X, Zhang Y, Rosenmund C, Bacaj T, Sudhof TC (2013b) Syntaxin-1 N-peptide and Habc-domain perform distinct essential functions in synaptic vesicle fusion. EMBO J 32:159–171

Zilly FE, Sorensen JB, Jahn R, Lang T (2006) Munc18-bound syntaxin readily forms SNARE complexes with synaptobrevin in native plasma membranes. PLoS Biol 4:e330

Chapter 5
Roles of Tomosyn in Neurotransmitter Release

Yasunori Yamamoto and Toshiaki Sakisaka

Abstract SNARE complexes and synaptotagmin mediate synaptic vesicle fusion with the plasma membrane of the active zone for the neurotransmitter release from presynaptic nerve terminals responding to neuronal signals. Many regulatory proteins for the SNARE complex formation have been identified. Among them, our originally identified protein, tomosyn, is likely to be a key molecule for the regulation of the SNARE complex-involved pre-fusion step and the Ca^{2+}-triggered synaptic vesicle fusion step. Tomosyn inhibits SNARE complex formation and thereby inhibits synaptic vesicle fusion by sequestering target SNAREs through its C-terminal VAMP-like domain in a Ca^{2+}-independent manner. The N-terminal WD40 repeats are the site for its binding to synaptotagmin-1, a Ca^{2+}-sensor protein, in a Ca^{2+}-dependent manner. The interaction negatively regulates the Ca^{2+}-dependent synaptic vesicle fusion mediated by synaptotagmin-1. Thus, tomosyn is a potent inhibitor, temporally and stepwisely regulating the synaptic vesicle fusion at the active zone, for the synchronized and fast neurotransmitter release.

Keywords Neurotransmitter release • Exocytosis • SNARE • Tomosyn • Synaptotagmin-1

5.1 Introduction

Tomosyn is a syntaxin-1-binding protein that we originally identified (Fujita et al. 1998). Tomosyn means tomo (friend in Japanese) of syntaxin-1. Tomosyn contains N-terminal WD40 repeats, a tail domain, and a C-terminal domain homologous to VAMP2. The C-terminal VAMP-like domain is responsible for binding to syntaxin-1 (Fujita et al. 1998) (Fig. 5.1a). Tomosyn belongs to the Lgl (lethal giant larvae) family that is conserved from yeast to human (Kagami et al. 1998; Hattendorf et al. 2007; Ashery et al. 2009) (Fig. 5.1b). The Lgl family is characterized by N-terminal fourteen WD40 repeats constituting two β-propeller structures (seven WD40 repeats constitute one β-propeller structure) (Hattendorf et al. 2007) and

Y. Yamamoto • T. Sakisaka (✉)
Division of Membrane Dynamics, Department of Physiology and Cell Biology, Kobe University Graduate School of Medicine, Kobe 650-0017, Japan
e-mail: sakisaka@med.kobe-u.ac.jp

S. Mochida (ed.), *Presynaptic Terminals*, DOI 10.1007/978-4-431-55166-9_5

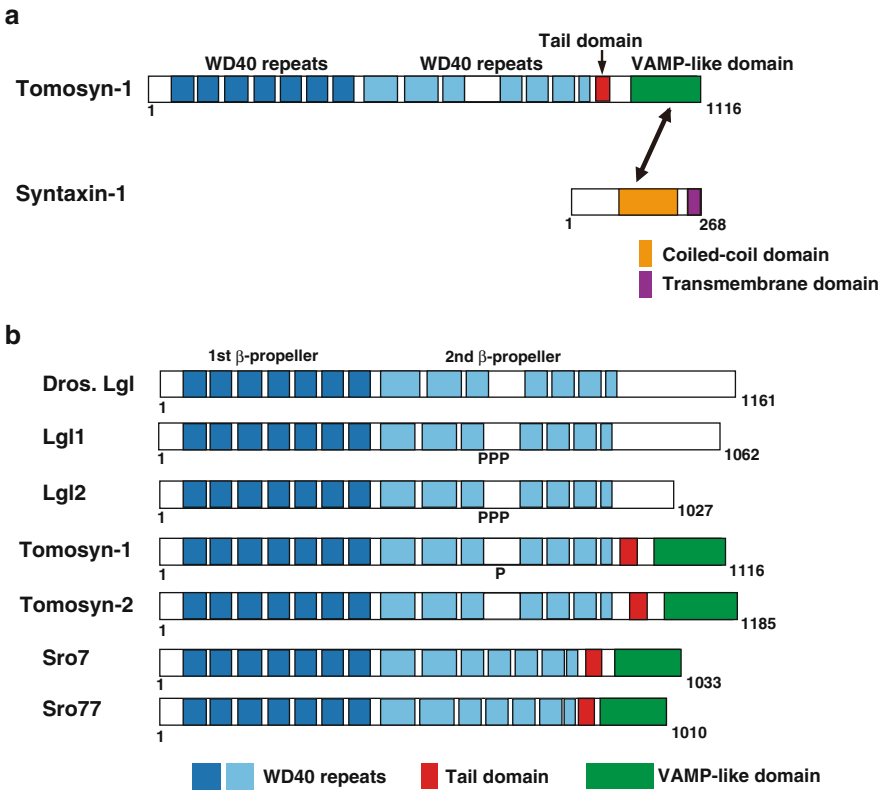

Fig. 5.1 Schematic representation of tomosyn. (**a**) Tomosyn is a syntaxin-1-binding protein. Tomosyn is composed of N-terminal WD40 repeats, a tail domain, and a C-terminal VAMP-like domain. Tomosyn binds to syntaxin-1 through the C-terminal VAMP-like domain. (**b**) Tomosyn belongs to the Lgl family. The Lgl family is characterized by the N-terminal WD40 repeats that constitute two β-propeller structures. Sro7 and Sro77 are yeast orthologues of tomosyn. Among the family members, only Lgl does not have the tail domain and the C-terminal VAMP-like domain. Phosphorylation sites are indicated with P

composed of Lgl, tomosyn, Sro7, and Sro77. Among the family members, only Lgl does not have a tail domain and a C-terminal domain homologous to VAMP2. Lgl, which was firstly identified as a tumor suppressor gene in *Drosophila*, has been shown to be involved in polarity formation and cell-cell adhesion in epithelial cells (Wirtz-Peitz and Knoblich 2006; Yamanaka and Ohno 2008). Sro7 and Sro77, yeast orthologues of tomosyn, have been shown to regulate exocytosis in yeast (Lehman et al. 1999). Lgl, tomosyn, and Sro7 directly interact with SNARE proteins (Fujita et al. 1998; Lehman et al. 1999; Müsch et al. 2002; Gangar et al. 2005), suggesting that the Lgl family plays roles in the SNARE-dependent vesicle trafficking. However, the common mode of action among the Lgl family members remains elucidated. Among the Lgl family members, the origins of tomosyn and Sro7 are evolutionally old (Kloepper et al. 2008). During the evolution from prokaryotes to metazoan, Lgl is thought to be generated by gene duplication of tomosyn (Kloepper et al. 2008).

Therefore, elucidating modes of action for tomosyn and Sro7 is important for understanding the function of the Lgl family. In this chapter we describe, based on our findings, roles of tomosyn in the synaptic vesicle fusion phase. In the vertebrate nervous system, tomosyn-1 is expressed dominantly in the whole brain, while tomosyn-2 is expressed in the restricted area of the brain (Groffen et al. 2005). From here, we refer to tomosyn-1 as tomosyn.

5.2 SNARE-Dependent Vesicle Fusion Machinery

Synaptic vesicles are transported to the active zone in the presynaptic plasma membrane where Ca^{2+} channels are located. Depolarization induces Ca^{2+} influx into the cytosol of nerve terminals through the Ca^{2+} channels, and this Ca^{2+} influx initiates the fusion of the vesicles with the plasma membrane, finally leading to exocytosis of neurotransmitters (Südhof 2004). Soluble *N*-ethylmaleimide-sensitive fusion protein (NSF) attachment protein (SNAP) receptors (SNAREs) are essential for the synaptic vesicle exocytosis (Sutton et al. 1998; Weber et al. 1998; Jahn and Scheller 2006; Rizo and Rosenmund 2008). Synaptic vesicles are endowed with vesicle-associated membrane protein 2 (VAMP2) as a vesicular SNARE (v-SNARE), whereas the presynaptic plasma membrane is endowed with syntaxin-1 and SNAP-25 as target SNAREs (t-SNAREs) (Fig. 5.2a). VAMP2 interacts with SNAP-25 and syntaxin-1 to form a stable SNARE complex (Trimble et al. 1988; Bennett et al. 1992; Söllner et al. 1993; Chen and Scheller 2001). The formation of the SNARE complex then brings synaptic vesicles and the plasma membrane into close apposition and provides the energy that drives the mixing of the two lipid bilayers (Weber et al. 1998; Chen and Scheller 2001; Jahn and Scheller 2006; Rizo and Rosenmund 2008). Although the previous study using the SNARE complex reconstituted on liposomes demonstrated that the SNARE complex was sufficient for membrane fusion, the fusion kinetics was very slow (Weber et al. 1998). These facts indicate in vivo existence of a special factor(s) to regulate SNARE assembly for fast membrane fusion characteristic of neurotransmitter release.

5.3 Tomosyn Regulates SNARE Complex Formation Through the C-Terminal VAMP-Like Domain

Tomosyn interacts with SNAP-25 and syntaxin-1 to form a stable tomosyn-SNARE complex through the C-terminal VAMP-like domain (Fujita et al. 1998) (Fig. 5.2b). The tomosyn-SNARE complex formation sequesters t-SNAREs on the presynaptic plasma membrane, leading to inhibition of the SNARE complex formation (Fujita et al. 1998; Hatsuzawa et al. 2003; Pobbati et al. 2004; Sakisaka et al. 2008;

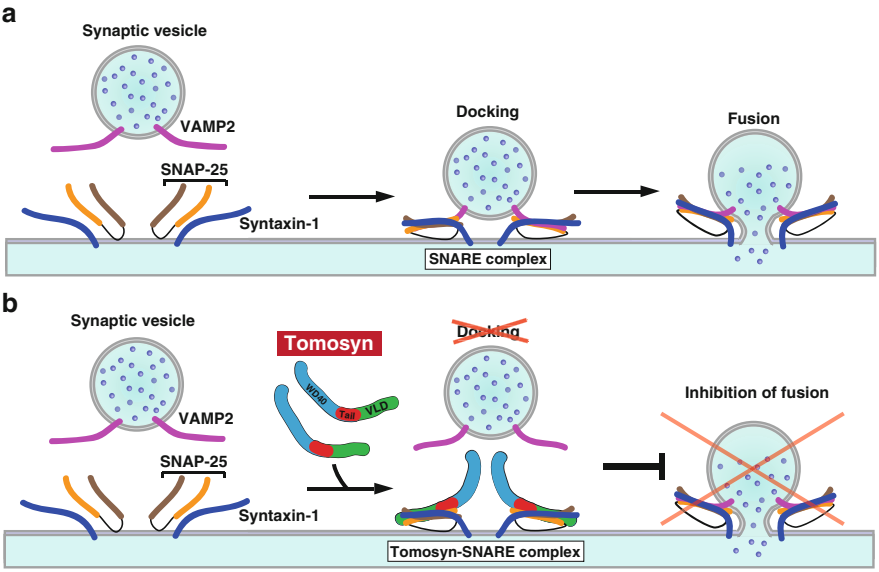

Fig. 5.2 Regulation of the SNARE complex formation by tomosyn. (**a**) Synaptic vesicle fusion driven by SNARE complexes. Syntaxin-1, SNAP-25, and VAMP2 form a ternary SNARE complex, allowing the synaptic vesicle to dock at the presynaptic membrane. Subsequently, the SNARE complexes catalyze membrane fusion in a zipper-like fashion. (**b**) Inhibition of the SNARE complex formation by tomosyn. Tomosyn binds to syntaxin-1 and SNAP-25 through the C-terminal VAMP-like domain (VLD), thereby forming a stable tomosyn-SNARE complex. The tomosyn-SNARE complex formation inhibits the SNARE complex formation by sequestering the t-SNAREs, leading to inhibition of membrane fusion

Yamamoto et al. 2009; Ashery et al. 2009). Consistent with the inhibitory activity on the SNARE complex formation, the genetic ablation of tomosyn in mice and *C. elegans* leads to enhancement of neurotransmitter release (Sakisaka et al. 2008; Gracheva et al. 2006; McEwen et al. 2006), and the overexpression in superior cervical ganglion (SCG) neurons inhibits neurotransmitter release induced by an action potential (Baba et al. 2005). Accumulating evidence suggests that tomosyn controls exocytotic efficacy of synaptic vesicles. Paired-pulse facilitation at mossy fiber synapses of hippocampi is decreased in the tomosyn-deficient mice (Sakisaka et al. 2008). In response to repetitive presynaptic action potentials, the tomosyn-overexpressing neurons show severe synaptic depression, in contrast to remarkable synaptic facilitation in control neurons (Baba et al. 2005). However, it is unclear how tomosyn controls the Ca^{2+}-dependent exocytosis, since the C-terminal VAMP-like domain sequesters the t-SNAREs in a Ca^{2+}-independent manner. We showed that tomosyn is directly phosphorylated by protein kinase A (PKA), which in turn reduces its interaction with syntaxin-1 and enhances the formation of the SNARE complex (Baba et al. 2005). In addition, Rho-associated serine/threonine kinase (ROCK) activated by Rho small G protein phosphorylates syntaxin-1, which in turn increases the affinity of syntaxin-1 for tomosyn and forms a stable complex with

tomosyn, resulting in inhibition of the formation of the SNARE complex during neurite extension (Sakisaka et al. 2004). Thus, the inhibitory activity of the C-terminal VAMP-like domain is regulated via the well-known signal transduction pathways.

5.4 Tomosyn Regulates SNARE Complex Formation Through the N-Terminal WD40 Repeats

The N-terminal WD40 repeats of tomosyn are also responsible for potent inhibition of neurotransmitter release. It has been demonstrated that catecholamine secretion is potently inhibited in chromaffin cells by overexpressing the N-terminal WD40 repeats (Yizhar et al. 2007). Intriguingly, the inhibitory activity of the N-terminal WD40 repeats in the chromaffin cells depends on Ca^{2+} concentration (Yizhar et al. 2004, 2007). We have also demonstrated that acetylcholine release from SCG neurons in long-term culture is potently inhibited by microinjecting the tomosyn fragment encompassing the N-terminal WD40 repeats (Sakisaka et al. 2008). Similarly to the inhibitory activity of the N-terminal WD40 repeats in the chromaffin cells, the inhibitory activity of tomosyn in the SCG neurons is influenced by Ca^{2+} concentration (Baba et al. 2005). In *C. elegans*, tomosyn associates with synaptic vesicles through the N-terminal WD40 repeats (McEwen et al. 2006), raising the possibility that the N-terminal WD40 repeats may negatively regulate the function of synaptic vesicle in a Ca^{2+}-dependent manner. While we have shown that tomosyn oligomerizes the SNARE complex through the N-terminal WD40 repeats (Sakisaka et al. 2008), this does not account for the Ca^{2+}-dependent inhibitory activity of the N-terminal WD40 repeats since the oligomerization takes place in a Ca^{2+}-independent manner. Therefore, the N-terminal WD40 repeats are expected to functionally interact with a Ca^{2+}-responsive protein(s) involved in the regulation of the synaptic vesicle fusion.

Indeed, we have demonstrated that tomosyn directly binds to synaptotagmin-1, a synaptic vesicle protein with two C_2 domains that both bind to Ca^{2+}, through the N-terminal WD40 repeats in a Ca^{2+}-dependent manner (Yamamoto et al. 2010a). Synaptotagmin-1 underlies Ca^{2+} responsiveness in the neurotransmitter release (Geppert et al. 1994; Sutton et al. 1995; Shao et al. 1998; Fukuda et al. 1999; Augustine 2001; Fernandez et al. 2001; Chapman 2008). Upon Ca^{2+} binding, synaptotagmin-1 induces positive curvature of the presynaptic membrane by inserting the hydrophobic loops in the C_2 domains into the presynaptic membrane, thereby catalyzing fast synaptic vesicle fusion in cooperation with the SNARE complex (Martens et al. 2007; Stein et al. 2007; Xue et al. 2008; Hui et al. 2009) (Fig. 5.3a). Importantly, the Ca^{2+}-dependent binding between tomosyn and synaptotagmin-1 impairs the synaptotagmin-1 catalysis (Yamamoto et al. 2010a) (Fig. 5.3b), indicating that tomosyn negatively regulates the synaptotagmin-1-mediated step of Ca^{2+}-dependent neurotransmitter release through the N-terminal WD40 repeats. Furthermore, the Ca^{2+}-dependent binding enhances the activity of the

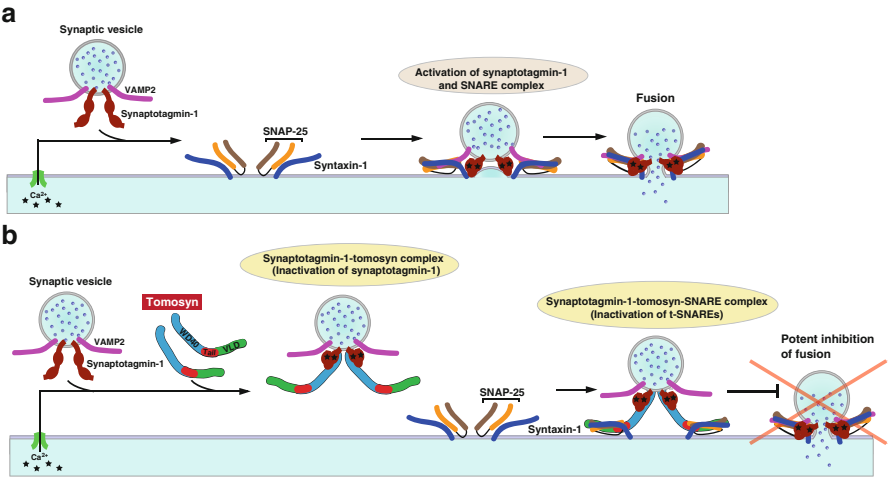

Fig. 5.3 Regulation of the Ca^{2+}-dependent synaptic vesicle fusion by tomosyn. (**a**) Synaptotagmin-1 is a Ca^{2+} sensor that cooperates with the SNARE complex toward membrane fusion. Upon Ca^{2+} influx, Ca^{2+} binds and activates synaptotagmin-1. Activated synaptotagmin-1 bends the presynaptic membrane, thereby facilitating the SNARE complex-driven membrane fusion. (**b**) Tomosyn acts as an alternative Ca^{2+} sensor that negatively regulates the synaptic vesicle fusion. Upon Ca^{2+} influx, tomosyn binds and inactivates Ca^{2+}-bound synaptotagmin-1 through the N-terminal WD40 repeats. The synaptotagmin-1 binding enhances sequestration of t-SNAREs by the C-terminal VAMP-like domain (VLD) of tomosyn. Eventually, membrane fusion is potently inhibited in a Ca^{2+}-dependent manner

C-terminal VAMP-like domain of tomosyn to sequester t-SNAREs (Yamamoto et al. 2010a) (Fig. 5.3b). These findings raise an attractive possibility that the interplay between tomosyn and synaptotagmin-1 underlies the inhibitory control of Ca^{2+}-dependent neurotransmitter release. In response to a rise in Ca^{2+} concentration, synaptotagmin-1 on the synaptic vesicle catches tomosyn and inactivates its own catalysis for membrane fusion. Simultaneously, the synaptotagmin-1-tomosyn complex enhances sequestering of t-SNAREs on the presynaptic membrane and blocks the SNARE assembly. Eventually, the Ca^{2+}-dependent synaptotagmin-1-tomosyn-SNARE complex formation will ensure inactivation of the fusion machineries on both the donor and target membranes and thereby inhibit priming of the synaptic vesicles. Synaptotagmin-1, cooperating with tomosyn on synaptic vesicles, would act as an alternative Ca^{2+} sensor to negatively control exocytotic efficacy of the synaptic vesicles, sensing Ca^{2+} concentration change near Ca^{2+} channel clusters.

5.5 The Tail Domain Regulates the Activity of Tomosyn

We have demonstrated that the tail domain of tomosyn acts as a regulatory domain for the C-terminal VAMP-like domain (Yamamoto et al. 2009, 2010b). The tail domain can directly bind to either the C-terminal VAMP-like domain or the

N-terminal WD40 repeats (Yamamoto et al. 2009). The binding of the tail domain to the C-terminal VAMP-like domain represses the activity of the C-terminal VAMP-like domain to inhibit the SNARE complex formation, which is restored by the binding of the tail domain to the N-terminal WD40 repeats (Yamamoto et al. 2009). Therefore, tomosyn will be in a state of equilibrium between two conformational states upon the tail domain binding. In one conformational state (state I), the tail domain binds to the N-terminal WD40 repeats, leading to exposure of the C-terminal VAMP-like domain. The exposed C-terminal VAMP-like domain efficiently forms the tomosyn-SNARE complex, resulting in the potent inhibition of the SNARE complex formation. In the other conformational state (state II), the tail domain masks the C-terminal VAMP-like domain and thereby blocks the tomosyn-SNARE complex formation (Yamamoto et al. 2009) or enables VAMP2 to displace tomosyn from the tomosyn-SNARE complex (Yamamoto et al. 2010b), resulting in loss of the inhibition of the SNARE complex formation. In support of the idea of equilibrium, full-length tomosyn moderately inhibited the SNARE-driven membrane fusion relative to the tomosyn fragment encompassing only the C-terminal VAMP-like domain in vitro (Yamamoto et al. 2010b), suggesting that full-length tomosyn exists in both states. Given that full-length tomosyn inhibited the SNARE-driven membrane fusion more than the tomosyn fragment encompassing both the tail domain and the C-terminal VAMP-like domain did in vitro (Yamamoto et al. 2010b), the state I may be a dominant state. What drives the conformational change from state I to state II? We previously reported that PKA phosphorylates tomosyn, resulting in reducing the binding of the C-terminal VAMP-like domain to syntaxin-1 (Baba et al. 2005). Therefore, PKA might be a possible regulator to drive the conformational change. The physiological meaning of the structural regulation of tomosyn upon the tail binding still remains elusive. We have generated tomosyn-deficient mice, characterized them electrophysiologically, and revealed that the tomosyn-deficient mice lacked short-term potentiation (Sakisaka et al. 2008). Therefore, the structural regulation of tomosyn may be important for the short-term memory. Further studies will be needed for understanding the regulation of the conformational change of tomosyn.

5.6 Tomosyn Regulates the Readily Releasable Pool Size

Evidence is accumulating that tomosyn regulates the readily releasable pool (RRP) size in response to repetitive presynaptic activity. Tomosyn-deficient mice show reduced paired-pulse facilitation in hippocampi (Sakisaka et al. 2008), suggesting that, without tomosyn, the first action potential depletes synaptic vesicles in the RRP. Tomosyn-overexpressing presynaptic SCG neurons evoke smaller excitatory postsynaptic potentials (EPSPs) but cannot respond to following repetitive action potentials, thus inducing severe synaptic depression (Baba et al. 2005). In addition,

synchronization for repeated transmitter release was lost under tomosyn loss-function by point mutations in the N-terminal WD40 repeats (Baba et al. 2005). The SCG neuron in culture forms synapses with many varicosities wrapping the cell soma (Baba et al. 2005; Ma et al. 2009); therefore, the RRP size is relatively large, 84–180 synaptic vesicles (Ma et al. 2009; unpublished data), calculated from the depletion of the RRP with a train of high-frequency action potentials. The averaged EPSP amplitude is ≈20 mV (Baba et al. 2005; Ma et al. 2009), suggesting that the number of releasable vesicles in response to an action potential is well controlled. From the averaged EPSP integral, we estimate that neurotransmitters are released from ≈50 synaptic vesicles in the GFP-overexpressing or non-transfected SCG neurons (Baba et al. 2005; Ma et al. 2009). However, only 14 synaptic vesicles in tomosyn-overexpressing neurons (unpublished data) and 12–20 synaptic vesicles in the tomosyn mutant–overexpressing neurons (Baba et al. 2005) can be exocytosed in response to an action potential. Therefore, tomosyn is a key molecule to determine the size of the RRP.

The size of the RRP in the central nervous system is extremely small (1–2 % of the total number of vesicles) (Schikorski and Stevens 2001; Sakaba et al. 2002; Rizzoli and Betz 2004). The vast majority of the synaptic vesicles are reserved at the presynaptic nerve terminals despite Ca^{2+} influx (Südhof 2000; Rizzoli and Betz 2005), and a subset of them is accordingly mobilized to prevent depletion of the RRP (Südhof 2000; Harata et al. 2001; Rizzoli and Betz 2005), resulting in maintaining of the RRP size. As mentioned above, tomosyn is the molecule that inactivates synaptotagmin-1 in response to Ca^{2+} influx and thereby perturbs the SNARE machinery activation (Yamamoto et al. 2010a) (Figs. 5.2b and 5.3b). Interestingly, the presynaptic interplay between tomosyn and synaptotagmin-1 controls the EPSP shape in the falling phase, enabling the neurons to respond to high-frequency action potentials (unpublished data). This result raises the possibility that the RRP size, i.e., exocytotic efficacy of release-ready synaptic vesicles, might be determined by the interaction between tomosyn and synaptotagmin-1 during arrivals of consecutive neuronal signals to the presynaptic terminal. The Ca^{2+}-dependent synaptotagmin-1-tomosyn-SNARE complex formation as depicted in Fig. 5.3b may ensure inactivation of the fusion machineries on both the donor and target membranes under high Ca^{2+} concentration accumulating with repetitive Ca^{2+} influxes to reserve the synaptic vesicles, leading to maintaining of the RRP size. By laser photolysis of caged calcium in a rat calyx of Held synapse, a rise in Ca^{2+} concentration to 1–2 μM readily evoked release (Bollmann et al. 2000; Felmy et al. 2003). Brief local Ca^{2+} rise to 10–25 μM is sufficient to achieve the amount and the kinetics of the physiological transmitter release (Schneggenburger and Neher 2005). An increase to >30 μM depleted the RRP in <0.5 ms (Bollmann et al. 2000). In addition, 40 μM is the peak concentration for synaptic vesicles at the release site during an action potential (half-width approximately 0.4 ms; Meinrenken et al. 2002). Therefore, in our scenario, the synaptotagmin-1-tomosyn-SNARE complex will be maximally formed in the high-range concentration (10–50 μM) of local Ca^{2+} rise that depletes the RRP (Meinrenken et al. 2002), in order to negatively control exocytotic efficacy of synaptic vesicle in a late phase

of transmitter release preventing the RRP depletion. Severe synaptic depression induced by repetitive action potentials in tomosyn-overexpressing neurons (Baba et al. 2005) supports that the synaptotagmin-1-tomosyn-SNARE complex strongly retains low efficacy of synaptic vesicles for exocytosis in the accumulated Ca^{2+} rise. By contrast, our in vitro Ca^{2+} titration analysis shows that the synaptotagmin-1-tomosyn-SNARE complex formation is not saturated at 10–50 μM and proceeds more as the Ca^{2+} concentration increases (unpublished data). However, this result does not debate on the physiological relevance of the synaptotagmin-1-tomosyn-SNARE complex formation. The in vitro biochemical reactions do not reconstitute the local Ca^{2+} rise as seen in neurons. It has been reported that a specific membrane lipid, PI(4,5)P2, increases Ca^{2+} affinity of synaptotagmin-1 (Radhakrishnan et al. 2009). Therefore, to address biochemically the precise dependency on Ca^{2+} concentration for the synaptotagmin-1-tomosyn-SNARE complex formation, it will be required to develop more physiologically relevant assay reflecting the local Ca^{2+} rise and the membranous environment.

5.7 Conclusions and Perspectives

The crystal structure of the N-terminal WD40 repeats of Sro7, the yeast orthologue of tomosyn, has been solved (Hattendorf et al. 2007). Based on the solved structure, Sro7 is suggested to bind to Sec9, a yeast counterpart of SNAP-25, through the N-terminal WD40 repeats and thereby inhibits the SNARE complex formation. While Sec9 binds to Sro7 through both the N-terminal region and the SNARE motifs, the N-terminal region of Sec9 is not conserved in mammalian SNAP-25 (Hattendorf et al. 2007). As far as we examined, the N-terminal WD40 repeats of tomosyn had no inhibitory activity on the SNARE complex formation. Therefore, the inhibitory activity of the N-terminal WD40 repeats of Sro7 on the SNARE complex formation may not be evolutionally conserved. On the other hand, the binding of tomosyn to synaptotagmin-1 is in good agreement with the association of tomosyn with the synaptic vesicles in *C. elegans* (McEwen et al. 2006), raising a possibility that the activity of the N-terminal WD40 repeats to inhibit the synaptotagmin-1 function might be evolutionally conserved between nematodes and mammals. The N-terminal WD40 repeats also have the activity to oligomerize the SNARE complex (Sakisaka et al. 2008). However, it remains to be elucidated whether the inhibitory activity on the synaptotagmin-1 function and the oligomerization activity on the SNARE complex are mutually exclusive or compatible. Tomosyn adopts two conformational states upon reciprocal intramolecular bindings of the tail domain (Yamamoto et al. 2009). In one conformational state where the tail domain binds to the N-terminal WD40 repeats, tomosyn potently inhibits the SNARE complex formation through the C-terminal VAMP-like domain. In the other conformational state where the tail domain binds to the C-terminal VAMP-like domain, the inhibitory activity of the C-terminal VAMP-like domain is decreased. The binding of synaptotagmin-1 to the N-terminal WD40 repeats

enhances the tomosyn-SNARE complex formation through the C-terminal VAMP-like domain (Yamamoto et al. 2010a). Therefore, synaptotagmin-1 binding may stabilize the former conformational state of tomosyn, leading to enhancement of the tomosyn-SNARE complex formation. Future structural studies of full-length tomosyn, the synaptotagmin-1-tomosyn complex, and the synaptotagmin-1-tomosyn-SNARE complex will be required to address these concerns.

References

Ashery U, Bielopolski N, Barak B, Yizhar O (2009) Friends and foes in synaptic transmission: the role of tomosyn in vesicle priming. Trends Neurosci 32:275–282

Augustine GJ (2001) How does calcium trigger neurotransmitter release? Curr Opin Neurobiol 11:320–326

Baba T, Sakisaka T, Mochida S, Takai Y (2005) PKA-catalyzed phosphorylation of tomosyn and its implication in Ca^{2+}-dependent exocytosis of neurotransmitter. J Cell Biol 170:1113–1125

Bennett MK, Calakos N, Scheller RH (1992) Syntaxin: a synaptic protein implicated in docking of synaptic vesicles at presynaptic active zones. Science 257:255–259

Bollmann JH, Sakmann B, Borst JG (2000) Calcium sensitivity of glutamate release in a calyx-type terminal. Science 289:953–957

Chapman ER (2008) How does synaptotagmin trigger neurotransmitter release? Annu Rev Biochem 77:615–641

Chen YA, Scheller RH (2001) SNARE-mediated membrane fusion. Nat Rev Mol Cell Biol 2:98–106

Felmy F, Neher E, Schneggenburger R (2003) The timing of phasic transmitter release is Ca^{2+}-dependent and lacks a direct influence of presynaptic membrane potential. Proc Natl Acad Sci U S A 100:15200–15205

Fernandez I, Araç D, Ubach J, Gerber SH, Shin O, Gao Y, Anderson RG, Südhof TC, Rizo J (2001) Three-dimensional structure of the synaptotagmin 1 C_2B-domain: synaptotagmin 1 as a phospholipid binding machine. Neuron 32:1057–1069

Fujita Y, Shirataki H, Sakisaka T, Asakura T, Ohya T, Kotani H, Yokoyama S, Nishioka H, Matsuura Y, Mizoguchi A, Scheller RH, Takai Y (1998) Tomosyn: a syntaxin-1-binding protein that forms a novel complex in the neurotransmitter release process. Neuron 20:905–915

Fukuda M, Kanno E, Mikoshiba K (1999) Conserved N-terminal cysteine motif is essential for homo- and heterodimer formation of synaptotagmins III, V, VI, and X. J Biol Chem 274:31421–31427

Gangar A, Rossi G, Andreeva A, Hales R, Brennwald P (2005) Structurally conserved interaction of Lgl family with SNAREs is critical to their cellular function. Curr Biol 15:1136–1142

Geppert M, Goda Y, Hammer RE, Li C, Rosahl TW, Stevens CF, Südhof TC (1994) Synaptotagmin I: a major Ca^{2+} sensor for transmitter release at a central synapse. Cell 79:717–727

Gracheva EO, Burdina AO, Holgado AM, Berthelot-Grosjean M, Ackley BD, Hadwiger G, Nonet ML, Weimer RM, Richmond JE (2006) Tomosyn negatively regulates CAPS-dependent peptide release at *Caenorhabditis elegans* synapses. PLoS Biol 4:e261

Groffen AJ, Jacobsen L, Schut D, Verhage M (2005) Two distinct genes drive expression of seven tomosyn isoforms in the mammalian brain, sharing a conserved structure with a unique variable domain. J Neurochem 92:554–568

Harata N, Pyle JL, Aravanis AM, Mozhayeva M, Kavalali ET, Tsien RW (2001) Limited numbers of recycling vesicles in small CNS nerve terminals: implications for neural signaling and vesicular cycling. Trends Neurosci 24:637–643

Hatsuzawa K, Lang T, Fasshauer D, Bruns D, Jahn R (2003) The R-SNARE motif of tomosyn forms SNARE core complexes with syntaxin 1 and SNAP-25 and down-regulates exocytosis. J Biol Chem 278:31159–31166

Hattendorf DA, Andreeva A, Gangar A, Brennwald PJ, Weis WI (2007) Structure of the yeast polarity protein Sro7 reveals a SNARE regulatory mechanism. Nature 446:567–571

Hui E, Johnson CP, Yao J, Dunning FM, Chapman ER (2009) Synaptotagmin-mediated bending of the target membrane is a critical step in Ca(2$^+$)-regulated fusion. Cell 138:709–721

Jahn R, Scheller RH (2006) SNAREs–engines for membrane fusion. Nat Rev Mol Cell Biol 7:631–643

Kagami M, Toh-e A, Matsui Y (1998) Sro7p, a *Saccharomyces cerevisiae* counterpart of the tumor suppressor l(2)gl protein, is related to myosins in function. Genetics 149:1717–1727

Kloepper TH, Kienle CN, Fasshauer D (2008) SNAREing the basis of multicellularity: consequences of protein family expansion during evolution. Mol Biol Evol 25:2055–2068

Lehman K, Rossi G, Adamo JE, Brennwald P (1999) Yeast homologues of tomosyn and lethal giant larvae function in exocytosis and are associated with the plasma membrane SNARE, Sec9. J Cell Biol 146:125–140

Ma H, Cai Q, Lu W, Sheng ZH, Mochida S (2009) KIF5B motor adaptor syntabulin maintains synaptic transmission in sympathetic neurons. J Neurosci 29:13019–13029

Martens S, Kozlov MM, McMahon HT (2007) How synaptotagmin promotes membrane fusion. Science 316:1205–1208

McEwen JM, Madison JM, Dybbs M, Kaplan JM (2006) Antagonistic regulation of synaptic vesicle priming by Tomosyn and UNC-13. Neuron 51:303–315

Meinrenken CJ, Borst JG, Sakmann B (2002) Calcium secretion coupling at calyx of held governed by nonuniform channel-vesicle topography. J Neurosci 22:1648–1667

Müsch A, Cohen D, Yeaman C, Nelson WJ, Rodriguez-Boulan E, Brennwald PJ (2002) Mammalian homolog of Drosophila tumor suppressor lethal (2) giant larvae interacts with basolateral exocytic machinery in Madin-Darby canine kidney cells. Mol Biol Cell 13:158–168

Pobbati AV, Razeto A, Boddener M, Becker S, Fasshauer D (2004) Structural basis for the inhibitory role of tomosyn in exocytosis. J Biol Chem 279:47192–47200

Radhakrishnan A, Stein A, Jahn R, Fasshauer D (2009) The Ca^{2+} affinity of synaptotagmin 1 is markedly increased by a specific interaction of its C2B domain with phosphatidylinositol 4,5-bisphosphate. J Biol Chem 284:25749–25760

Rizo J, Rosenmund C (2008) Synaptic vesicle fusion. Nat Struct Mol Biol 15:665–674

Rizzoli SO, Betz WJ (2004) The structural organization of the readily releasable pool of synaptic vesicles. Science 303:2037–2039

Rizzoli SO, Betz WJ (2005) Synaptic vesicle pools. Nat Rev Neurosci 6:57–69

Sakaba T, Schneggenburger R, Neher E (2002) Estimation of quantal parameters at the calyx of Held synapse. Neurosci Res 44:343–356

Sakisaka T, Baba T, Tanaka S, Izumi G, Yasumi M, Takai Y (2004) Regulation of SNAREs by tomosyn and ROCK: implication in extension and retraction of neurites. J Cell Biol 166:17–25

Sakisaka T, Yamamoto Y, Mochida S, Nakamura M, Nishikawa K, Ishizaki H, Okamoto-Tanaka-M, Miyoshi J, Fujiyoshi Y, Manabe T, Takai Y (2008) Dual inhibition of SNARE complex formation by tomosyn ensures controlled neurotransmitter release. J Cell Biol 183:323–337

Schikorski T, Stevens CF (2001) Morphological correlates of functionally defined synaptic vesicle populations. Nat Neurosci 4:391–395

Schneggenburger R, Neher E (2005) Presynaptic calcium and control of vesicle fusion. Curr Opin Neurobiol 15:266–274

Shao X, Fernandez I, Südhof TC, Rizo J (1998) Solution structures of the Ca^{2+}-free and Ca^{2+}-bound C_2A domain of synaptotagmin I: does Ca^{2+} induce a conformational change? Biochemistry 37:16106–16115

Söllner T, Bennett MK, Whiteheart SW, Scheller RH, Rothman JE (1993) A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. Cell 75:409–418

Stein A, Radhakrishnan A, Riedel D, Fasshauer D, Jahn R (2007) Synaptotagmin activates membrane fusion through a Ca^{2+}-dependent trans interaction with phospholipids. Nat Struct Mol Biol 14:904–911

Südhof TC (2000) The synaptic vesicle cycle revisited. Neuron 28:317–320

Südhof TC (2004) The synaptic vesicle cycle. Annu Rev Neurosci 27:509–547

Sutton RB, Davletov BA, Berghuis AM, Südhof TC, Sprang SR (1995) Structure of the first C_2 domain of synaptotagmin I: a novel Ca^{2+}/phospholipid-binding fold. Cell 80:929–938

Sutton RB, Fasshauer D, Jahn R, Brunger AT (1998) Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 Å resolution. Nature 395:347–353

Trimble WS, Cowan DM, Scheller RH (1988) VAMP-1: a synaptic vesicle-associated integral membrane protein. Proc Natl Acad Sci U S A 85:4538–4542

Weber T, Zemelman BV, McNew JA, Westermann B, Gmachl M, Parlati F, Söllner TH, Rothman JE (1998) SNAREpins: minimal machinery for membrane fusion. Cell 92:759–772

Wirtz-Peitz F, Knoblich JA (2006) Lethal giant larvae take on a life of their own. Trends Cell Biol 16:234–241

Xue M, Ma C, Craig TK, Rosenmund C, Rizo J (2008) The Janus-faced nature of the C_2B domain is fundamental for synaptotagmin-1 function. Nat Struct Mol Biol 15:1160–1168

Yamamoto Y, Mochida S, Kurooka T, Sakisaka T (2009) Reciprocal intramolecular interactions of tomosyn control its inhibitory activity on SNARE complex formation. J Biol Chem 284:12480–12490

Yamamoto Y, Mochida S, Miyazaki N, Kawai K, Fujikura K, Kurooka T, Iwasaki K, Sakisaka T (2010a) Tomosyn inhibits synaptotagmin-1-mediated step of Ca^{2+}-dependent neurotransmitter release through its N-terminal WD40 repeats. J Biol Chem 285:40943–40955

Yamamoto Y, Fujikura K, Sakaue M, Okimura K, Kobayashi Y, Nakamura T, Sakisaka T (2010b) The tail domain of tomosyn controls membrane fusion through tomosyn displacement by VAMP2. Biochem Biophys Res Commun 399:24–30

Yamanaka T, Ohno S (2008) Role of Lgl/Dlg/Scribble in the regulation of epithelial junction, polarity and growth. Front Biosci 13:6693–6707

Yizhar O, Matti U, Melamed R, Hagalili Y, Bruns D, Rettig J, Ashery U (2004) Tomosyn inhibits priming of large dense-core vesicles in a calcium-dependent manner. Proc Natl Acad Sci U S A 101:2578–2583

Yizhar O, Lipstein N, Gladycheva SE, Matti U, Ernst SA, Rettig J, Stuenkel EL, Ashery U (2007) Multiple functional domains are involved in tomosyn regulation of exocytosis. J Neurochem 103:604–616

Chapter 6
Regulation of SNAREs: Complexin

Hiroshi Tokumaru

Abstract Complexin (also called as synaphin), a small cytoplasmic protein, binds to SNAP receptor (SNARE) complex (membrane fusion machinery) and regulates neurotransmitter release at the post-priming stage. Several lines of evidence have demonstrated that complexin has at least two independent roles: clamping of the *trans*-SNARE complex and activation of Ca^{2+}-triggered synchronous release of neurotransmitter. Both functions require its central SNARE binding domain. Recent studies suggest that complexin contains several functional domains that either stimulate or inhibit neurotransmitter release. Thus, the function of complexin is likely more complex than expected from its small size.

Keywords Complexin • SNARE complex • Exocytosis • Synaptic vesicles • Synaptotagmin 1

6.1 Introduction

Action potential-evoked neurotransmitter release is triggered by Ca^{2+} influx through voltage-gated calcium channels at the active zone. The increase in Ca^{2+} concentration initiates rapid signaling cascades that lead to the exocytosis of synaptic vesicles. Two soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins from the presynaptic membrane, syntaxin-1 and SNAP25, and one SNARE protein from the synaptic vesicle membrane, VAMP2 (also known as synaptobrevin-2), form a four-helix bundle (called the *trans*-SNARE complex or SNAREpin) that catalyzes membrane fusion (Kasai et al. 2012). The synaptic vesicle protein synaptotagmin 1 serves as a major Ca^{2+} sensor for fast action potential-evoked synaptic vesicle exocytosis. The rapid interactions between synaptotagmin 1, the SNARE complex, and membrane phospholipids induced by Ca^{2+} are critical for membrane fusion.

The evoked neurotransmitter release is regulated by several cytosolic proteins including complexin. Complexin and its binding to the SNARE complex are critical

H. Tokumaru (✉)
Faculty of Pharmaceutical Sciences at Kagawa, Tokushima Bunri University, Kagawa 769-2193, Japan
e-mail: tokumaruh@kph.bunri-u.ac.jp

S. Mochida (ed.), *Presynaptic Terminals*, DOI 10.1007/978-4-431-55166-9_6

for fast neurotransmitter release. Microinjection of the SNARE binding site peptide of complexin into the squid presynaptic terminal almost completely abolished neurotransmitter release, demonstrating the requirement of complexin binding to SNARE complex for synaptic vesicle exocytosis (Tokumaru et al. 2001). Several lines of evidence have demonstrated that complexin has at least two independent roles: clamping of the *trans*-SNARE complex to prevent membrane fusion and activation of Ca^{2+}-triggered synchronous release of neurotransmitter.

6.2 Complexin Binds to the SNARE Complex

Complexins are soluble proteins (134 residues) identified as SNARE complex binding proteins (Ishizuka et al. 1995; Takahashi et al. 1995; McMahon et al. 1995). Four isoforms of complexin are present in mammals. Complexin-1 and complexin-2 are major brain isoforms. They are very similar (134 residues, 86 % identical), but their distributions are quite different (Ishizuka et al. 1999). Complexin-1 was predominantly expressed in the cerebral cortex (the IV cortical layer), thalamus, locus coeruleus, gigantocellular reticular field, cuneate nucleus, and cerebellar basket and stellate cells, whereas complexin-2 was predominant in the cerebral cortex (the II, III, and VI cortical layers), claustrum, hippocampal CA2 and CA3, granule cell layer of the dentate gyrus, entorhinal cortex, amygdaloid nuclei, substantia nigra pars compacta, superior colliculus, pontine reticulotegmental nucleus, and inferior olive. On the other hand, complexin-3 (158 residues) and complexin-4 (160 residues) are membrane-bound miner isoforms and are predominantly expressed in the retina (Reim et al. 2005). Complexin contains an α-helical domain subdivided into the accessory helix (residues 23–47) and the central helix (residues 48–70), flanked by N- and C-terminal unstructured regions (Chen et al. 2002; Bracher et al. 2002). The central α-helix mediates the interaction with the assembled SNARE complex. The crystal structure of the complexin/SNARE complex revealed that the central helix sits in the middle of the assembled SNARE complex, near the central zero-layer surface groove between VAMP2 and syntaxin-1 in an antiparallel fashion (Fig. 6.1).

6.3 Complexin Regulates Synchronous Ca^{2+}-Dependent Ultrafast Exocytosis

Complexin-1/2 double knockout or double knockdown mice show 60–70 % reduction of Ca^{2+}-triggered synchronous ultrafast release at hippocampal glutamatergic synapses (Reim et al. 2001; Maximov et al. 2009). Likewise, disruption of the only gene for complexin in *Drosophila* results in marked reduction of neurotransmitter release (Huntwork and Littleton 2007). Both complexin-1/2 double knockout and

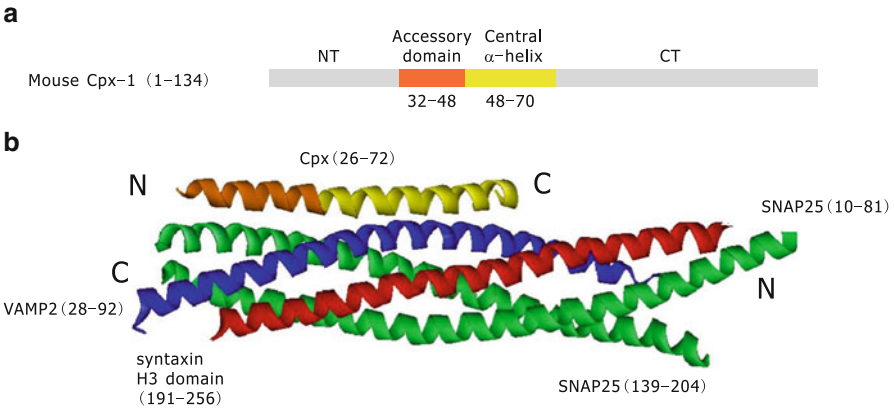

Fig. 6.1 (**a**) The primary structure of mouse complexin-1. NT, NH_2-terminal region; CT, COOH-terminal region. (**b**) The crystal structure of a SNARE complex and complexin (Cpx) (Adapted from Kasai et al. 2012)

double knockdown neurons can be rescued by overexpression of complexin-1 (Xue et al. 2007). However, complexin mutants that cannot bind to the SNARE complex do not restore synaptic activity. The central helix peptide (residues 46–74) competes with full-length complexin for SNARE complex, and microinjection of the peptide into the squid presynaptic terminal completely inhibits synaptic transmission (Tokumaru et al. 2001). Interestingly, the phenotype of complexin-1/2 double knockout mice is apparently similar to that of synaptotagmin 1-deficient mice (Geppert et al. 1994). However, unlike in synaptotagmin 1 double knockout mice, deficits in synchronous release in complexin-1/2 double knockout mice can be overcome by increased external Ca^{2+} concentration (12 mM) (Reim et al. 2001). Complexin does not bind Ca^{2+}. Therefore, complexin and synaptotagmin 1 likely play distinct roles closely associated with each other in synaptic vesicle exocytosis.

6.4 Complexin Clamps *trans*-SNARE Complex

The formation of the SNARE complex starts from the N-terminal of SNARE motifs, gradually proceeding toward the C-terminal. The resulting *trans*-SNARE complex would then bring the membranes gradually into close apposition. If the SNARE complex is a universal fusion machinery for both regulated and constitutive exocytosis, a fusion clamp molecule would be necessary to prevent zippering of the SNARE complex, which automatically causes membrane fusion. The fusion clamp model predicts that an abundance of complexins may inhibit fusion activities. As expected, overexpression of complexin-1/2 inhibits acetylcholine and dopamine release from PC12 cells (Itakura et al. 1999). Likewise, excess amount of complexin decreases insulin secretion from cultured insulin-secreting cell lines

(Abderrahmani et al. 2004) and inhibits liposome fusion (Schaub et al. 2006) and fusion of intact cells mediated by flipped SNAREs (Giraudo et al. 2006). In addition, the complexin-1/2 double knockdown in cultured mouse cortical neurons increases 3–4-fold the frequency of spontaneous neurotransmitter release (synaptic vesicle exocytosis) (Maximov et al. 2009). These results have led to the suggestion of a clamping role of complexin to prevent the full zippering of the *trans*-SNARE complex (complexin clamping model) (Giraudo et al. 2006; Schaub et al. 2006; Tang et al. 2006). Complexin binds to the partially assembled *trans*-SNARE complex through its central helix. Then, the N-terminal accessory helix (residues 26–42) may form an alternative four-helix bundle with syntaxin/SNAP25 instead of VAMP2 C-terminus near the membrane to prevent full zippering (Giraudo et al. 2009). Biochemical studies predict that the complexin clamp would be competitively relieved by Ca^{2+}-bound synaptotagmin 1 (Tang et al. 2006). When Ca^{2+} signal arrives, synaptotagmin 1 relieves the clamp by competing or cooperating with complexin, thereby inducing fast Ca^{2+}-evoked fusion of synaptic vesicle and plasma membrane (complexin-synaptotagmin 1 switch model).

However, both the complexin clamping and the complexin-synaptotagmin 1 switch models remain speculative, and several pieces of data are incompatible with these models. First, deletion of clamp molecules, complexin-1/2, does not enhance neurotransmitter release (Reim et al. 2001). Second, overexpression of complexin (>10-fold) does not inhibit glutamate release from cultured hippocampal neurons (Xue et al. 2007). Third, complexin stimulates SNARE-mediated liposome fusion (Malsam et al. 2009). This stimulatory effect required a preincubation at low temperature. Fourth, immunoprecipitation studies reveal that synaptotagmin 1 and complexin coexist in SNARE complex (Tokumaru et al. 2008). Finally, complexin dissociates orders of magnitude too slowly to be displaced from the SNARE complex after Ca^{2+} enters the nerve terminal. The complexin-synaptotagmin 1 switch model requires that Ca^{2+}-bound synaptotagmin 1 displaces complexin from the SNARE complex within 1 ms (Llinas et al. 1981; Augustine et al. 1985) between the time that Ca^{2+} enters the presynaptic terminal and the neurotransmitter is released. The kinetic analyses show that complexin binds to the SNARE complex rapidly and dissociates slowly (Pabst et al. 2002; Li et al. 2007). Two studies estimate the dissociation constant of complexin/SNARE complex to be 0.31 and 2.5 s^{-1}. These values indicate that the time required for unclamping with Ca^{2+}-synaptotagmin 1 is approximately 3.2–4 s.

A recent study shows a compensatory increase in complexin-3/4 mRNA levels in complexin-1/2 double knockdown neurons (Yang et al. 2013). Further, overexpression of membrane-bound complexin-3 increased spontaneous exocytosis. Thus, the increased spontaneous release in complexin-1/2 double knockdown neuron could simply result from increased complexin-3. However, this hypothesis is not compatible with the finding that overexpression of mouse complexin-3 or complexin-4 rescues the phenotype of complexin-1/2 double knockout hippocampal neurons (Reim et al. 2005).

6.5 Complexin Enhances Synaptic Vesicle Exocytosis

As described above, the central helix peptide (residues 46–74) has a SNARE complex binding activity similar to full-length complexin. Presynaptically microinjected, the central helix peptide nevertheless inhibits neurotransmitter release (Tokumaru et al. 2001). Thus, N- (amino acids 1–45) and/or C-terminal (amino acids 75–134) regions of complexin very likely mediate functions other than SNARE complex binding. In chromaffin cells, N-terminal domain (residues 1–27) of complexin-2 accelerates fusion of primed vesicle, whereas C-terminal domain (residues 74–134) prevents premature exocytosis (Dhara et al. 2014). The function of N-terminal domain and C-terminal domain in neurons is controversial. Reduced synchronous neurotransmitter release (synaptic vesicle exocytosis) in complexin-1/2 double knockout neurons is rescued by overexpression of complexin, but not with the complexin mutant lacking the N-terminal part ($Cpx_{27–134}$), suggesting that the extreme N-terminal domain (residues 1–26) is effective for recovering synaptic vesicle exocytosis (Xue et al. 2007). Interestingly, the region (residues 29–47) adjacent to the extreme N-terminal domain inhibits synaptic vesicle exocytosis (Xue et al. 2007). There is also a report that C-terminal part of the complexin contributes to the stimulation of neurotransmitter release. Malsam et al. (2009) demonstrated that stimulation of liposome fusion is no longer seen when the C-terminal domain of complexin is deleted ($Cpx_{1–77}$), or serine 115 is mutated. In this case, N-terminal portion (residues 1–75) of complexin seems to work inhibitory in liposome fusion. The N-terminal lacking mutants ($Cpx_{27–134}$ and $Cpx_{41–134}$) promote liposome fusion more strongly than wild-type complexin.

We found that complexin directly associates with synaptotagmin 1, a major calcium sensor for ultrafast neurotransmitter release, through its C-terminal region (Tokumaru et al. 2008). The binding is Ca^{2+}-independent and is enhanced in the presence of the SNARE complex. Because synaptotagmin 1 does not bind to the SNARE complex in the absence of Ca^{2+} (Lai et al. 2011; Zhou et al. 2013), it is likely that complexin recruits synaptotagmin 1 to *trans*-SNARE complex before Ca^{2+} influx (Fig. 6.2).

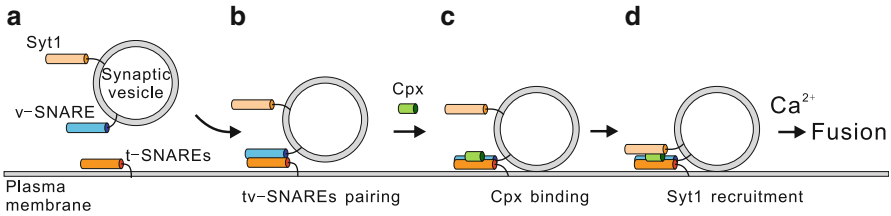

Fig. 6.2 Model for complexin function. The assembly of the SNARE complex between v-SNARE (VAMP2) and t-SNAREs (syntaxin 1/SNAP25) (**a** and **b**) is followed by rapid binding of complexin to the complex (see Pabst et al. 2002; Li et al. 2007) (**c**). Then synaptotagmin 1 is recruited to the complex of complexin/SNAREs (**d**). The complexin/SNAREs/synaptotagmin 1 complex awaits Ca^{2+} influx to trigger membrane fusion (Adapted from Tokumaru et al. 2008)

6.6 Conclusion

A large body of evidence suggests that complexin is important for Ca^{2+}-evoked SNARE-mediated membrane fusion, having at least two independent roles: clamping of the *trans*-SNARE complex and activation of Ca^{2+}-triggered synchronous release of neurotransmitter. Both functions require its central SNARE binding domain. Recent studies suggest that complexin contains several functional domains that either stimulate or inhibit neurotransmitter release. It is still not clear how complexin works. As described in Sect. 6.5, similar experiments produce opposite results. The difference may be due to the concentration of complexin used in each study. Higher concentration of complexin may produce nonphysiological interactions. If SNARE complex has nonphysiological, low-affinity binding site for complexin near the synaptotagmin 1 binding site, higher concentrations of complexin may inhibit synaptotagmin 1 binding to the SNARE complex.

References

Abderrahmani A, Niederhauser G, Plaisance V et al (2004) Complexin I regulates glucose-induced secretion in pancreatic beta-cells. J Cell Sci 117:2239–2247

Augustine GJ, Charlton MP, Smith SJ (1985) Calcium entry and transmitter release at voltage-clamped nerve terminals of squid. J Physiol 367:163–181

Bracher A, Kadlec J, Betz H, Weissenhorn W (2002) X-ray structure of a neuronal complexin-SNARE complex from squid. J Biol Chem 277:26517–26523

Chen X, Tomchick DR, Kovrigin E et al (2002) Three-dimensional structure of the complexin/SNARE complex. Neuron 33:397–409

Dhara M, Yarzagaray A, Schwarz Y et al (2014) Complexin synchronizes primed vesicle exocytosis and regulates fusion pore dynamics. J Cell Biol 204:1123–1140

Geppert M, Goda Y, Hammer RE et al (1994) Synaptotagmin I: a major Ca^{2+} sensor for transmitter release at a central synapse. Cell 79:717–727

Giraudo CG, Eng WS, Melia TJ, Rothman JE (2006) A clamping mechanism involved in SNARE-dependent exocytosis. Science 313:676–680

Giraudo CG, Garcia-Diaz A, Eng WS (2009) Alternative zippering as an on-off switch for SNARE-mediated fusion. Science 323:512–516

Huntwork S, Littleton JT (2007) A complexin fusion clamp regulates spontaneous neurotransmitter release and synaptic growth. Nat Neurosci 10:1235–1237

Ishizuka T, Saisu H, Odani S, Abe T (1995) Synaphin: a protein associated with the docking/fusion complex in presynaptic terminals. Biochem Biophys Res Commun 213:1107–1114

Ishizuka T, Saisu H, Odani S et al (1999) Distinct regional distribution in the brain of messenger RNAs for the two isoforms of synaphin associated with the docking/fusion complex. Neuroscience 88:295–306

Itakura M, Misawa H, Sekiguchi M et al (1999) Transfection analysis of functional roles of complexin I and II in the exocytosis of two different types of secretory vesicles. Biochem Biophys Res Commun 265:691–696

Kasai H, Takahashi N, Tokumaru H (2012) Distinct initial SNARE configurations underlying the diversity of exocytosis. Physiol Rev 92:1915–1964

Lai AL, Huang H, Herrick DZ et al (2011) Synaptotagmin 1 and SNAREs form a complex that is structurally heterogeneous. J Mol Biol 405:696–706

Li Y, Augustine GJ, Weninger K (2007) Kinetics of complexin binding to the SNARE complex: correcting single molecule FRET measurements for hidden events. Biophys J 93:2178–2187

Llinas R, Steinberg IZ, Walton K (1981) Relationship between presynaptic calcium current and postsynaptic potential in squid giant synapse. Biophys J 33:323–351

Malsam J, Seiler F, Schollmeier Y et al (2009) The carboxy-terminal domain of complexin I stimulates liposome fusion. Proc Natl Acad Sci U S A 106:2001–2006

Maximov A, Tang J, Yang X et al (2009) Complexin controls the force transfer from SNARE complexes to membranes in fusion. Science 323:516–521

McMahon HT, Missler M, Li C, Sudhof TC (1995) Complexins: cytosolic proteins that regulate SNAP receptor function. Cell 83:111–119

Pabst S, Margittai M, Vainius D et al (2002) Rapid and selective binding to the synaptic SNARE complex suggests a modulatory role of complexins in neuroexocytosis. J Biol Chem 277:7838–7848

Reim K, Mansour M, Varoqueaux F et al (2001) Complexins regulate a late step in Ca^{2+}-dependent neurotransmitter release. Cell 104:71–81

Reim K, Wegmeyer H, Brandstatter JH et al (2005) Structurally and functionally unique complexins at retinal ribbon synapses. J Cell Biol 169:669–680

Schaub JR, Lu X, Doneske B et al (2006) Hemifusion arrest by complexion is relieved by Ca^{2+}-Synaptotagmin I. Nat Struct Mol Biol 13:748–750

Takahashi S, Yamamoto H, Matsuda Z (1995) Identification of two highly homologous presynaptic proteins distinctly localized at the dendritic and somatic synapses. FEBS Lett 368:455–460

Tang J, Maximov A, Shin OH et al (2006) A complexin/synaptotagmin 1 switch controls fast synaptic vesicle exocytosis. Cell 126:1175–1187

Tokumaru H, Umayahara K, Pellegrini LL et al (2001) SNARE complex oligomerization by synaphin/complexin is essential for synaptic vesicle exocytosis. Cell 104:421–432

Tokumaru H, Shimizu-Okabe C, Abe T (2008) Direct interaction of SNARE complex binding protein synaphin/complexin with calcium sensor synaptotagmin 1. Brain Cell Biol 36:173–189

Xue M, Reim K, Chen X et al (2007) Distinct domains of complexin I differentially regulate neurotransmitter release. Nat Struct Mol Biol 14:949–958

Yang X, Cao P, Südhof TC (2013) Deconstructing complexin function in activating and clamping Ca^{2+}-triggered exocytosis by comparing knockout and knockdown phenotypes. Proc Natl Acad Sci U S A 110:20777–20782

Zhou A, Brewer KD, Rizo J (2013) Analysis of SNARE complex/synaptotagmin-1 interactions by one-dimensional NMR spectroscopy. Biochemistry 52:3446–3456

Chapter 7
The Synaptic Vesicle V-ATPase: A Regulatory Link Between Loading and Fusion?

Oussama El Far and Michael Seagar

Abstract The vacuolar proton pump (V-ATPase) is a huge multi-subunit complex composed of two distinct non-covalently associated sectors. The cytosolic V1 sector hydrolyses ATP, providing the energy for the V0 membrane sector to translocate protons into the vesicle lumen. The proton gradient is then used by vesicular transporters to load synaptic vesicles with specific neurotransmitters. The primary role of the V-ATPase in vesicle loading is widely accepted. However, multiple studies in a variety of model organisms point to an additional general role of the V0 sector in downstream events, notably in regulating SNARE-mediated membrane fusion. This chapter outlines the molecular pharmacology of the V-ATPase and its role in the synaptic vesicle cycle. It then focuses specifically on molecular interactions between V0 subunits and synaptic vesicle trafficking proteins and reviews their relevance to late steps in neurotransmitter release. While this secondary role for the V-ATPase membrane sector is not yet fully established, we speculate that it could provide a regulatory link between vesicle filling and fusion, acting as a filter that allows loaded vesicles to engage the fusion machinery.

Keywords V-ATPase • SNARE proteins • Membrane fusion • Neurotransmitter release • Proton pump

7.1 Introduction

Early observations (Del Castillo and Katz 1954; Heuser et al. 1974; Peper et al. 1974) established the quantal theory, whereby neurotransmitters are released in discrete packets, and proposed synaptic vesicle content to be the functional unit

O. El Far (✉)
Neurobiologie des canaux Ioniques et de la Synapse (UNIS),
INSERM, UMR_S 1072, Marseille 13015, France
e-mail: oussama.el-far@inserm.fr

M. Seagar
Neurobiologie des canaux Ioniques et de la Synapse (UNIS),
Aix-Marseille Université, Marseille 13015, France

S. Mochida (ed.), *Presynaptic Terminals*, DOI 10.1007/978-4-431-55166-9_7

of neurotransmission. Nerve endings contain synaptic vesicles, spherical organelles of 30–40 nm in diameter, that fuse with the plasmalemma in response to an increase in intra-terminal calcium concentration, releasing neurotransmitters into the synaptic cleft. Vesicles dock with the presynaptic plasma membrane and acquire precise molecular characteristics that render them responsive to sudden local increase in calcium concentration (Neher and Sakaba 2008), the amplitude of which is determined both by the distance that separates the calcium channel from the releasable vesicle and the calcium-buffering properties of the cytoplasm. Membrane fusion is an extremely rapid process, leading to merging of the synaptic vesicle and plasma membranes and exocytosis of the luminal contents into the synaptic cleft.

An average synaptic vesicle contains 1–2 copies of the vacuolar proton pump (V-ATPase). The V-ATPase consumes ATP to pump protons into the synaptic vesicle lumen. Specific vesicular transporters then use the proton gradient to load vesicles with different neurotransmitters (glutamate, acetylcholine, GABA, glycine, etc.). Following vesicle filling, findings from a variety of model systems suggest that the V0 membrane sector plays an additional role at late steps in exocytosis, notably by interacting with SNAREs. Thus, the V-ATPase potentially provides a regulatory link between loading and exocytosis that is common to all synaptic vesicles, in spite of the diversity of their contents. In this chapter, we will highlight studies to investigate the implication of the membrane sector (V0) of the V-ATPase in SNARE-dependent neurotransmitter release.

7.2 V-ATPase: Molecular Structure and Pharmacology of a Vesicular Proton Pump

V-ATPase is a specialised 900 kDa enzyme complex (Fig. 7.1), containing at least 14 different subunits, some of which are present as multiple copies. Its primary function is to translocate protons and acidify intracellular compartments, using a rotatory mechanism driven by ATP hydrolysis. The V-ATPase complex is made of two distinct sectors V0 and V1. The former is a membrane-embedded 260 kDa oligomer, which constitutes a discontinuous transmembrane pathway for proton transport. It is reversibly associated with a 650 kDa cytosolic V1 sector that carries the ATPase activity (Nishi and Forgac 2002). Electron microscopy studies show V0 has a flexible hexameric doughnut-like structure, (Wilkens et al. 1999; Clare et al. 2006; Wilkens and Forgac 2001), associated with a spherical extramembranous V1 sector (Wilkens et al. 1999). As this chapter will focus on the role of the V0 sector in vesicle trafficking and fusion, we will briefly outline the molecular properties of the V0 subunits.

V-ATPase subunits (Fig. 7.1a) are conventionally designated by ATP6V1 or ATP6V0 followed by letters, using the upper case for V1 (*A–H*) and the lower case

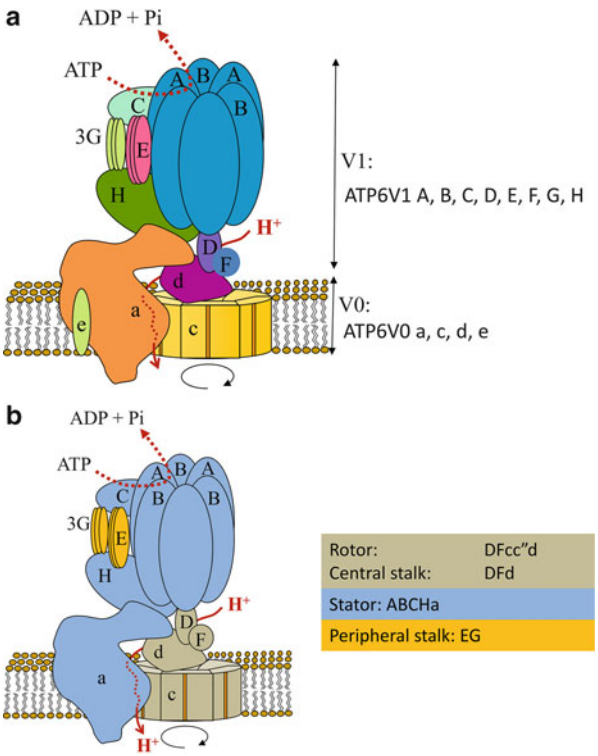

Fig. 7.1 Subunit structure of V-ATPase. (**a**) Schematic representation of V-ATPase subunit structure. The V-ATPase is composed of two reversibly attached sectors, ATP6V1 (extramembranous) and ATP6V0 (transmembranous). (**b**) Schematic representation of V-ATPase functional subdomains. The stator (*blue*) ATP6V1ABCHV0a, the rotor ATP6V1DFV0cc″d and central stalk ATP6V1DFV0d (*grey*) and the peripheral stalk ATP6V1EG (*yellow*). ATP hydrolysis drives rotation of the rotor relative to the stator. The rotation of the c-ring relative to a subunit translocates protons through discontinuous channels from the cytosol into the synaptic vesicle lumen without opening a continuous transmembrane pore

for V0 (a, c, d, e). In higher eukaryotes the V0 sector contains five distinct subunit types a, c, c″, d and e subunits, plus accessory proteins.

V0a subunits (100 kDa) contain a hydrophilic cytosolic N-terminal domain and a C-terminal domain with multiple transmembrane regions (TMR). The membrane topology of the a subunit is still controversial, although recent studies in yeast strongly indicate 8 TMRs. Vertebrate V0a subunits (a1–a4) are coded by four genes. Four a1 splice variants have been identified, and the a1-I form is specifically targeted to nerve terminals (Morel et al. 2003).

V0c (4 TMR, 16 kDa) and c″ subunits (5 TMR, 23 kDa), sometimes called proteolipids, are exceptionally hydrophobic proteins with high sequence similarity that assemble into an oligomeric ring structure containing five copies of c and one copy of c″. Proton transport across the membrane occurs at the interface between transmembrane helices of the a and c subunits. c-subunit helices also constitute the

binding site for the V-ATPase inhibitors bafilomycin and concanamycin (Bowman et al. 2006).

V0d subunit is a soluble 38 kDa protein, which has two isoforms in mammals (d1 and d2), although only the d1 isoform is found in the brain (Nishi et al. 2003). It binds peripherally to the cytoplasmic side of the c-subunit ring and participates in forming the central rotational stalk connecting V1 to the c-subunit ring. This subunit was independently identified as a binding partner for the synaptic vesicle membrane protein synaptophysin and designated physophyllin (Thomas and Betz 1990).

Mammalian V0 may also contain peripheral e1 or e2 subunit isoforms and the accessory proteins Ac45 and M8-9. However, the stoichiometry of these subunits remains to be clarified.

Five principal classes of mammalian V-ATPase inhibitors have been described: plecomacrolide antibiotics, archazolids, benzolactone enamides, indolyls and the so-called late-generation inhibitors (Perez-Sayans et al. 2009). Plecomacrolide antibiotics, mainly bafilomycin and concanamycin, are the most frequently used and best characterised inhibitors. They are active at nanomolar concentrations, bind to the V0c subunit and disrupt proton pumping by inhibiting rotation of transmembrane helices relative to each other in the c-subunit ring.

7.3 The Synaptic Vesicle Cycle: Loading, Release Modes and SNARE Proteins

The classical view of the nerve terminal describes three functionally distinct synaptic vesicle pools (Rizzoli and Betz 2005). (1) The readily releasable pool (≈1 %) corresponds to a few loaded, docked and primed vesicles that await only Ca^{2+} influx to trigger exocytosis of their contents. (2) The recycling pool (10–15 %), under stimulation at physiological frequencies, undergoes continuous cycles of docking, fusion, endocytosis and loading. (3) Finally the reserve pool accounts for 80–90 % of the total population and corresponds to vesicles that only respond to intense stimulation. This pool is probably mobilised when the recycling pool is depleted.

Vesicles in the recycling pool are loaded by concerted action of the V-ATPase and vesicular neurotransmitter transporters (see following Sect. 7.4). Their entry into the readily releasable pool involves SNARE interactions at the vesicle/plasma membrane interface. Briefly, syntaxin 1 and SNAP-25 in the plasma membrane bind to VAMP2 (synaptobrevin) in the vesicle membrane. Trimeric SNARE complexes in a trans configuration constitute a minimal fusion machine, in which helical zippering overcomes lipid repulsion, pulling opposing membranes into close proximity. This is thought to result in hemifusion (Hernandez et al. 2012; Schaub et al. 2006), in which the outer leaflet of the vesicle has merged with the inner leaflet of the plasma membrane. Hence the lumen of a readily releasable

vesicle is thought to be separated from the synaptic cleft by only a single lipid bilayer. However, recent reports indicate that Ca^{2+}-triggered fusion events must start from point contacts that are not hemifused (Diao et al. 2013) and that local bilayer protrusions from t-SNARE vesicles facing v-SNARE vesicles have an increased membrane curvature that might reduce the kinetic barrier to fusion (Bharat et al. 2014). Throughout these events SNARE interactions are chaperoned, stabilised and regulated by a variety of accessory SNARE-binding proteins (see Chap. 4 by Ira Milosevic and Jakob Balslev Sørensen).

Experiments in vitro with pure recombinant proteins in a controlled lipid environment have furthered our understanding of SNARE function. Upon reconstitution into artificial vesicles, trans-assembly of v-SNAREs and t-SNAREs triggers membrane fusion (Di Giovanni et al. 2010b; Karatekin et al. 2010; Weber et al. 1998). Furthermore when SNARE proteins are expressed on the cell surface (Giraudo et al. 2005), they induce intercellular fusion. Similar to synaptic vesicle fusion with the plasma membrane, in vitro SNARE-mediated fusion can be modulated by different SNARE-interacting proteins such as complexin, Munc13 and Munc18 or calcium sensors such as synaptotagmin and calmodulin (Di Giovanni et al. 2010b; Ma et al. 2013; Schaub et al. 2006; Tucker et al. 2004).

Complexins (also called synaphins) are a family of small soluble acidic proteins. Complexin I and II are mainly neuronal and III and IV are retina specific (Reim et al. 2005). They inhibit full SNARE assembly by binding partially assembled trimeric SNARE complexes (Chen et al. 2002; Reim et al. 2001; Tokumaru et al. 2001) and are involved in controlling neurotransmitter release (Huntwork and Littleton 2007; Maximov et al. 2009; Tokumaru et al. 2001; Xue et al. 2007, 2010). The C-terminal domains of complexins I and II have lipid-binding properties (Diao et al. 2013; Malsam et al. 2009; Wragg et al. 2013) and directly bind synaptotagmin 1 in a calcium-enhanced manner (Tokumaru et al. 2008). Although synaptotagmin can directly bind to the assembled SNARE complex, independently from its interactions with complexin (Davis et al. 1999; Tokumaru et al. 2008), complexin recruits Ca^{2+}/synaptotagmin through its C-terminal domain to trigger membrane fusion (Tokumaru et al. 2008). This interaction is thought to release complexin-induced inhibition after Ca^{2+} entry (Schaub et al. 2006; Tang et al. 2006).

In the recent years a complex network of dynamic protein interactions at the active zone, which control different steps in the synaptic vesicle cycle, has been elucidated. These findings, which will be described in detail elsewhere (see Chap. 4 by Ira Milosevic and Jakob Balslev Sørensen), have led to a profound breakthrough in the understanding of synaptic vesicle docking and maturation, as well as the calcium dependency of exocytosis and endocytosis. However, the precise molecular events that result in membrane fusion are less clear.

Synaptic vesicle contents initially escape via a channel-like structure or fusion pore, which perforates the lipid diaphragm separating the vesicle lumen from the surface of the nerve terminal. Using SNARE proteins in opposing lipid membranes, coarse-grained molecular dynamic simulations showed that splayed lipids represent the main barrier in membrane fusion and the trans SNARE complex probably plays a crucial role in the consecutive steps from membrane contact to fusion pore

expansion (Risselada and Grubmüller 2012). Although the implication of SNARE proteins in membrane fusion was part of the discoveries rewarded by the 2013 Nobel Prize in Physiology or Medicine, the detailed molecular processes that take place during the final step in synaptic vesicle/plasma membrane fusion remain enigmatic. Functionally distinct SNARE-mediated release modes have been described, although the molecular mechanisms that underlie transitions between these different release modes are unknown. A "full fusion" mode leading to a complete collapse of the synaptic vesicle membrane into the nerve terminal plasma membrane contrasts with the so-called kiss and run fusion mode in which synaptic vesicles partially release their contents through a fusion pore that can open and close. In order to account for the reversal of the fusion pore opening, it has been suggested that a protein-based scaffold might inhibit its expansion or even constitute a fusion pore gating mechanism (Almers and Tse 1990; Chernomordik and Kozlov 2008). In 2001, Peters et al. suggested that the V0 sector of the V-ATPase could play this kind of role in yeast vacuole fusion. As basic membrane fusion events are conserved from yeast to man and ruled by similar molecular mechanisms (Ferro-Novick and Jahn 1994), this idea has been extrapolated to synaptic vesicle fusion (Morel et al. 2001). However, the conceptual leap from yeast to the nerve terminal must also account for numerous specialisations, including transitions between different release modes, spontaneous and asynchronous release as well as the extreme rapidity of synchronous synaptic release.

7.4 V-ATPase and Vesicular Transporters in Vesicle Loading

The eukaryotic V-ATPase is located on intracellular membrane compartments, including synaptic vesicles, where it couples ATP hydrolysis to proton pumping into the lumen. However, the V-ATPase can also be expressed either constitutively (Breton et al. 1996; Schlesinger et al. 1997; Wagner et al. 2004) or in a transient manner (Zhang et al. 2010) at the plasma membrane of certain specialised cells where it contributes to extracellular acidification (Breton et al. 1996; Schlesinger et al. 1997; Wagner et al. 2004) and intracellular alkalinisation (Zhang et al. 2010).

As well as the two reversibly associated V1 and V0 sectors, the V-ATPase can be divided into functional subdomains (Fig. 7.1b). A V1DFV0cc″d complex constitutes the rotor: comprised of the c-ring (V0cc″) and the central stalk (V1DFV0d). The rotor is surrounded by the stator: comprised of the catalytic V1AB hexamer and the collar (V1CHV0a). The catalytic hexamer and the collar are connected by peripheral stalk V1EG complexes. Hydrolysis of ATP by the V1AB hexamer drives rotation of the rotor relative to stator. Rotation of the V0c-ring relative to V0a subunit translocates protons at their interface, from the cytosol into the synaptic vesicle lumen. Exactly how protons transit the membrane is not known. However, the dominant hypothesis postulates two offset

hemi-channels. The first hemi-channel delivers protons from the cytosol to a conserved glutamate residue located in c-subunit TMR 4 at the periphery of the c-ring, the second from the c-ring to the lumen. Thus transport is achieved without opening a continuous transmembrane pore.

Proton influx acidifies intracellular compartments (pH 5.2–5.5). Furthermore the proton gradient also actively participates in generating synaptic vesicle membrane potential ($\Delta\Psi$). Specific neurotransmitter transporters in the vesicle membrane use either $\Delta\Psi$ for glutamate loading or proton cotransport for GABA, acetylcholine and monoamine uptake (Liu and Edwards 1997; Maycox et al. 1990; Moriyama et al. 1992; Nelson and Lill 1994). Acidification by V-ATPase is an important factor in vesicular trafficking and the dynamics of cellular compartments. It regulates membrane protein sorting (Nishi and Forgac 2002) (Marshansky and Futai 2008) and endo-membranous degradation and is involved in autophagy (Williamson and Hiesinger 2010; Williamson et al. 2010).

Reversible dissociation of V1 and V0 sectors arrests proton transport and is a physiological process used to regulate ATPase activity and luminal acidification (Kane 1995; Qi et al. 2007; Sumner et al. 1995). The dissociation of sectors is very sensitive to cellular environment and occurs principally in response to nutrient depletion, probably via a pH-dependent mechanism (Qi et al. 2007). The subunit composition of the V1 sector, namely, V1A isoforms and V1C (Perez-Sayans et al. 2012), determines coupling efficiency and therefore regulates proton transport. The factors that regulate assembly/disassembly have been mainly studied in the lower eukaryotes and in yeast, the a subunit (Vph1 and Stv1) plays a crucial role. Disassembly involves microtubules while assembly relies on the cytosolic Rabconnectins or RAVE (Regulator of H^+-ATPase of Vacuolar and Endosomal membranes) complex. The glycolytic enzyme aldolase, possibly acting as a glucose sensor, also regulates V-ATPase activity by direct interaction with the b subunit and disruption of binding results in disassembly of V1/V0 (Lu et al. 2001, 2007). Furthermore V-ATPase and aldolase can also form a ternary complex with ARNO/Arf6 (Merkulova et al. 2011), proteins which have been implicated in linking intravesicular pH with the regulation of trafficking in the endocytic pathway (Hurtado-Lorenzo et al. 2006). Specifically the recruitment of the small GTPase Arf6 and ARNO (cytohesin-2) from the cytosol to the endosome membrane surface is dependent on acidification of the lumen by V-ATPase. Arf6 and ARNO bind to V0c and V0a subunits, respectively, and disruption of binding inhibited endocytosis, suggesting that intraluminal pH sensing by the V-ATPase can impact membrane trafficking. The relevance of these different pathways (Fig. 7.2) to the regulation of V-ATPase activity and membrane trafficking in the nerve terminal has not yet been explored in detail. However, studies in zebrafish hair cells have shown that rabconnectin3α modulates V-ATPase activity in synaptic vesicles by promoting assembly of V1 and V0 (Einhorn et al. 2012).

Vesicle loading with transmitter thus depends on the V-ATPase, which is a universal component of all synaptic vesicles, acting upstream of the transporter (glutamate, GABA, etc.) that defines specific vesicle content. Thus the V-ATPase is ideally situated to provide quality control of vesicle filling by monitoring and regulating the proton gradient. Presumably it is more cost-effective for the nerve

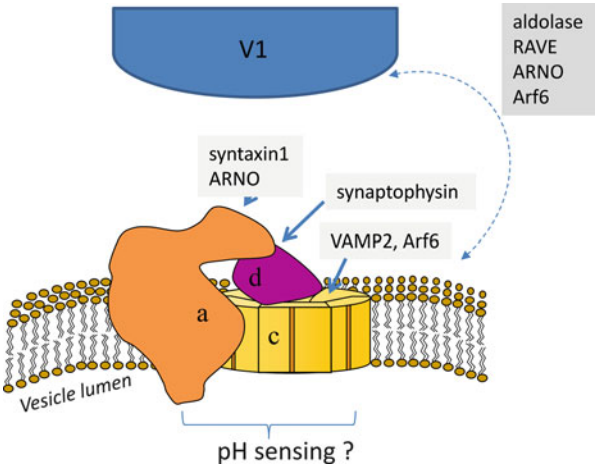

Fig. 7.2 Interactions of metabolic and trafficking proteins with V0 subunits. Schematic representation of molecular interactions involving the V-ATPase V0 sector. Interactions (*arrows*) are represented in two functional categories: those that are implicated in vesicle trafficking and exocytosis (VAMP2, syntaxin1, synaptophysin, ARNO and Arf6) and others involved in control of V1/V0 interactions and pH sensing (ARNO, Arf6, RAVE and aldolase)

terminal to avoid the exocytosis of empty or partially filled vesicles. But how could the trafficking and fusion machinery know when a synaptic vesicle is full? It has been suggested (Morel et al. 2003) that when a vesicle is fully loaded, proton efflux from the lumen stops, because it is no longer required to compensate neurotransmitter influx. Hence maximal acidification is attained, which would result in dissociation of V1. In this way vesicles could sense luminal pH and translate it into a "full" signal at the vesicle surface. Thus isolated V0 sectors, unmasked by V1 assembly, would become available to engage the docking and fusion machinery (Morel et al. 2003) (Fig. 7.3). While this attractive concept seems compatible with the quantal nature of transmitter release, it leads to further challenging questions. Can empty vesicles fuse? When proton transport is blocked, does exocytosis still occur?

Vesicular neurotransmitter concentration itself does not seem to be a limiting factor for fusion (Edwards 2007), as vesicles with a reduced neurotransmitter content still fuse (Zhou et al. 2000). Inhibition of proton transport by bafilomycin A1 leads to diminished activity when postsynaptic responses are used to monitor presynaptic exocytosis (Di Giovanni et al. 2010a; Zhou et al. 2000). However, this does not rule out the possibility that empty vesicles still fuse. More recent studies in the calyx of Held (Xue et al. 2013) using FM dye to monitor vesicle exocytosis showed that in the presence of bafilomycin A1, vesicles fuse and fully participate in the recycling synaptic vesicle pool. These results suggest that unacidified neurotransmitter-free synaptic vesicles can still accomplish fusion. In apparent contradiction with this finding, data from Poëa-Guyon and collaborators support the view that luminal acidification is a prerequisite for fusion (Poea-Guyon et al. 2013). They argue that the V-ATPase has a role in intraluminal pH sensing like it does in endosomes (Hurtado-Lorenzo et al. 2006). However, bafilomycin,

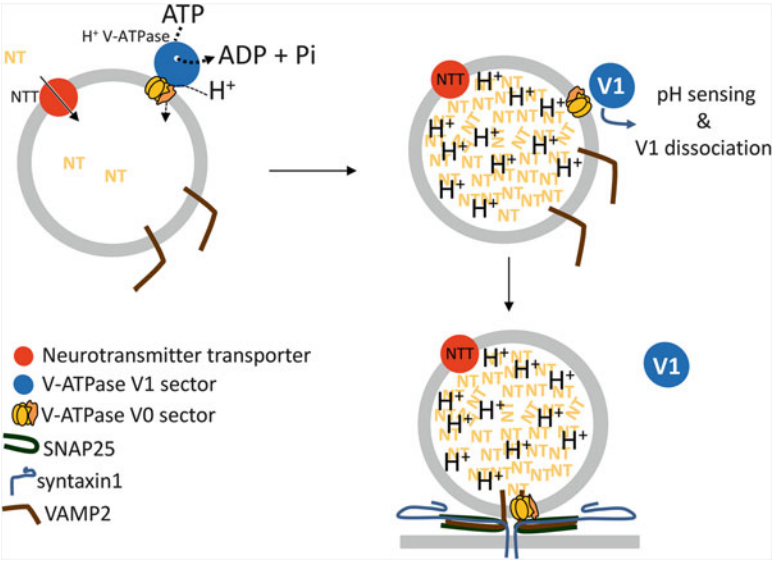

Fig. 7.3 Regulatory links between loading and fusion. ATP-driven H^+ transport through fully assembled V-ATPase at synaptic vesicle membranes is crucial for synaptic vesicle loading with neurotransmitters (NTT). Completion of synaptic vesicle loading leads to dissociation of the V1 sector and renders V0 accessible to bind SNARE proteins. V0 sector is speculatively located near the fusion pore

which binds to V-ATPase c-subunit, promotes V1 disassembly and stabilises a conformation of V0 that mimics a maximally acidified "ready for exocytosis" state.

7.5 A Role for V0 Subunits in Membrane Trafficking and Exocytosis

In the preceding section we reviewed evidence that V0 subunits may carry molecular signals (domains unmasked by dissociation of V1, bound ARNO/Arf6, conformational changes intrinsic to V0 subunits) that report intraluminal pH and thus indirectly monitor loading. In this section we address the possibility that V0 subunits play a direct role in docking and fusion downstream of loading. This role can be viewed from different angles: pore-formers and/or lipid destabilisers and SNARE partners.

7.5.1 Pore-Formers and/or Lipid Destabilisers

From the early 1980s, reports appeared in the literature initially suggesting that the V0 sector of the V-ATPase has a direct role in acetylcholine release. In an attempt at identifying a putative presynaptic translocator of acetylcholine (ACh) that could directly release ACh molecules from the cytoplasm into the synaptic cleft, Israel and collaborators purified a 150–200 kDa protein complex from *Torpedo marmorata* electric organ (Birman et al. 1986) (Israel et al. 1986). This complex was found to be composed of a single very hydrophobic 17 kDa polypeptide, which upon reconstitution in proteoliposomes showed calcium-dependent pore properties. The 17 kDa protein, initially designated "mediatophore", finally turned out to be the V0c subunit of the V-ATPase (Birman et al. 1990). These findings were not pursued by other laboratories since the authors advocated a controversial non-vesicular release model, incompatible with the mainstream view of synaptic vesicle exocytosis.

Although V0c is intrinsically expressed in release-incompetent neuroblastoma cells, overexpression of V0c conferred vesicle-mediated calcium-induced release properties (Bugnard et al. 1999). Most interestingly, freeze-fracture images from these cells were similar to synaptic vesicle fusion events observed much earlier at frog neuromuscular junction (Heuser et al. 1979) or Torpedo synaptic terminals upon neurotransmitter release (Muller et al. 1987). Immunolabelling of freeze-fractured Torpedo electric organ synaptosomes showed the presence of this protein on synaptic vesicles at the docking interface with the plasma membrane (Morel et al. 2001).

Overexpression of V0c in substantia nigra also increased dopamine release. Although the precise molecular mechanism underlying DA release is not yet clear, it has been suggested that overexpressing V0c might be a useful rescue strategy, in addition to enzymes of the dopamine synthetic pathway, for the gene therapy of Parkinson's disease (Jin et al. 2012).

In an independent investigation into how Ca^{2+}/calmodulin (Ca^{2+}/CaM) signals completion of docking and triggering in yeast vacuolar fusion (Peters and Mayer 1998), Peters et al. (2001) reported that the V0 sector is implicated in vacuolar fusion, independently of its proton transport activity, and displays pore-like properties (Almers 2001; Morel et al. 2001; Zimmerberg 2001). They showed that V0c binds Ca^{2+}/CaM and that CaM-dependent V0-V0 trans-complexes assembled after vacuolar docking and downstream of SNARE complex pairing. The presence of yeast V0a subunit, on both vacuoles destined to fuse, was later shown to be required for over 80 % of fusion events (Bayer et al. 2003) and that this protein is involved in calcium efflux from vacuoles, without being part of the Ca^{2+} release channel (Bayer et al. 2003). In photoreceptor synapses, V0a was shown to directly interact with Ca^{2+}/CaM. While binding is not necessary for synaptic targeting of V0a, this investigation uncovered functional regulation of V-ATPase by CaM (Zhang et al. 2008). Furthermore impairment of CaM binding to V0a resulted in dose-dependent neuronal toxicity. It is interesting also to note that V0c was also reported

to be part of connexon-free gap junctions in crustaceans (Dermietzel et al. 1989; Finbow and Pitts 1993). V0a has a regulatory role in insulin secretion as deficiency in the V0a subunit in insulin-containing granules (V0a3) drastically impaired secretion from beta cells in mouse pancreatic islets (Sun-Wada et al. 2006).

Exosomes are small vesicular structures (30–100 nm) contained in endosome-derived multivesicular bodies (MVB) (van Niel et al. 2006). In a variety of cell types including neurons (Faure et al. 2006), MVBs fuse with the plasma membrane and release exosomes into the extracellular milieu. A study on cuticle formation in *C. elegans* uncovered the involvement of vha5 (V0a of *C. elegans*) in MVB fusion with the apical membrane during release of exosomes and hedgehog-related cuticle components (Liegeois et al. 2006). Furthermore microglial phagosome/lysosome fusion in zebrafish was shown to be mediated by V0a, independently of its proton transport activity (Peri and Nusslein-Volhard 2008). In a different register, bone-resorbing osteoclasts are multinucleated, giant cells of haematopoietic origin, formed by the fusion of mononuclear pre-osteoclasts derived from myeloid cells. Deficiency in V0d2 subunit dramatically inhibited pre-osteoclast fusion and therefore bone resorption, independently of V-ATPase proton pump activity (Lee et al. 2006). This study suggests that V-ATPase V0 components are not only involved in intracellular fusion mechanisms but can also be required for cellular fusion.

7.5.2 SNARE Partners

In a search for synaptic vesicle-binding proteins, early studies by Thomas and Betz suggested an interaction of the soluble V-ATPase V0d subunit with synaptophysin, a synaptic vesicle signature protein of unknown function that interacts with VAMP2 (Thomas and Betz 1990). The association of V-ATPase components with SNARE proteins was first reported using immunoprecipitation from rat brain extracts (Galli et al. 1996). In this study using anti-VAMP2 antibodies, several V0 subunits and synaptophysin were co-immunoprecipitated. In elegant differential co-immunoprecipitation studies, a complex of V0a and V0c was found to associate with assembled SNARE complexes, as well as with VAMP2 (Morel et al. 2003). In an unbiased screen for genes implicated in synaptic function in *Drosophila* photoreceptors, V0a subunit was found to be involved in a late step of synaptic vesicle exocytosis and its deficit induces severe defects in evoked synaptic transmission. Interestingly, these effects were independent of V0a action in acidification (Hiesinger et al. 2005). Further investigation of the molecular mechanism underlying these defects demonstrated a direct interaction of the N-terminal part of the t-SNARE syntaxin with V0a. In an attempt to dissect SNARE protein interactions with V0c, the most hydrophobic component of the V-ATPase V0 sector, a direct interaction with the v-SNARE VAMP2 was uncovered (Di Giovanni et al. 2010a). Interaction details were mapped on both binding partners and shown to involve the V0c cytosolic loop that links TMR 3 and TMR 4 and the juxtamembrane VAMP2 tryptophan residues 89 and 90, formerly shown by the same group to mediate

binding to Ca^{2+}/CaM (Di Giovanni et al. 2010a, b). Furthermore, VAMP2 interactions with Ca^{2+}/CaM and V0c are mutually exclusive. Mutating these tryptophan residues inhibited Ca^{2+}-dependent exocytosis in PC12 cells (Quetglas et al. 2002) as well as in neurons (Maximov et al. 2009). Most interestingly, perturbing V0c/VAMP2 binding, using interfering peptides from V0c loop 3.4 or VAMP2 juxtamembrane domain, inhibited glutamatergic as well as cholinergic neurotransmitter release from neurons in cortical slices and cultured SCG. Inhibition was not observed if both peptides were co-injected (unpublished data) or upon mutation of the tryptophan residues in VAMP2 peptide or scrambling the V0c loop 3.4 peptide sequence (Di Giovanni et al. 2010a, b). The 1 VAMP2: 1 V0c binding stoichiometry suggests that the c-subunit ring could organise v-SNAREs into a radial array and determine the number of SNARE complexes that assemble around the fusion pore (El Far and Seagar 2011). In this case, due to intrinsic hydrophilic properties of V0d, the V0a N-terminal domain as well as the extravesicular loops of V0c, hemifusion intermediates are unlikely in fusion events involving V0.

7.6 Conclusions and Future Directions

In this chapter we have highlighted over 30 years of research that converges to support a role for the V0 sector of the V-ATPase in diverse forms of SNARE-mediated fusion: organelle fusion (yeast vacuoles, phagosomes/lysosomes), regulated secretion, exosomal release from MVBs and synaptic neurotransmitter release. These findings are consistent with a model in which V0 function goes beyond that of an ATP-dependent proton pump, to include monitoring of vesicle pH, loading with neurotransmitters and membrane fusion. Implicit in these observations is the hypothesis that intraluminal pH promotes conformational transitions of V0 subunits, which modulate direct interactions with SNAREs and impact the fusion process. If how and when V0 subunits act in membrane fusion are still open to debate. At the interface of membranes destined to fuse, the presence of a structure that geometrically organises a fixed number of trans SNARE complexes could be a simple way to standardise the energy resources available to drive membrane fusion. Alternatively, partial SNARE complex assembly could pin membranes together and provide precisely located scaffolding for proteins that act downstream to form a channel-like pore or catalyse rearrangements of lipid pore intermediates. Models of this kind are not yet energetically delimited, but certainly deserve theoretical investigation. Progress will require answers to some of the following questions. Do V0 subunits provide signals indicating that SNARE assembly can begin? Can they organise SNAREs into radial oligomeric arrays with appropriate properties to overcome energy barriers to membrane fusion? Might this also involve V0 subunits destabilising bilayers to promote opening of a lipid-lined fusion pore? Can pure recombinant V0 subunits form a protein-lined pore? Future investigations will hopefully address some of these issues.

References

Almers W (2001) Fusion needs more than SNAREs. Nature 409:567–568

Almers W, Tse FW (1990) Transmitter release from synapses: does a preassembled fusion pore initiate exocytosis? Neuron 4:813–818

Bayer MJ, Reese C, Buhler S, Peters C, Mayer A (2003) Vacuole membrane fusion: V0 functions after trans-SNARE pairing and is coupled to the Ca^{2+}-releasing channel. J Cell Biol 162:211–222

Bharat TA, Malsam J, Hagen WJ, Scheutzow A, Sollner TH, Briggs JA (2014) SNARE and regulatory proteins induce local membrane protrusions to prime docked vesicles for fast calcium-triggered fusion. EMBO Rep 15:308–314

Birman S, Israel M, Lesbats B, Morel N (1986) Solubilization and partial purification of a presynaptic membrane protein ensuring calcium-dependent acetylcholine release from proteoliposomes. J Neurochem 47:433–444

Birman S, Meunier FM, Lesbats B, Le Caer JP, Rossier J, Israel M (1990) A 15 kDa proteolipid found in mediatophore preparations from Torpedo electric organ presents high sequence homology with the bovine chromaffin granule protonophore. FEBS Lett 261:303–306

Bowman BJ, McCall ME, Baertsch R, Bowman EJ (2006) A model for the proteolipid ring and bafilomycin/concanamycin-binding site in the vacuolar ATPase of Neurospora crassa. J Biol Chem 281:31885–31893

Breton S, Smith PJ, Lui B, Brown D (1996) Acidification of the male reproductive tract by a proton pumping (H^+)-ATPase. Nat Med 2:470–472

Bugnard E, Sors P, Roulet E, Bloc A, Loctin F, Dunant Y (1999) Morphological changes related to reconstituted acetylcholine release in a release-deficient cell line. Neuroscience 94(1):329–338

Chen X, Tomchick DR, Kovrigin E, Arac D, Machius M, Sudhof TC, Rizo J (2002) Three-dimensional structure of the complexin/SNARE complex. Neuron 33:397–409

Chernomordik LV, Kozlov MM (2008) Mechanics of membrane fusion. Nat Struct Mol Biol 15:675–683

Clare DK, Orlova EV, Finbow MA, Harrison MA, Findlay JBC, Saibil HR (2006) An expanded and flexible form of the vacuolar ATPase membrane sector. Structure 14:1149–1156

Davis AF, Bai J, Fasshauer D, Wolowick MJ, Lewis JL, Chapman ER (1999) Kinetics of synaptotagmin responses to Ca^{2+} and assembly with the core SNARE complex onto membranes. Neuron 24:363–376

Del Castillo J, Katz B (1954) Quantal components of the end-plate potential. J Physiol 124:560–573

Dermietzel R, Volker M, Hwang TK, Berzborn RJ, Meyer HE (1989) A 16 kDa protein co-isolating with gap junctions from brain tissue belonging to the class of proteolipids of the vacuolar H^+-ATPases. FEBS Lett 253:1–5

Di Giovanni J, Boudkkazi S, Mochida S, Bialowas A, Samari N, Leveque C, Youssouf F, Brechet A, Iborra C, Maulet Y, Moutot N, Debanne D, Seagar M, El Far O (2010a) V-ATPase membrane sector associates with synaptobrevin to modulate neurotransmitter release. Neuron 67:268–279

Di Giovanni J, Iborra C, Maulet Y, Leveque C, El Far O, Seagar M (2010b) Calcium-dependent regulation of SNARE-mediated membrane fusion by calmodulin. J Biol Chem 285:23665–23675

Diao J, Cipriano DJ, Zhao M, Zhang Y, Shah S, Padolina MS, Pfuetzner RA, Brunger AT (2013) Complexin-1 enhances the on-rate of vesicle docking via simultaneous SNARE and membrane interactions. J Am Chem Soc 135:15274–15277

Edwards RH (2007) The neurotransmitter cycle and quantal size. Neuron 55:835–858

Einhorn Z, Trapani JG, Liu Q, Nicolson T (2012) Rabconnectin3alpha promotes stable activity of the H^+ pump on synaptic vesicles in hair cells. J Neurosci 32:11144–11156

EL Far O, Seagar M (2011) A role for V-ATPase subunits in synaptic vesicle fusion? J Neurochem 117:603–612

Faure J, Lachenal G, Court M, Hirrlinger J, Chatellard-Causse C, Blot B, Grange J, Schoehn G, Goldberg Y, Boyer V, Kirchhoff F, Raposo G, Garin J, Sadoul R (2006) Exosomes are released by cultured cortical neurones. Mol Cell Neurosci 31:642–648

Ferro-Novick S, Jahn R (1994) Vesicle fusion from yeast to man. Nature 370:191–193

Finbow ME, Pitts JD (1993) Is the gap junction channel–the connexon–made of connexin or ductin? J Cell Sci 106:463–471

Galli T, McPherson PS, De Camilli P (1996) The V0 sector of the V-ATPase, synaptobrevin, and synaptophysin are associated on synaptic vesicles in a Triton X-100-resistant, freeze-thawing sensitive, complex. J Biol Chem 271:2193–2198

Giraudo CG, Hu C, You D, Slovic AM, Mosharov EV, Sulzer D, Melia TJ, Rothman JE (2005) SNAREs can promote complete fusion and hemifusion as alternative outcomes. J Cell Biol 170:249–260

Hernandez JM, Stein A, Behrmann E, Riedel D, Cypionka A, Farsi Z, Walla PJ, Raunser S, Jahn R (2012) Membrane fusion intermediates via directional and full assembly of the SNARE complex. Science 336:1581–1584

Heuser JE, Reese TS, Landis DM (1974) Functional changes in frog neuromuscular junctions studied with freeze-fracture. J Neurocytol 3:109–131

Heuser JE, Reese TS, Dennis MJ, Jan Y, Jan L, Evans L (1979) Synaptic vesicle exocytosis captured by quick freezing and correlated with quantal transmitter release. J Cell Biol 81:275–300

Hiesinger PR, Fayyazuddin A, Mehta SQ, Rosenmund T, Schulze KL, Zhai RG, Verstreken P, Cao Y, Zhou Y, Kunz J, Bellen HJ (2005) The v-ATPase V(0) subunit a1 is required for a late step in synaptic vesicle exocytosis in Drosophila. Cell 121:607–620

Huntwork S, Littleton JT (2007) A complexin fusion clamp regulates spontaneous neurotransmitter release and synaptic growth. Nat Neurosci 10:1235–1237

Hurtado-Lorenzo A, Skinner M, El Annan J, Futai M, Sun-Wada GH, Bourgoin S, Casanova J, Wildeman A, Bechoua S, Ausiello DA, Brown D, Marshansky V (2006) V-ATPase interacts with ARNO and Arf6 in early endosomes and regulates the protein degradative pathway. Nat Cell Biol 8:124–136

Israel M, Morel N, Lesbats B, Birman S, Manaranche R (1986) Purification of a presynaptic membrane protein that mediates a calcium-dependent translocation of acetylcholine. Proc Natl Acad Sci U S A 83:9226–9230

Jin D, Muramatsu S-i, Shimizu N, Yokoyama S, Hirai H, Yamada K, Liu H-X, Higashida C, Hashii M, Higashida A, Asano M, Ohkuma S, Higashida H (2012) Dopamine release via the vacuolar ATPase V0 sector c-subunit, confirmed in N18 neuroblastoma cells, results in behavioral recovery in hemiparkinsonian mice. Neurochem Int 61:907–912

Kane PM (1995) Disassembly and reassembly of the yeast vacuolar H^+-ATPase in vivo. J Biol Chem 270:17025–17032

Karatekin E, Di Giovanni J, Iborra C, Coleman J, O'Shaughnessy B, Seagar M, Rothman JE (2010) A fast, single-vesicle fusion assay mimics physiological SNARE requirements. Proc Natl Acad Sci U S A 107:3517–3521

Lee SH, Rho J, Jeong D, Sul JY, Kim T, Kim N, Kang JS, Miyamoto T, Suda T, Lee SK, Pignolo RJ, Koczon-Jaremko B, Lorenzo J, Choi Y (2006) v-ATPase V0 subunit d2-deficient mice exhibit impaired osteoclast fusion and increased bone formation. Nat Med 12:1403–1409

Liegeois S, Benedetto A, Garnier JM, Schwab Y, Labouesse M (2006) The V0-ATPase mediates apical secretion of exosomes containing Hedgehog-related proteins in *Caenorhabditis elegans*. J Cell Biol 173:949–961

Liu Y, Edwards RH (1997) The role of vesicular transport proteins in synaptic transmission and neural degeneration. Annu Rev Neurosci 20:125–156

Lu M, Holliday LS, Zhang L, Dunn WA Jr, Gluck SL (2001) Interaction between aldolase and vacuolar H^+-ATPase: evidence for direct coupling of glycolysis to the ATP-hydrolyzing proton pump. J Biol Chem 276:30407–30413

Lu M, Ammar D, Ives H, Albrecht F, Gluck SL (2007) Physical interaction between aldolase and vacuolar H^+-ATPase is essential for the assembly and activity of the proton pump. J Biol Chem 282:24495–24503

Ma C, Su L, Seven AB, Xu Y, Rizo J (2013) Reconstitution of the vital functions of Munc18 and Munc13 in neurotransmitter release. Science 339:421–425

Malsam J, Seiler F, Schollmeier Y, Rusu P, Krause JM, Sollner TH (2009) The carboxy-terminal domain of complexin I stimulates liposome fusion. Proc Natl Acad Sci U S A 106:2001–2006

Marshansky V, Futai M (2008) The V-type H^+-ATPase in vesicular trafficking: targeting, regulation and function. Curr Opin Cell Biol 20:415–426

Maximov A, Tang J, Yang X, Pang ZP, Sudhof TC (2009) Complexin controls the force transfer from SNARE complexes to membranes in fusion. Science 323:516–521

Maycox PR, Hell JW, Jahn R (1990) Amino acid neurotransmission: spotlight on synaptic vesicles. Trends Neurosci 13:83–87

Merkulova M, Hurtado-Lorenzo A, Hosokawa H, Zhuang Z, Brown D, Ausiello DA, Marshansky V (2011) Aldolase directly interacts with ARNO and modulates cell morphology and acidic vesicle distribution. Am J Physiol 300:C1442–C1455

Morel N, Dunant Y, Israel M (2001) Neurotransmitter release through the V0 sector of V-ATPase. J Neurochem 79:485–488

Morel N, Dedieu JC, Philippe JM (2003) Specific sorting of the a1 isoform of the V-H^+ATPase a subunit to nerve terminals where it associates with both synaptic vesicles and the presynaptic plasma membrane. J cell sci 116:4751–4762

Moriyama Y, Maeda M, Futai M (1992) The role of V-ATPase in neuronal and endocrine systems. J Exp Biol 172:171–178

Muller D, Garcia-Segura LM, Parducz A, Dunant Y (1987) Brief occurrence of a population of presynaptic intramembrane particles coincides with transmission of a nerve impulse. Proc Natl Acad Sci U S A 84:590–594

Neher E, Sakaba T (2008) Multiple roles of calcium ions in the regulation of neurotransmitter release. Neuron 59:861–872

Nelson N, Lill H (1994) Porters and neurotransmitter transporters. J Exp Biol 196:213–228

Nishi T, Forgac M (2002) The vacuolar (H^+)-ATPases – nature's most versatile proton pumps. Nat Rev Mol Cell Biol 3:94–103

Nishi T, Kawasaki-Nishi S, Forgac M (2003) Expression and function of the mouse V-ATPase d subunit isoforms. J Biol Chem 278:46396–46402

Peper K, Dreyer F, Sandri C, Akert K, Moor H (1974) Structure and ultrastructure of the frog motor endplate. A freeze-etching study. Cell Tissue Res 149:437–455

Perez-Sayans M, Somoza-Martin JM, Barros-Angueira F, Rey JMG, Garcia-Garcia A (2009) V-ATPase inhibitors and implication in cancer treatment. Cancer Treat Rev 35:707–713

Perez-Sayans M, Suarez-Penaranda JM, Barros-Angueira F, Diz PG, Gandara-Rey JM, Garcia-Garcia A (2012) An update in the structure, function, and regulation of V-ATPases: the role of the C subunit. Braz J Biol 72:189–198

Peri F, Nusslein-Volhard C (2008) Live imaging of neuronal degradation by microglia reveals a role for v0-ATPase a1 in phagosomal fusion in vivo. Cell 133:916–927

Peters C, Mayer A (1998) Ca^{2+}/calmodulin signals the completion of docking and triggers a late step of vacuole fusion. Nature 396:575–580

Peters C, Bayer MJ, Buhler S, Andersen JS, Mann M, Mayer A (2001) Trans complex formation by proteolipid channels in the terminal phase of membrane fusion. Nature 409:581–588

Poea-Guyon S, Ammar MR, Erard M, Amar M, Moreau AW, Fossier P, Gleize V, Vitale N, Morel N (2013) The V-ATPase membrane domain is a sensor of granular pH that controls the exocytotic machinery. J Cell Biol 203:283–298

Qi J, Wang Y, Forgac M (2007) The vacuolar (H^+)-ATPase: subunit arrangement and in vivo regulation. J Bioenerg Biomembr 39:423–426

Quetglas S, Iborra C, Sasakawa N, De Haro L, Kumakura K, Sato K, Leveque C, Seagar M (2002) Calmodulin and lipid binding to synaptobrevin regulates calcium-dependent exocytosis. EMBO J 21:3970–3979

Reim K, Mansour M, Varoqueaux F, McMahon HT, Sudhof TC, Brose N, Rosenmund C (2001) Complexins regulate a late step in Ca^{2+}-dependent neurotransmitter release. Cell 104:71–81

Reim K, Wegmeyer H, Brandstatter JH, Xue M, Rosenmund C, Dresbach T, Hofmann K, Brose N (2005) Structurally and functionally unique complexins at retinal ribbon synapses. J Cell Biol 169:669–680

Risselada HJ, Grubmüller H (2012) How SNARE molecules mediate membrane fusion: recent insights from molecular simulations. Curr Opin Struct Biol 22:187–196

Rizzoli SO, Betz WJ (2005) Synaptic vesicle pools. Nat Rev 6:57–69

Schaub JR, Lu X, Doneske B, Shin YK, McNew JA (2006) Hemifusion arrest by complexin is relieved by Ca^{2+}-synaptotagmin I. Nat Struct Mol Biol 13:748–750

Schlesinger PH, Blair HC, Teitelbaum SL, Edwards JC (1997) Characterization of the osteoclast ruffled border chloride channel and its role in bone resorption. J Biol Chem 272:18636–18643

Sumner J-P, Dow JAT, Earley FGP, Klein U, JÃ¤ger D, Wieczorek H (1995) Regulation of plasma membrane V-ATPase activity by dissociation of peripheral subunits. J Biol Chem 270:5649–5653

Sun-Wada GH, Toyomura T, Murata Y, Yamamoto A, Futai M, Wada Y (2006) The a3 isoform of V-ATPase regulates insulin secretion from pancreatic beta-cells. J Cell Sci 119:4531–4540

Tang J, Maximov A, Shin OH, Dai H, Rizo J, Sudhof TC (2006) A complexin/synaptotagmin 1 switch controls fast synaptic vesicle exocytosis. Cell 126:1175–1187

Thomas L, Betz H (1990) Synaptophysin binds to physophilin, a putative synaptic plasma membrane protein. J Cell Biol 111:2041–2052

Tokumaru H, Umayahara K, Pellegrini LL, Ishizuka T, Saisu H, Betz H, Augustine GJ, Abe T (2001) SNARE complex oligomerization by synaphin/complexin is essential for synaptic vesicle exocytosis. Cell 104:421–432

Tokumaru H, Shimizu-Okabe C, Abe T (2008) Direct interaction of SNARE complex binding protein synaphin/complexin with calcium sensor synaptotagmin 1. Brain Cell Biol 36:173–189

Tucker WC, Weber T, Chapman ER (2004) Reconstitution of Ca^{2+}-regulated membrane fusion by synaptotagmin and SNAREs. Science 304:435–438

van Niel G, Porto-Carreiro I, Simoes S, Raposo G (2006) Exosomes: a common pathway for a specialized function. J Biochem 140:13–21

Wagner CA, Finberg KE, Breton S, Marshansky V, Brown D, Geibel JP (2004) Renal vacuolar H^+-ATPase. Physiol Rev 84:1263–1314

Weber T, Zemelman BV, McNew JA, Westermann B, Gmachl M, Parlati F, Sollner TH, Rothman JE (1998) SNAREpins: minimal machinery for membrane fusion. Cell 92:759–772

Wilkens S, Forgac M (2001) Three-dimensional structure of the vacuolar ATPase proton channel by electron microscopy. J Biol Chem 276:44064–44068

Wilkens S, Vasilyeva E, Forgac M (1999) Structure of the vacuolar ATPase by electron microscopy. J Biol Chem 274:31804–31810

Williamson WR, Hiesinger PR (2010) On the role of v-ATPase V0a1-dependent degradation in Alzheimer disease. Commun Integr Biol 3:604–607

Williamson WR, Wang D, Haberman AS, Hiesinger PR (2010) A dual function of V0-ATPase a1 provides an endolysosomal degradation mechanism in Drosophila melanogaster photoreceptors. J Cell Biol 189:885–899

Wragg RT, Snead D, Dong Y, Ramlall TF, Menon I, Bai J, Eliezer D, Dittman JS (2013) Synaptic vesicles position complexin to block spontaneous fusion. Neuron 77:323–334

Xue M, Reim K, Chen X, Chao HT, Deng H, Rizo J, Brose N, Rosenmund C (2007) Distinct domains of complexin I differentially regulate neurotransmitter release. Nat Struct Mol Biol 14:949–958

Xue M, Craig TK, Xu J, Chao H-T, Rizo J, Rosenmund C (2010) Binding of the complexin N terminus to the SNARE complex potentiates synaptic-vesicle fusogenicity. Nat Struct Mol Biol 17:568–575

Xue L, Sheng J, Wu XS, Wu W, Luo F, Shin W, Chiang HC, Wu LG (2013) Most vesicles in a central nerve terminal participate in recycling. J Neurosci 33:8820–8826

Zhang W, Wang D, Volk E, Bellen HJ, Hiesinger PR, Quiocho FA (2008) V-ATPase V0 sector subunit a1 in neurons is a target of calmodulin. J Biol Chem 283(1):294–300

Zhang Z, Nguyen KT, Barrett EF, David G (2010) Vesicular ATPase inserted into the plasma membrane of motor terminals by exocytosis alkalinizes cytosolic pH and facilitates endocytosis. Neuron 68:1097–1108

Zhou Q, Petersen CC, Nicoll RA (2000) Effects of reduced vesicular filling on synaptic transmission in rat hippocampal neurones. J Physiol 525:195–206

Zimmerberg J (2001) How can proteolipids be central players in membrane fusion? Trends Cell Biol 11:233–235

Chapter 8
Ca^{2+} Sensors: Synaptotagmins

Tei-ichi Nishiki, Kentaro Kuroki, Toshio Masumoto, and Hideki Matsui

Abstract Synaptotagmin is a synaptic vesicle membrane protein that is postulated to function as a calcium (Ca^{2+}) sensor for neurotransmitter release. This protein contains two Ca^{2+}-binding domains (C_2A, C_2B) in its cytoplasmic region. Genetic studies provide strong evidence supporting the idea that synaptotagmin is the Ca^{2+} sensor for the fast synchronous component of evoked transmitter release. In addition, synaptotagmin appears to be involved in docking and priming (by clamping spontaneous fusion) of synaptic vesicles and regulating fusion pores as well as vesicle endocytosis. In vitro studies demonstrate that synaptotagmin binds to phospholipid membranes in the presence and absence of Ca^{2+}. Furthermore, in response to Ca^{2+}, the tips of synaptotagmin C_2 domains penetrate lipid bilayers, altering the membrane curvature. Synaptotagmin also binds to the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex that is believed to be the core fusion machinery, although the findings on the Ca^{2+} dependency of binding of synaptotagmin to SNARE have been inconsistent and controversial. Taken together, synaptotagmin appears to trigger a fast synchronous component of transmitter release by binding to Ca^{2+}, probably through the interactions with both membranes and SNAREs.

Keywords Exocytosis • Phospholipids • SNARE • Synaptic vesicle • Synchronous release

T. Nishiki (✉) • K. Kuroki • H. Matsui
Department of Physiology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kita-ku, Okayama 700-8558, Japan
e-mail: nishiki@okayama-u.ac.jp

T. Masumoto
Department of Physiology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kita-ku, Okayama 700-8558, Japan

Brain Science Institute, RIKEN, Saitama 351-0198, Japan

S. Mochida (ed.), *Presynaptic Terminals*, DOI 10.1007/978-4-431-55166-9_8

8.1 Introduction

Signal transmission at chemical synapses is mediated by neurotransmitters stored in synaptic and dense-core vesicles in nerve terminals. When an action potential reaches the nerve terminal, neurotransmitters are exocytosed at the active zone by the calcium ion (Ca^{2+})-dependent fusion of these vesicles with presynaptic plasma membranes. This vesicle fusion is mediated by soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) localized on both vesicular and presynaptic plasma membranes (see Chap. 4). The complex formation of SNAREs between these two membranes is believed to drive membrane fusion. While SNAREs are constitutively active and have no Ca^{2+} sensitivity in triggering membrane fusion, neurotransmitter release is highly regulated by Ca^{2+}. Therefore, some other molecule must serve as a Ca^{2+} sensor, transducing the Ca^{2+} signals to the SNARE complex.

With respect to Ca^{2+} influx into presynaptic nerve terminals, evoked neurotransmitter release can be classified into two kinetically distinct components: a fast synchronous component and a slow asynchronous component (Goda and Stevens 1994). These two components of transmitter release may be differentially regulated by two distinct Ca^{2+} sensors with different Ca^{2+}-binding affinities. A low-affinity Ca^{2+} sensor senses a local increase in Ca^{2+} concentration in the vicinity of open Ca^{2+} channels. It can trigger a fast transmitter release that is synchronized within sub-millisecond time scales after Ca^{2+} influx. In contrast, a Ca^{2+} sensor with higher affinity mainly responds to a more diffuse increase in Ca^{2+} concentration during a transient change in Ca^{2+} or during repetitive action potential firing. This sensor can induce slow, long-lasting transmitter release.

The best candidate for the low-affinity Ca^{2+} sensor for fast synchronous release is synaptic vesicle membrane proteins, synaptotagmins I and II. Synaptotagmins are a large family of membrane proteins characterized by one transmembrane region and two Ca^{2+}-binding domains. Synaptotagmins are highly conserved across evolution (from nematodes to humans), and there are at least 17 isoforms in mice and humans (Craxton 2010). Among them, eight synaptotagmins (I, II, III, V, VI, VII, IX, and X) bind to Ca^{2+}. In this chapter, following overviews of the structure and function of synaptotagmin, we review our current knowledge of the protein with a particular focus on the interaction of synaptotagmin I with phospholipids and SNAREs. Furthermore, we discuss the possible functional roles of synaptotagmins in evoked neurotransmitter release. We apologize to the authors of many important studies that we could not cite because of the scope and space limitations of this article.

8.2 Overview of Synaptotagmins

8.2.1 Synaptotagmin I, II, and IX Subfamily

Synaptotagmin I (referred to as synaptotagmin without other mentioning) is the best characterized isoform in the synaptotagmin family (Matthew et al. 1981; Perin

et al. 1990; Takahashi et al. 1991). Among other isoforms in the family, synaptotagmin II is the most closely related to synaptotagmin I (76 % sequence identity in mice) (Geppert et al. 1991). Synaptotagmin IX, which was originally cloned as synaptotagmin V (Craxton and Goedert 1995; Hudson and Birnbaum 1995), shares a high sequence identity (49–54 %) with synaptotagmins I and II. These three synaptotagmins are classified in an evolutionarily conserved subfamily that is involved in Ca^{2+}-dependent secretory vesicle exocytosis (Fukuda 2003).

Synaptotagmins I and II are the most widely distributed isoforms in the nervous system as well as the endocrine system (Marquèze et al. 1995; Fox and Sanes 2007; Matsuoka et al. 2011). In the nervous system, synaptotagmin I is primarily expressed in rostral brain regions (i.e., olfactory bulb, cerebral cortex, and hippocampus), while synaptotagmin II is abundantly expressed in caudal brain neurons (i.e., spinal cord, brainstem, and cerebellum) (Matthew et al. 1981; Geppert et al. 1991; Takahashi et al. 1991; Marquèze et al. 1995; Mittelsteadt et al. 2009). Synaptotagmin IX is also expressed at high levels in the brain (limbic system and striatum) but not in the spinal cord or adrenal glands (Craxton and Goedert 1995; Hudson and Birnbaum 1995; Xu et al. 2007).

Synaptotagmins I and II are localized on synaptic vesicles in neurons. On the synaptic vesicle surface, synaptotagmin I reportedly exists at a high local concentration. In highly purified synaptic vesicles, the average copy number of synaptotagmin per vesicle is calculated to be 15.2 (Takamori et al. 2006). Assuming that the diameter of synaptic vesicles is approximately 40 nm and the size of a typical compact globular protein is approximately 10 nm, the volume of the synaptic vesicle surface where vesicle proteins are localized is calculated as approximately 79,547 nm^3 (the volume of a sphere with diameter 60 nm minus the volume of a sphere with diameter 40 nm = 113,040 nm^3 − 33,493 nm^3). These calculations suggest that the local concentration of synaptotagmin on the synaptic vesicle surface is approximately 317 μM.

8.2.2 Structure

Members of the synaptotagmin family share a domain configuration: an amino (N)-terminal intravesicular region followed by a single transmembrane domain, a linker region, and two carboxyl (C)-terminal C_2 domains (Perin et al. 1991; Fig. 8.1a). The C_2 domain is a Ca^{2+}- and phospholipid-binding motif consisting of approximately 130–150 residues that was originally identified in the Ca^{2+}-dependent isoforms of protein kinase C (Nishizuka 1988). The two synaptotagmin C_2 domains are referred to as C_2A and C_2B. X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy analysis revealed that both C_2 domains of synaptotagmin I are composed of a β-sandwich formed by two β-sheets, each consisting of four antiparalleled β-strands, with three flexible loops on top and

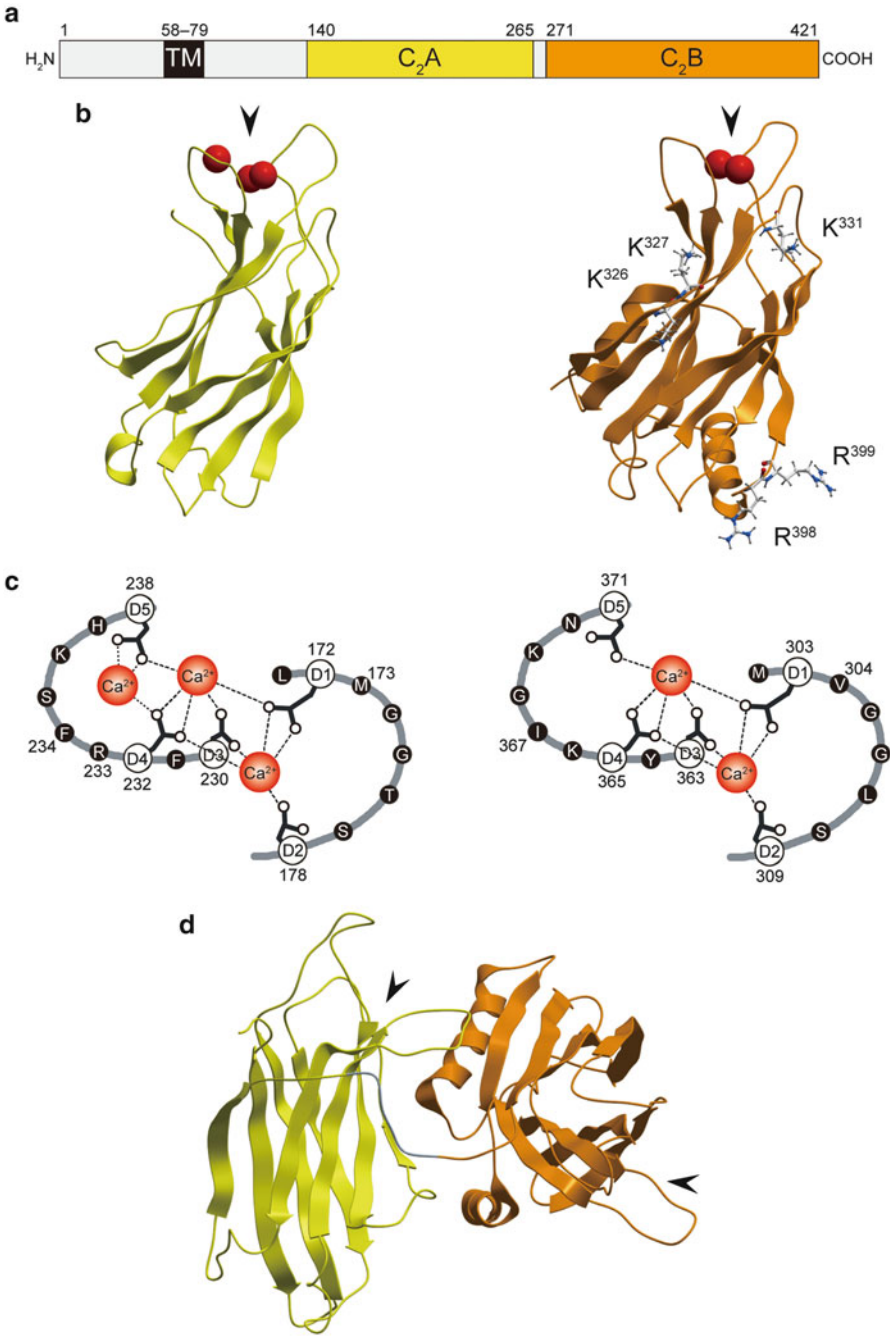

Fig. 8.1 Structures of rat (**a**–**c**) and human (**d**) synaptotagmins. (**a**) Domain diagram of rat synaptotagmin I. TM, transmembrane. (**b**) Side views of ribbon diagrams of the structures of the C_2A (*left*; PDB file 1BYN; Shao et al. 1998) and C_2B (*right*; PDB file 1K5W; Fernandez et al. 2001) domains in the presence of Ca^{2+} (*red spheres*). Indicated amino acid residues are shown as *ball* and *sticks*. (**c**) Ca^{2+}-binding sites in the C_2A (*left*) and C_2B (*right*) domains, modified

four loops at the bottom (Sutton et al. 1995; Shao et al. 1996; Fernandez et al. 2001; Fig. 8.1b).

In the top face of the C_2 domains, two loops of three contain five negatively charged aspartate (Asp, D) residues forming Ca^{2+}-binding sites (D^{172}, D^{178}, D^{230}, D^{232}, and D^{238} in rat C_2A and D^{303}, D^{309}, D^{363}, D^{365}, and D^{371} in rat C_2B) (Shao et al. 1998; Fernandez et al. 2001; Fig. 8.1c). However, X-ray crystal structure analysis indicated that Asp^{371} in the C_2B domain does not bind to Ca^{2+} (Cheng et al. 2004). The C_2A domain of synaptotagmin I binds three Ca^{2+} ions with an estimated affinity of 60–75 μM, 400–500 μM, and >1 mM (Ubach et al. 1998). The isolated C_2B domain binds two Ca^{2+} ions with similar calcium affinities in the range of 300–600 μM (Fernandez et al. 2001). Thus, intrinsic Ca^{2+}-binding affinities of both C_2 domains are low but dramatically increased in the presence of negatively charged phospholipids (5–50 μM) (Brose et al. 1992; Fernández-Chacón et al. 2001; Radhakrishnan et al. 2009). The binding of Ca^{2+} changes only the electrostatic potential of the isolated domains without inducing large conformational change in these domains (Shao et al. 1998; Fernandez et al. 2001).

The bottom face of the C_2B domain has two additional α-helices that are absent in the C_2A domain (Fernandez et al. 2001) and a basic region containing two conserved arginine (Arg, R) residues (R^{398} and R^{399} in rats; Araç et al. 2006). Another basic area on the surface of the C_2B domain, which is called a polylysine (Lys, K) region (K^{326}, K^{327}, and K^{331} in rats), is located in the fourth β-strand on the edge of a concaved side of its β-sandwich. Thus, the surface of the C_2B domain possesses distinctly basic (top) and acidic (side, bottom) areas, which are postulated to be important for the Ca^{2+}-independent interactions of synaptotagmin through the C_2B domain (Fig. 8.1b, c).

While considering the mechanism underlying the function of synaptotagmin in neurotransmitter release, the two synaptotagmin C_2 domains are the most interesting features. Because the tandem C_2A and C_2B domains are connected by a short flexible linker, the relative orientation of the two C_2 domains, especially Ca^{2+}-binding sites, is critical. In some cases, the image of the structure of the synaptotagmin I cytoplasmic region (referred to as the C_2AB fragment) has been drawn by connecting the NMR structure of each C_2 domain with a manually drawn line based on the crystal structure of synaptotagmin III cytoplasmic region in the absence of Ca^{2+} (Sutton et al. 1999). In such images, the Ca^{2+}-binding regions of both synaptotagmin C_2 domains are directed toward each other. Crystal structural analysis of the C_2AB fragment of human synaptotagmin I, in the absence of Ca^{2+}, revealed that its conformation is in contrast to that of synaptotagmin III. The C_2A and C_2B domains of synaptotagmin I are related by ~108° rotation about the axis of

Fig. 8.1 (continued) from Fernandez et al. (2001). (**d**) Ribbon diagram of the structure of human synaptotagmin I (PDB file 2R83; Fuson et al. 2007). ICM Browser software (Molsoft L.L.C., San Diego, CA) was used to generate three-dimensional structures. Ca^{2+}-binding pockets are indicated by *arrowheads*. The same color coding is used for each C_2 domain (C_2A in *yellow*, C_2B in *orange*) in all figures

a linker connecting the two C_2 domains (Fuson et al. 2007). In this configuration, the Ca^{2+}-binding sites of the C_2A domain interact with an α-helix at the bottom of the C_2B domain (Fig. 8.1d). Therefore, this structure of synaptotagmin, in the absence of Ca^{2+}, is called the closed form.

In contrast to crystallographic analysis, NMR and electron paramagnetic resonance (EPR) spectroscopy of the synaptotagmin C_2AB fragment demonstrate that the two C_2AB domains are flexibly linked and do not interact with each other in solution (Araç et al. 2006; Herrick et al. 2009). EPR spectroscopy also shows that the Ca^{2+}-binding sites of each C_2 domain face in roughly opposite directions when synaptotagmin binds to SNARE complexes in solution (Lai et al. 2011). These findings suggest that the Ca^{2+}-binding sites in one C_2 domain interact with synaptic vesicle membranes, and those in the other C_2 domain bind to presynaptic membranes when synaptotagmin binds to a SNARE complex between two fusion partners (termed a *trans* complex) at nerve terminals.

The structure of synaptotagmin bound to the SNARE complex embedded in membranes appears to be different from that in solution. Single-molecule Förster resonance energy transfer (also called fluorescence resonance energy transfer, FRET) analysis revealed the structure of synaptotagmin bound to a SNARE complex in the *cis* (on the same membrane) conformation in lipid bilayers in the presence of Ca^{2+}. Binding to the membrane-embedded *cis*-SNARE complex stabilizes the conformation variability of the synaptotagmin C_2AB fragment and arranges the Ca^{2+}-binding loops of both C_2A and C_2B domains such that they face away from the SNARE complex. This configuration allows the two C_2 domains to interact with the same membrane (Choi et al. 2010). In the complex between synaptotagmin and SNAREs, the C_2B bottom face interacts with the SNARE complex. However, the conserved Arg residues are sufficiently exposed to allow potential interaction with membranes. Further studies are required to determine the structure of synaptotagmin when the protein binds to the *trans*-SNARE complex that is ideally formed between two membranes.

8.3 Multiple Roles of Synaptotagmin in Ca^{2+}-Dependent Neurotransmitter Release

8.3.1 A Ca^{2+} Sensor for Neurotransmitter Release

As described in the above section, synaptotagmin I is an integral synaptic vesicle membrane protein. Multiple Ca^{2+} ions bind to its cytoplasmic C_2 domains that are known to interact with membrane lipids at physiological concentrations ($\geq$1 μM) in the presence of negatively charged phospholipids (Brose et al. 1992). Based on its role in evoked neurotransmitter and the aforementioned features, the protein has been postulated to be a Ca^{2+} sensor of synaptic vesicle exocytosis. Synaptotagmin I

is also postulated to function as a Ca^{2+} sensor for spontaneous release as well as evoked release (Xu et al. 2009).

Pioneering work on genetically mutated animals revealed a critical role for synaptotagmin I in Ca^{2+}-dependent evoked transmitter release (DiAntonio et al. 1993; Littleton et al. 1993; Nonet et al. 1993; Geppert et al. 1994). In agreement with these findings, microinjection of synthetic C_2 domain inhibitory peptides or antibodies against synaptotagmin into presynaptic neurons or endocrine cells inhibits neurotransmitter release (Bommert et al. 1993; Elferink et al. 1993; Fukuda et al. 1995; Mochida et al. 1997; Ohara-Imaizumi et al. 1997). Somewhat surprisingly, synaptotagmin is not required for Ca^{2+}-dependent transmitter release in response to a stimulus per se. This is because, in the absence of synaptotagmin, vesicle exocytosis continues to occur at a normal level but with slower kinetics (Shoji-Kasai et al. 1992; Shin et al. 2003; Nishiki and Augustine 2004a). Most notably, in primary cultured hippocampal neurons of mice lacking synaptotagmin I, synchronous transmitter release is attenuated by more than 95 %, leaving only asynchronous release as the main component of transmitter release (Shin et al. 2003; Nishiki and Augustine 2004a). In the synaptotagmin-deficient *Drosophila* neuromuscular junction (NMJ), synchronous release is also abolished and asynchronous release is increased (Yoshihara and Littleton 2002). These findings suggest that synaptotagmin I regulates evoked neurotransmitter release in two different ways: triggering synchronous release and suppressing asynchronous release. It was recently reported that, in mice, synchronous release triggered by synaptotagmin I in the hippocampus CA1 region is important for recognizing recent fear memories, while that in the prefrontal cortex is required for storage and/or retrieval of distant fear memories (Xu et al. 2012).

In some synapses (i.e., inhibitory synapses between basket cells and granule cells in the dentate gyrus of the hippocampus), synaptotagmin I is not absolutely required for synchronous release (Kerr et al. 2008). This suggests the existence of other fast Ca^{2+} sensors. Genetic studies in mice strongly indicate that synaptotagmins II and IX also function as the Ca^{2+} sensor for the synchronous component of transmitter release, in addition to synaptotagmin I (Pang et al. 2006a, b; Sun et al. 2007; Xu et al. 2007). Synaptotagmins I, II, and IX can also function as Ca^{2+} sensors for fast secretion in endocrine cells (Voets et al. 2001; Iezzi et al. 2005; Nagy et al. 2006; Lynch and Martin 2007; Zhu et al. 2007). Thus, synaptotagmins I, II, and IX are the best current candidates to serve as Ca^{2+} sensors triggering synchronous transmitter release. Although the Ca^{2+} sensor for asynchronous release is still under debate (Yao et al. 2011), it has recently been reported that synaptotagmin VII is involved in triggering asynchronous transmitter release (Bacaj et al. 2013).

Although the two synaptotagmin C_2 domains share a high degree of sequence homology with each other (41 % identity) (Perin et al. 1991), their physiological roles in neurotransmitter release clearly are unequal and nonredundant. Many physiological studies of several different research groups have clearly shown that the binding of Ca^{2+} to the C_2B domain is essential for synaptotagmin to trigger synchronous release (Littleton et al. 1994, 2001; Mackler et al. 2002; Nishiki and

Augustine 2004b; Shin et al. 2009; Yoshihara et al. 2010; Lee et al. 2013). Especially, among multiple Asp residues coordinating Ca^{2+}-binding sites in the C_2B domain, the second and third Asp (D^{309} (D2) and D^{363} (D3) in rats) residues are essential for synchronous release (Nishiki and Augustine 2004b).

It remains controversial as to whether the binding of Ca^{2+} to the C_2A domain is involved in transmitter release. Neutralizing negative charges of the second Asp (D2) residue in the C_2A domain (D^{229} in *Drosophila*), which is essential for the binding of Ca^{2+} to the synaptotagmin C_2A domain, by substituting with asparagine (Asn, N) does not alter synaptic transmission in *Drosophila* NMJ (Robinson et al. 2002). In contrast, the substitution of Asp^{229} with glutamate in the *Drosophila* synaptotagmin C_2A domain impairs transmitter release (Striegel et al. 2012). The substitution of $Asp^{232}Asn$ (D4N in the C_2A domain) in mouse synaptotagmin enhances synaptic transmission (Stevens and Sullivan 2003). Substitution of positively charged R^{233} in the Ca^{2+}-binding loop of the C_2A domain with glutamine (Gln, Q) reduces the Ca^{2+}-binding affinity of synaptotagmin and causes a corresponding reduction in the Ca^{2+} sensitivity of transmitter release (Fernández-Chacón et al. 2001). However, R^{233} is not directly involved in forming Ca^{2+}-binding sites in the C_2A domain. Taken together, these findings suggest that the C_2A domain may regulate exocytosis, but its specific function remains unclear.

8.3.2 Docking of Secretory Vesicles

Although synaptotagmin has been extensively studied as a Ca^{2+} sensor for neurotransmitter release, this is not the only function of synaptotagmin. It has been postulated that synaptotagmin also plays multiple roles in synaptic vesicle exocytosis. For example, synaptotagmin is involved in vesicle docking and fusion clamping before Ca^{2+} influx when synaptic vesicles are preparing for Ca^{2+}-triggered exocytosis. Neither synaptic vesicle depletion nor decreased vesicle docking is observed in hippocampal neurons cultured from synaptotagmin-deficient mice (Geppert et al. 1994). However, several studies show that synaptotagmin is critical for synaptic vesicle docking and maintaining docked vesicles at some synapses in *Caenorhabditis elegans*, *Drosophila,* and squid (Jorgensen et al. 1995; Reist et al. 1998; Fukuda et al. 2000; Loewen et al. 2006). Studies in neuroendocrine cells also show that synaptotagmin is implicated in vesicle docking through its binding to SNAREs located on presynaptic plasma membranes (Chieregatti et al. 2002; de Wit et al. 2009; Mohrmann et al. 2013). However, synaptotagmin is not absolutely necessary for vesicle docking because even in the synapses of *Drosophila* mutants lacking synaptotagmin, docked vesicles were observed at the release site and spontaneous transmitter release was recorded (Reist et al. 1998; Loewen et al. 2006).

The physical distance of primed synaptic vesicles to the sites of Ca^{2+} influx significantly influences the kinetics of vesicle exocytosis. To release synaptic vesicles synchronously and rapidly (within 100 μs) in response to Ca^{2+} influx

(Sabatini and Regehr 1996), they should be positioned adjacent to Ca^{2+} channels. Synaptotagmin has been reported to interact directly or indirectly with Ca^{2+} channels in vitro, and this interaction has been postulated to position synaptic vesicles to Ca^{2+} entry sites (Bennett et al. 1992; Leveque et al. 1992; Yoshida et al. 1992; Lévêque et al. 1994; Charvin et al. 1997; Sheng et al. 1997) (see Sect. 9.4.2). Recent studies in the calyx of Held demonstrate that synaptotagmin II harboring mutations in R^{399} and R^{400} greatly inhibits synchronous release, while the intrinsic Ca^{2+} sensitivity of synaptic vesicle for release is unchanged. This suggests that the protein positions releasable vesicles near Ca^{2+} channels and facilitates transmitter release (Young and Neher 2009). Similar mutations in synaptotagmin I ($R^{398}Q$ and $R^{399}Q$) also reduce synchronous release from hippocampal neurons (Xue et al. 2008). Nevertheless, the involvement of these amino acid residues in the binding of synaptotagmin to molecules at release sites, especially Ca^{2+} channels, remains to be elucidated.

8.3.3 Clamp for Spontaneous Vesicle Fusion

After docking, synaptic vesicles need to wait in a primed and fusion-competent state for evoked release. Therefore, spontaneous fusion of vesicle membranes with presynaptic membranes must be suppressed until Ca^{2+} influx. This process maintains the size of the pool of primed vesicles. Early physiological studies indicate that synaptotagmin has a fusion clamp role that blocks spontaneous vesicle exocytosis at resting Ca^{2+} levels in nerve terminals (DeBello et al. 1993; DiAntonio et al. 1993; Littleton et al. 1993; Nonet et al. 1993; Popov and Poo 1993; Broadie et al. 1994; Morimoto et al. 1995). If synaptotagmin acts as a fusion clamp, direct or indirect Ca^{2+}-independent binding of synaptotagmin to the fusion machinery (probably the *trans*-SNARE complex) would be expected. The clamping function for spontaneous release appears to be mediated by the poly-Lys region in the C_2B domain (K^{327}, K^{328}, and K^{332} in rat synaptotagmin II; Kochubey and Schneggenburger 2011) and the C_2A domain (K^{240}, K^{241}, K^{242}, and K^{243} in *Drosophila*, Mace et al. 2009).

In a recently proposed model, complexin, a presynaptic soluble protein, functions as a primary fusion clamp by binding to the *trans*-SNARE complex in a Ca^{2+}-independent manner (see Chap. 6). Subsequent binding of Ca^{2+} to synaptotagmin releases the clamp from the SNARE complex, and this triggers membrane fusion (Südhof and Rothman 2009). However, synaptotagmin can compete with complexin for binding to SNARE in the presence or absence of Ca^{2+} (Tang et al. 2006). Therefore, Ca^{2+}-independent binding of synaptotagmin appears to displace complexin from the *trans*-SNARE complex (Tang et al. 2006; Krishnakumar et al. 2011). Thus, synaptotagmin takes over the clamping role of complexin and functions as a secondary fusion clamp. Alternatively, complexin may indirectly associate with the *trans*-SNARE complex by binding to synaptotagmin (Tokumaru et al. 2008). In the latter case, synaptotagmin and

complexin may function synergistically to enhance their clamp activities (Giraudo et al. 2006). The Ca^{2+}-independent functions of synaptotagmin in fusion clamping, as well as in vesicle docking, increase a pool of release-ready vesicles, from which rapid synchronous release can occur after Ca^{2+}-dependent clamp removal. Unraveling these Ca^{2+}-independent functions of synaptotagmin will be necessary to understand how the protein works in transmitter release.

8.3.4 Fusion Pore Regulation

Ca^{2+} influx into presynaptic nerve terminals causes the vesicle membrane to fuse with the presynaptic membrane. An aqueous fusion pore is formed, and this pore connects the lumen of a vesicle with the space of the synaptic cleft. Although opening and dilating the fusion pore allows the release of neurotransmitters from vesicles, it is difficult to elucidate the exocytosis mechanism of a synaptic vesicle because its lifetime is extremely short. To analyze the nature of fusion pores electrophysiologically, detailed amperometric measurements have been performed using adrenal chromaffin cells and rat pheochromocytoma PC12 cells.

Mutations in the Ca^{2+}-binding sites of synaptotagmin alter fusion pore lifetimes and the transition of an open fusion pore to a dilating fusion pore (Wang et al. 2001, 2003a, 2006; Bai et al. 2004b). Mutations of lipid-penetrating residues in the Ca^{2+}-binding loops of synaptotagmin (see Sect. 8.4.1) also alter fusion pore dilation (Lynch et al. 2007). Furthermore, mutations that selectively impair the binding of synaptotagmin to SNAREs can destabilize open fusion pores (Bai et al. 2004b). A single-vesicle SNARE-mediated fusion assay revealed that the opening and expansion of the fusion pore also require synaptotagmin and Ca^{2+} (Lai et al. 2013). These results suggest that, during exocytosis, the assembly of synaptotagmin and SNARE complexes opens and stabilizes fusion pores by preventing them from closing. Although fusion pore dynamics are apparently regulated by Ca^{2+}-bound synaptotagmin, further studies are needed to unravel the mechanisms underlying these synaptotagmin functions.

8.3.5 Endocytosis of Synaptic Vesicles

Synaptotagmin is postulated to play a role as a Ca^{2+} sensor for endocytosis and exocytosis of synaptic vesicles. Genetic disruption of synaptotagmin in *C. elegans* demonstrates its involvement in vesicle recycling (Jorgensen et al. 1995). In *Drosophila* and mouse neurons lacking synaptotagmin, endocytosis is significantly impaired and the endocytic rate is severely lowered (Poskanzer et al. 2003; Nicholson-Tomishima and Ryan 2004). Synaptotagmin appears to control the endocytic rate through the binding of Ca^{2+} to both C_2A and C_2B domains (Yao et al. 2012a). Similar findings are observed for clathrin-mediated endocytosis in

synaptotagmin-deficient adrenal chromaffin cells (Yao et al. 2012b, but see Lee et al. 2013). Although the molecular mechanisms underlying the function of synaptotagmin in Ca^{2+}-regulated vesicle endocytosis remain unknown, it is interesting that synaptotagmin binds to proteins involved in clathrin-mediated vesicle endocytosis such as the AP-2 adapter complex (Zhang et al. 1994) and stonin 2 (Martina et al. 2001; Walther et al. 2001) (see Chap. 10). Taken together, these findings suggest that synaptotagmin plays a role as a Ca^{2+} sensor for slow clathrin-mediated endocytosis. However, synaptotagmin may not be essential for this process.

8.4 Interaction with Lipids, Inositol Polyphosphates, and Presynaptic Proteins

During synaptic vesicle exocytosis, several interactions of synaptotagmin are important mechanisms that underpin the physiological functions of the protein in neurotransmitter release. Synaptotagmin interacts with phospholipids, inositol high-polyphosphates (IHPS), and SNAREs. A critical issue is whether these interactions are Ca^{2+} independent or dependent. The original clone harbors an Asp residue at position 374, instead of glycine that is highly conserved among the synaptotagmin family (Osborne et al. 1999; Desai et al. 2000). This mutation causes misfolding of the C_2B domain (Ubach et al. 2001). Therefore, binding experiments with recombinant synaptotagmin, which were conducted before 2000, may be affected by this mutation and need to be reassessed.

8.4.1 Interaction with Lipid Membranes

Both C_2A and C_2B domains of synaptotagmin interact with phospholipid membranes in a Ca^{2+}-dependent manner (Davletov and Südhof 1993; Fernandez et al. 2001). Negatively charged phospholipids such as phosphatidylserine (PS) enhance this interaction and increase the apparent Ca^{2+} affinity of C_2 domains and thus enable them to bind Ca^{2+} in the physiological range that triggers transmitter release (Brose et al. 1992). Upon Ca^{2+} binding, hydrophobic residues in the Ca^{2+}-binding loops of the C_2 domains (methionine 173 and phenylalanine 234 in rat C_2A and valine 304 and isoleucine 367 in rat C_2B; Fig. 8.1c) penetrate the membrane (Chapman and Davis 1998; Davis et al. 1999; Herrick et al. 2006; Hui et al. 2006). These amino acids are important for the synaptotagmin C_2AB fragment to induce membrane curvature changes and to tubulate membranes in the presence of Ca^{2+} (Martens et al. 2007; Hui et al. 2009). The tubulation of liposomes by the C_2AB fragment requires the C_2B domain and its Ca^{2+}-binding sites (Martens et al. 2007; Hui et al. 2009). Atomic force microscopy reveals that the binding of

the C_2AB fragment perturbs the bilayer structure and induces the formation of stable indentations in bilayers (Shahin et al. 2008). These hydrophobic amino acids in the Ca^{2+}-binding loops seem to be important for the Ca^{2+} sensitivity of transmitter release (Rhee et al. 2005). At the *Drosophila* NMJ, membrane penetration of the C_2B domain is more important for transmitter release than that of the C_2A domain (Paddock et al. 2011).

In the absence of Ca^{2+}, synaptotagmin also binds to phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) through its C_2B domain (Schiavo et al. 1996). The interaction with PtdIns(4,5)P_2 markedly enhances the Ca^{2+}-binding affinity of synaptotagmin (Radhakrishnan et al. 2009; van den Bogaart et al. 2012). A conserved poly-Lys region of the C_2B domain (K^{326} and K^{327} in rats), critical for synchronous release (Mackler and Reist 2001; Borden et al. 2005; Li et al. 2006), is involved in the binding of synaptotagmin to PtdIns(4,5)P_2 (Bai et al. 2004a; Li et al. 2006; van den Bogaart et al. 2012). However, one study reports that mutations in the same Lys residues have no significant effect on binding to PtdIns(4,5)P_2 (Radhakrishnan et al. 2009). Two conserved Arg residues at the bottom of the C_2B domain, which are critical for synchronous release (Xue et al. 2008; Young and Neher 2009), also interact with the negatively charged phospholipid head groups in the absence of Ca^{2+} (Araç et al. 2006).

There is increasing evidence supporting the idea that synaptotagmin has an ability to bridge two membranes through Ca^{2+}-dependent and Ca^{2+}-independent lipid binding. Several different models have been proposed. In the presence of Ca^{2+}, the C_2B domain alone can bind to membranes by the Ca^{2+}-binding loops at the top and two conserved Arg residues at the bottom of the C_2B domain. This brings two membranes into close proximity within 4 nm (Araç et al. 2006). In contrast, tandem C_2 domains, but not individual domains, cross-link apposed membranes by binding individual C_2 domains to each membrane with a distance of approximately 9 nm in the presence of Ca^{2+} (Connell et al. 2008; van den Bogaart et al. 2011). While this type of model is called the direct-binding model, another model is called the oligomerization model (Seven et al. 2013). In this latter model, synaptotagmin bridges membranes through *trans* protein–protein interactions between synaptotagmins bound to different membranes via their Ca^{2+}-binding loops (Hui et al. 2011). Thus, although the mechanism underlying membrane bridging by synaptotagmin is controversial, these Ca^{2+}-dependent and Ca^{2+}-independent interactions between synaptotagmin and membrane lipids appear to be crucial for the regulation of SNARE-mediated membrane fusion.

8.4.2 Interaction with Inositol Polyphosphate

Inositol polyphosphates play important roles in cellular functions. Synaptotagmins I and II bind IHPS including inositol 1,3,4,5-tetrakisphosphate; inositol 1,3,4,5,6-pentakisphosphate; and 1,3,4,5,6-hexakisphosphate ($InsP_6$) through their C_2B domain (Fukuda et al. 1994; Niinobe et al. 1994; Mehrotra et al. 1997; Mikoshiba

et al. 1999). In neurons and adrenal chromaffin cells, the binding of IHPS to the C_2B domain appears to be important for synaptotagmin to keep the primed vesicles away from spontaneous fusion at resting levels of intracellular Ca^{2+} (Llinás et al. 1994; Ohara-Imaizumi et al. 1997; Yang et al. 2012). Recently, NMR analysis revealed that the $InsP_6$-binding site in the C_2B domain is overlapped with the AP-2-binding region (Joung et al. 2012). Indeed, the interaction between synaptotagmin with AP-2 is altered by $InsP_6$ (Mizutani et al. 1997). Likewise, the binding of IHPS to the synaptotagmin C_2B domain is suggested to be involved in endocytosis of synaptic vesicles in rat superior cervical ganglion neurons (Mochida et al. 1997). Thus, intracellular levels of IHPS appear to regulate synaptotagmin functions in synaptic vesicle cycling.

8.4.3 Ca^{2+} Dependency of Interactions with SNAREs

Synaptic vesicle exocytosis requires three neuronal SNARE proteins: syntaxin and synaptosomal-associated protein of 25 kDa (SNAP-25) on the plasma membrane (t-SNAREs) and synaptobrevin (also called vesicle-associated membrane protein) on the synaptic vesicles (v-SNARE). Therefore, the interactions between synaptotagmin and the neuronal SNAREs are important for synaptotagmin to trigger the SNARE-mediated vesicle exocytosis. Despite extensive studies over the last two decades, how synaptotagmin and SNAREs couple Ca^{2+} binding to vesicle fusion remains unclear.

Binding between native proteins has been monitored by immunoprecipitation of brain detergent extracts with antibodies against synaptotagmin or SNAREs. These studies demonstrate that synaptotagmin and SNAREs form Ca^{2+}-independent constitutive complexes (Bennett et al. 1992; Yoshida et al. 1992; Söllner et al. 1993; Rickman and Davletov 2003; Nagy et al. 2006; Masumoto et al. 2012). Some of these studies show that the interactions between native synaptotagmin and SNAREs are unaltered by Ca^{2+} (Söllner et al. 1993; Masumoto et al. 2012). Thus, these biochemical properties of the binding of synaptotagmin to SNAREs provide strong support for the hypothesis that synaptotagmin is involved in docking of vesicles and clamping of vesicle fusion in presynaptic nerve terminals at resting Ca^{2+} levels.

In contrast to studies using native proteins, there are many reports using recombinant proteins for binding experiments. In most of them, *Escherichia coli* has been routinely used as a host cell for the production of proteins fused with various affinity tags (e.g., glutathione *S*-transferase (GST) and polyhistidine), whereas baculovirus–insect cell expression systems and purification methods for full-length synaptotagmin have only been established recently (Vrljic et al. 2011). However, somewhat surprisingly, the results of binding experiments differ substantially from those for brain-derived proteins. Bacterially expressed proteins are used in solution, and the binding of bacterially expressed recombinant proteins is highly regulated by Ca^{2+} (Earles et al. 2001; Fernández-Chacón et al. 2001; Littleton et al. 2001; Zhang et al. 2002; Wang et al. 2003b; Bai et al. 2004b; Tang et al. 2006; Dai et al. 2007;

Lynch et al. 2007; Hui et al. 2009; but see Ernst and Brunger 2003). The kinetics of these Ca^{2+}-dependent interactions between synaptotagmin and SNAREs, as well as the Ca^{2+} concentration required, are within the physiological range of transmitter release (Davis et al. 1999; Tang et al. 2006). However, Ca^{2+}-induced binding of synaptotagmin to syntaxin depends on which protein is immobilized onto the beads as bait through an affinity tag in a pull-down assay (Kee and Scheller 1996). Moreover, although the interaction between synaptotagmin and SNAREs is highly sensitive to the ionic strength (Tang et al. 2006), many binding experiments have been conducted at ionic strengths that are lower than physiological conditions (50–100 mM NaCl or KCl), and more Ca^{2+}-dependent binding is observed. More recently, one-dimensional NMR spectroscopy revealed that the primary binding mode of synaptotagmin to the SNARE complex is formed by a poly-Lys region in the C_2B domain. In contrast, Ca^{2+} causes the aggregation of synaptotagmin–SNARE complex assemblies through two Arg residues at the bottom of the C_2B domain, enhancing the binding of synaptotagmin to the SNARE complex (Zhou et al. 2013). Although there are discrepancies, Ca^{2+}-dependent interactions with SNAREs in vitro have been extensively characterized because such interactions provide an attractive mechanism by which Ca^{2+} influxes could be coupled directly to membrane fusion.

8.4.4 Interaction with t-SNAREs

Brain-derived SNAREs can exist in several forms in detergent-containing solutions of binding experiments, e.g., ternary SNARE complexes, t-SNARE heterodimers consisting of syntaxin and SNAP-25, and isolated individual SNAREs. The assembly of the synaptic fusion complex probably begins with the formation of the binary complex, consisting of syntaxin and SNAP-25, on the presynaptic plasma membrane. Immunoprecipitation from brain extracts cannot tell us which SNARE protein binds to synaptotagmin in the absence of Ca^{2+}. To identify the synaptotagmin-binding partner in SNARE complexes, purified native proteins and/or recombinant proteins are required for binding experiments. Rickman and colleagues (Rickman and Davletov 2003; Rickman et al. 2004) conducted binding experiments using purified native t-SNAREs. The group combined immunoaffinity chromatography, preparative SDS-PAGE, and electroelution techniques to purify proteins from the brain. Their binding experiments show that synaptotagmin binds to the t-SNARE heterodimer in the absence of Ca^{2+} (Rickman and Davletov 2003).

The t-SNARE-binding site on synaptotagmin appears to involve the poly-Lys region in its C_2B domain as well as a tyrosine (Tyr, Y) residue at 311 (Rickman et al. 2004, 2006) corresponding to the Tyr residue that is well characterized in the *Drosophila* mutant AD3 (DiAntonio and Schwarz 1994). In contrast, the nature of the synaptotagmin-binding site on the t-SNARE heterodimer is ambiguous. When recombinant proteins expressed in the mammalian cell culture system (HEK293, human embryonic kidney cells) are used, synaptotagmin binds to syntaxin, but not

to SNAP-25, through the poly-Lys region of its C_2B domain. This suggests that synaptotagmin binds to a t-SNARE dimer through an interaction between its C_2B domain and syntaxin (Masumoto et al. 2012). However, the GST-fused C_2AB fragment of synaptotagmin shows no ability to bind to brain-derived syntaxin or SNAP-25, whereas GST-C_2AB binds to the t-SNARE heterodimer consisting of the brain-derived proteins at a 1:1:1 stoichiometrical molar ratio (Rickman and Davletov 2003). This discrepancy may be due to the denaturation of brain-derived syntaxin during preparative SDS-PAGE and its refolding through binding to SNAP-25.

Binding of the synaptotagmin GST-C_2A domain to syntaxin in brain extracts is greatly enhanced by Ca^{2+} (Li et al. 1995). Mutating Asp residues in the Ca^{2+}-binding sites of the synaptotagmin C_2A domain completely abolishes its syntaxin-binding ability (Shao et al. 1997; Davis et al. 1999). However, synaptotagmin with the same mutation is still able to trigger transmitter release at the *Drosophila* NMJ (Robinson et al. 2002). Taken together, these findings suggest that the physiological significance of the Ca^{2+}-enhanced synaptotagmin–syntaxin interaction is in processes other than Ca^{2+}-dependent synaptic vesicle exocytosis.

Ca^{2+} also increases the binding affinity of GST-SNAP-25 to the C_2AB fragment of recombinant synaptotagmin but does not alter the stoichiometry of the binding at saturation (Gerona et al. 2000). This is confirmed by cross-linking experiments (Lynch et al. 2007). C-terminal acidic amino acid residues in SNAP-25 (especially D^{179}, D^{186}, and D^{193}) are required for the Ca^{2+}-dependent binding of synaptotagmin to the SNARE complex (Gerona et al. 2000; Zhang et al. 2002; Lynch et al. 2007). Although mutations in D^{179} and D^{186} inhibit the Ca^{2+}-dependent binding of synaptotagmin to the SNARE complex by approximately 50 % (Zhang et al. 2002), mutations in the same amino acids only caused a subtle reduction in vesicle exocytosis from chromaffin cells (Sørensen et al. 2002; Mohrmann et al. 2013). While both synaptotagmin C_2 domains are required for Ca^{2+}-regulated binding to SNAP-25, the C_2A domain is partly involved in Ca^{2+}-dependent binding activity (Gerona et al. 2000). Moreover, whereas mutations in R^{199} and K^{200} in the synaptotagmin C_2A domain greatly reduce the Ca^{2+}-regulated binding (Lynch et al. 2007), these amino acids are not involved in forming Ca^{2+}-binding sites. Regardless of these findings, the physiological significance of the Ca^{2+}-enhanced synaptotagmin I–SNAP-25 interaction in Ca^{2+}-dependent synaptic vesicle exocytosis remains unclear.

8.4.5 Interactions with Membrane-Reconstituted SNAREs

The incorporation of bacterially expressed SNAREs and/or synaptotagmin to lipid membranes appears to influence their protein–protein interactions and changes their Ca^{2+} dependency. In single-molecule FRET analysis, the synaptotagmin C_2AB fragment binds to the t-SNARE heterodimer that is formed in bilayers containing full-length syntaxin in the absence of Ca^{2+} (Weninger et al. 2008). Liposomes

containing full-length synaptotagmin bind to liposomes containing binary t-SNARE complexes and PtdIns(4,5)P_2 in the absence of Ca^{2+} (Kim et al. 2012). These observations are consistent with the findings that synaptotagmin is involved in vesicle docking through binding to t-SNARE in chromaffin cells (Chieregatti et al. 2002; de Wit et al. 2009; Mohrmann et al. 2013), as discussed in Sect. 8.3.2.

In single-molecule FRET analysis, the low-affinity interaction between the synaptotagmin C_2AB fragment and trimeric *cis*-SNARE complexes in lipid bilayers is dramatically increased with Ca^{2+} addition (Bowen et al. 2005). Similar results are obtained when the C_2AB fragment binds to the *cis*-SNARE complexes reconstituted in lipid bilayers deposited within microfluidic channels (Dai et al. 2007). However, it is unclear whether Ca^{2+} enhances the binding of synaptotagmin to SNARE complexes in nerve terminals, because synaptotagmin is able to insert the Ca^{2+}-binding loops of both C_2 domains into phospholipid bilayers even in the presence of *cis*-SNARE complexes (Dai et al. 2007). Indeed, two-photon fluorescence cross-correlation spectroscopy, a powerful technique to quantify interactions between two individual fluorescently labeled liposomes, has revealed that Ca^{2+} does not significantly enhance the binding of membrane-anchored synaptotagmin to liposomes containing the *cis*-SNARE complex (Vennekate et al. 2012). Thus, although the significance of Ca^{2+}-dependent binding of synaptotagmin to membrane-embedded SNAREs remains unclear, synaptotagmin appears to initially bind to the t-SNARE heterodimer, and/or the *trans*-SNARE complex, after vesicle docking in the resting state and might still bind to the *cis*-SNARE complex during or immediately after vesicle fusion. When residual concentrations of Ca^{2+} in the release site return toward the resting level, synaptotagmin may dissociate from the *cis*-SNARE complex.

8.5 In Vitro Reconstitution of SNARE-Mediated Fusion Triggered by Ca^{2+}–Synaptotagmin

8.5.1 Fusion Between t-SNARE and v-SNARE Liposomes

Dozens of studies have attempted to establish an in vitro reconstituted fusion system consisting of only lipids and four proteins (synaptotagmin, synaptobrevin, syntaxin, and SNAP-25). However, despite the physiological evidence for synaptotagmin as the primary Ca^{2+} sensor for synaptic vesicle exocytosis, in vitro reconstitution of synaptotagmin function has proven to be very difficult. Tucker et al. (2004) and Martens et al. (2007) showed that Ca^{2+} and the synaptotagmin C_2AB fragment increase the speed of fusion between neuronal t-SNARE- and v-SNARE-reconstituted liposomes. Under these experimental conditions, the C_2AB fragment binds to t-SNAREs before Ca^{2+} addition, suppressing SNARE-mediated liposome fusion (Chicka et al. 2008). However, mutations in the Ca^{2+}-binding sites of the C_2B domain have no effect on liposome fusion, whereas mutations in the C_2A

domain completely inhibit the acceleration effect of the C_2AB fragment (Stein et al. 2007). This is opposite to the roles of synaptotagmin C_2 domains in transmitter release, as discussed in Sect. 8.3.1.

In contrast to the C_2AB fragment, some in vitro liposome fusion assays using full-length synaptotagmin reconstituted into liposomes with synaptobrevin have failed to observe a Ca^{2+}-dependent acceleration of SNARE-mediated fusion. Similar results are also obtained in a "flipped" SNARE cell–cell fusion assay (Giraudo et al. 2006). These studies have yielded ambiguous results in that while Ca^{2+} has no effect on SNARE-mediated fusion in the presence of full-length synaptotagmin (Mahal et al. 2002; Giraudo et al. 2006), Ca^{2+}–synaptotagmin inhibits fusion when both t-SNARE and v-SNARE liposomes contain PS (Stein et al. 2007). In another study, fusion is enhanced at 10 μM Ca^{2+} but decreased at higher Ca^{2+} concentrations (100 μM) (Lee et al. 2010). Moreover, one report indicates that Ca^{2+} accelerates liposome fusion only at $\geq$2 mM (Kyoung et al. 2011). At (almost) physiological ionic strength (100–150 mM potassium salts), full-length synaptotagmin increases fusion between t-SNARE and v-SNARE liposomes in a Ca^{2+}-independent manner, although Ca^{2+}–synaptotagmin triggers SNARE-mediated liposome fusion at low ionic strength (5 mM KCl) (Mahal et al. 2002; Stein et al. 2007; van den Bogaart et al. 2011).

Wang et al. (2011) recently reported that reconstituted full-length synaptotagmin can trigger the acceleration of SNARE-mediated liposome fusion in a Ca^{2+} concentration-dependent manner ($[Ca^{2+}]_{1/2} = 250$ μM). This occurs when t-SNARE-reconstituted liposomes contain PS and $PtdIns(4,5)P_2$ and are preincubated with v-SNARE-reconstituted liposomes before the addition of Ca^{2+} to the fusion assay. In their fusion assay system, the binding of Ca^{2+} to the C_2B domain is more important than that to the C_2A domain, which is very similar in neurons. Full-length, membrane-embedded synaptotagmin has no ability to clamp SNARE-mediated fusion in the absence of Ca^{2+} (Wang et al. 2011), which is different from the findings for the C_2AB fragment (Chicka et al. 2008). In Ca^{2+}-dependent neurotransmitter release, it is thought that synaptic vesicles are required to be docked and primed at the release site by a mechanism that is not yet understood. For instance, complexin, which binds to assembled SNAREs, has been proposed to clamp the SNARE complex until Ca^{2+} influx, suggesting a cooperative action with synaptotagmin (Südhof and Rothman 2009). Obviously, more work is needed for deciphering the clamping function of synaptotagmin in vitro.

8.5.2 Fusion of Synaptic Vesicles with t-SNARE Liposomes

Instead of using v-SNARE liposomes reconstituted with full-length synaptotagmin, secretory vesicles isolated from animal tissue have been used for the in vitro fusion assay. Synaptic vesicles purified from the rat brain can fuse constitutively with t-SNARE liposomes containing syntaxin and SNAP-25 (Holt et al. 2008). However, fusion of synaptic vesicles with t-SNARE liposomes under these experimental

conditions shows no Ca^{2+} dependency (but see Hu et al. 2002). This may be due to the *cis* association of synaptotagmin to synaptic vesicle membranes, which contains 15 % PS (Takamori et al. 2006), because when synaptotagmin is incorporated in a PS-containing membrane, the protein binds to its own membrane by Ca^{2+} and prevents itself from binding to target membranes (Stein et al. 2007; Vennekate et al. 2012). This is in agreement with findings that the C_2AB fragment of synaptotagmin is inserted into t-SNARE liposomes by Ca^{2+} to accelerate the fusion of these liposomes with v-SNARE liposomes (Chicka et al. 2008). In vivo, this *cis* association of synaptotagmin to secretory vesicles may be suppressed by ATP (Park et al. 2012).

8.6 Molecular Mechanisms of Action of Synaptotagmin During Synaptic Vesicle Docking, Priming, and Fusion

As described above, extensive evidence indicates that synaptotagmin acts as a Ca^{2+} sensor in neurotransmitter release, but its mechanism of action, especially the role of the interaction between synaptotagmin and the SNARE complex, remains unclear. Figure 8.2 illustrates one of the current models for the possible roles of synaptotagmin in transmitter release. In this model, synaptotagmin binds to the t-SNARE heterodimer through the interaction between its C_2B domain and syntaxin at resting Ca^{2+} levels during or after vesicle docking.

During vesicle priming, synaptotagmin suppresses spontaneous SNARE-mediated vesicle fusion until Ca^{2+} enters. One possible mechanism would be that synaptotagmin indirectly prevents the assembly of the SNARE complex by regulating the distance between the vesicles and the plasma membrane (the 2nd in Fig. 8.2). When synaptotagmin binds to t-SNAREs, the conserved two Arg residues at the bottom of the C_2B domain appear to face toward either of the two membranes and bind to the membrane through the interaction between positive charges of these Arg residues and negative charges of the surface of the membranes. In this case, Ca^{2+}-binding sites on the top of the C_2B domain would face toward the other membrane. Because the Ca^{2+}-binding sites are coordinated by negatively charged multiple Asp residues, repulsive forces would generate between these residues and phospholipid membranes, suppressing the assembly of the SNARE complex. After Ca^{2+} binding, these negative charges on the top of the C_2B domain are neutralized and the repulsive forces between the two membranes disappear (the 3rd in Fig. 8.2). Simultaneously, the tips of the C_2 domains penetrate the membranes upon Ca^{2+} binding and locally bend the membrane, inducing curvature. These Ca^{2+}-dependent interactions between synaptotagmin and the membranes could remove the fusion clamp from the *trans*-SNARE complex, accelerating SNARE-mediated fusion (the 4th in Fig. 8.2).

Alternatively or concomitantly, synaptotagmin acts as a fusion clamp by directly blocking the full assembly of the partially assembled *trans*-SNARE complex. In

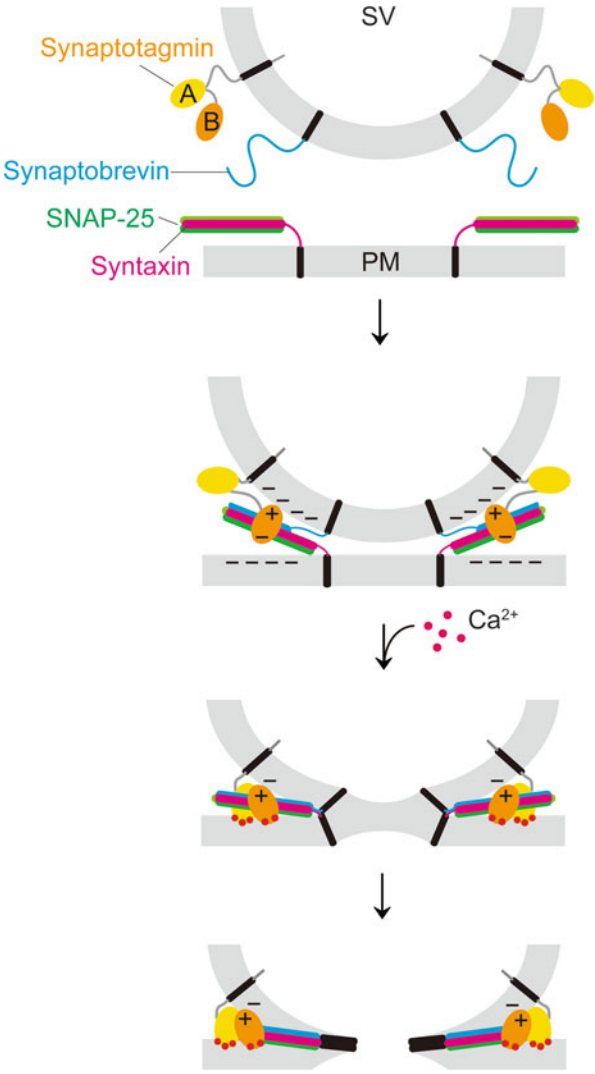

Fig. 8.2 A model for the possible roles of synaptotagmin during neurotransmitter release. *SV* indicates a synaptic vesicle, and *PM* indicates the presynaptic plasma membrane; symbols represent C_2A (*A*) and C_2B (*B*) domains of synaptotagmin; + and − indicate positive and negative charges, respectively. Ca^{2+} ions are shown as *small red spheres*. The N-terminal half (Habc) region of syntaxin and the linker region of SNAP-25 are not shown for simplicity

this case, synaptotagmin and complexin may function synergistically to enhance their clamp activities (Giraudo et al. 2006). Upon Ca^{2+} binding, synaptotagmin may relieve the clamp by disengaging from SNAREs (Leveque et al. 2000) or by Ca^{2+}-dependent higher-affinity binding to SNAREs, thus displacing the primary clamp complexin (Yang et al. 2010).

8.7 Conclusions

We have summarized the structure and function of synaptotagmin and its interactions with phospholipids and SNAREs. Extensive studies over the past quarter century strongly suggest that synaptotagmin serves as the Ca^{2+} sensor for the synchronous component of evoked neurotransmitter release. Although the mechanisms mediating the function of synaptotagmin in Ca^{2+}-dependent evoked release remain unknown, the binding of synaptotagmin to SNAREs and phospholipids appears to be involved. Evidence summarized in this review suggests that synaptotagmin most likely acts on the *trans*-SNARE complex during or after vesicle docking in a Ca^{2+}-independent manner and synchronizes SNARE-mediated vesicle fusion at least by interacting with phospholipid membranes upon Ca^{2+} binding. Several important questions remain to be answered. For example, does synaptotagmin directly act on SNARE complexes, alter their conformation, and facilitate their full assembly in a Ca^{2+}-dependent manner to trigger membrane fusion? How do synaptotagmin and complexin work together to clamp SNARE-mediated fusion? How do synaptotagmin, a Ca^{2+} sensor for synchronous release, and Ca^{2+} sensors for asynchronous release regulate the balance of these two components of transmitter release? Most importantly, would it be possible for synaptotagmin to exert its function as a Ca^{2+} sensor within 100 μs after Ca^{2+} influx in the nerve terminal? Further in vitro and in vivo studies will surely provide new exciting insights into the detailed mechanism of the molecular machinery governing evoked transmitter release.

Acknowledgment This work was supported by JSPS KAKENHI 26460297 to TN. The authors would like to thank Enago (www.enago.jp) for the English language review.

References

Araç D, Chen X, Khant HA, Ubach J, Ludtke SJ, Kikkawa M, Johnson AE, Chiu W, Südhof TC, Rizo J (2006) Close membrane-membrane proximity induced by Ca^{2+}-dependent multivalent binding of synaptotagmin-1 to phospholipids. Nat Struct Mol Biol 13:209–217

Bacaj T, Wu D, Yang X, Morishita W, Zhou P, Xu W, Malenka RC, Südhof TC (2013) Synaptotagmin-1 and synaptotagmin-7 trigger synchronous and asynchronous phases of neurotransmitter release. Neuron 80:947–959

Bai J, Tucker WC, Chapman ER (2004a) PIP_2 increases the speed of response of synaptotagmin and steers its membrane-penetration activity toward the plasma membrane. Nat Struct Mol Biol 11:36–44

Bai J, Wang CT, Richards DA, Jackson MB, Chapman ER (2004b) Fusion pore dynamics are regulated by synaptotagmin•t-SNARE interactions. Neuron 41:929–942

Bennett MK, Calakos N, Scheller RH (1992) Syntaxin: a synaptic protein implicated in docking of synaptic vesicles at presynaptic active zones. Science 257:255–259

Bommert K, Charlton MP, DeBello WM, Chin GJ, Betz H, Augustine GJ (1993) Inhibition of neurotransmitter release by C2-domain peptides implicates synaptotagmin in exocytosis. Nature 363:163–165

Borden CR, Stevens CF, Sullivan JM, Zhu Y (2005) Synaptotagmin mutants Y311N and K326/327A alter the calcium dependence of neurotransmission. Mol Cell Neurosci 29:462–470

Bowen ME, Weninger K, Ernst J, Chu S, Brunger AT (2005) Single-molecule studies of synaptotagmin and complexin binding to the SNARE complex. Biophys J 89:690–702

Broadie K, Bellen HJ, DiAntonio A, Littleton JT, Schwarz TL (1994) Absence of synaptotagmin disrupts excitation-secretion coupling during synaptic transmission. Proc Natl Acad Sci USA 91:10727–10731

Brose N, Petrenko AG, Südhof TC, Jahn R (1992) Synaptotagmin: a calcium sensor on the synaptic vesicle surface. Science 256:1021–1025

Chapman ER, Davis AF (1998) Direct interaction of a Ca^{2+}-binding loop of synaptotagmin with lipid bilayers. J Biol Chem 273:13995–14001

Charvin N, L'Eveque C, Walker D, Berton F, Raymond C, Kataoka M, Shoji-Kasai Y, Takahashi M, De Waard M, Seagar MJ (1997) Direct interaction of the calcium sensor protein synaptotagmin I with a cytoplasmic domain of the alpha1A subunit of the P/Q-type calcium channel. EMBO J 16:4591–4596

Cheng Y, Sequeira SM, Malinina L, Tereshko V, Söllner TH, Patel DJ (2004) Crystallographic identification of Ca^{2+} and Sr^{2+} coordination sites in synaptotagmin I C_2B domain. Protein Sci 10:2665–2672

Chicka MC, Hui E, Liu H, Chapman ER (2008) Synaptotagmin arrests the SNARE complex before triggering fast, efficient membrane fusion in response to Ca^{2+}. Nat Struct Mol Biol 15:827–835

Chieregatti E, Witkin JW, Baldini G (2002) SNAP-25 and synaptotagmin 1 function in Ca^{2+}-dependent reversible docking of granules to the plasma membrane. Traffic 3:496–511

Choi UB, Strop P, Vrljic M, Chu S, Brunger AT, Weninger KR (2010) Single-molecule FRET-derived model of the synaptotagmin 1–SNARE fusion complex. Nat Struct Mol Biol 17:318–324

Connell E, Giniatullina A, Lai-Kee-Him J, Tavare R, Ferrari E, Roseman A, Cojoc D, Brisson AR, Davletov B (2008) Cross-linking of phospholipid membranes is a conserved property of calcium-sensitive synaptotagmins. J Mol Biol 380:42–50

Craxton M (2010) A manual collection of *Syt*, *Esyt*, *Rph3a*, *Rph3al*, *Doc2*, and *Dblc2* genes from 46 metazoan genomes – an open access resource for neuroscience and evolutionary biology. BMC Genomics 11:37

Craxton M, Goedert M (1995) Synaptotagmin V: a novel synaptotagmin isoform expressed in rat brain. FEBS Lett 361:196–200

Dai H, Shen N, Araç D, Rizo J (2007) A quaternary SNARE–synaptotagmin–Ca^{2+}–phospholipid complex in neurotransmitter release. J Mol Biol 367:848–863

Davis AF, Bai J, Fasshauer D, Wolowick MJ, Lewis JL, Chapman ER (1999) Kinetics of synaptotagmin responses to Ca^{2+} and assembly with the core SNARE complex onto membranes. Neuron 24:363–376

Davletov BA, Südhof TC (1993) A single C_2 domain from synaptotagmin I is sufficient for high affinity Ca^{2+}/phospholipid binding. J Biol Chem 268:26386–26390

de Wit H, Walter AM, Milosevic I, Gulyas-Kovacs A, Riedel D, Sørensen JB, Verhage M (2009) Synaptotagmin-1 docks secretory vesicles to syntaxin-1/SNAP-25 acceptor complexes. Cell 138:935–946

DeBello WM, Betz H, Augustine GJ (1993) Synaptotagmin and neurotransmitter release. Cell 74:947–950

Desai RC, Vyas B, Earles CA, Littleton JT, Kowalchyck JA, Martin TF, Chapman ER (2000) The C2B domain of synaptotagmin is a Ca^{2+}-sensing module essential for exocytosis. J Cell Biol 150:1125–1136

DiAntonio A, Schwarz TL (1994) The effect on synaptic physiology of *synaptotagmin* mutations in Drosophila. Neuron 12:909–920

DiAntonio A, Parfitt KD, Schwarz TL (1993) Synaptic transmission persists in *synaptotagmin* mutants of Drosophila. Cell 73:1281–1290

Earles CA, Bai J, Wang P, Chapman ER (2001) The tandem C2 domains of synaptotagmin contain redundant Ca^{2+} binding sites that cooperate to engage t-SNAREs and trigger exocytosis. J Cell Biol 154:1117–1123

Elferink LA, Peterson MR, Scheller RH (1993) A role for synaptotagmin (p65) in regulated exocytosis. Cell 72:153–159

Ernst JA, Brunger AT (2003) High resolution structure, stability, and synaptotagmin binding of a truncated neuronal SNARE complex. J Biol Chem 278:8630–8636

Fernandez I, Araç D, Ubach J, Gerber SH, Shin O, Gao Y, Anderson RG, Südhof TC, Rizo J (2001) Three-dimensional structure of the synaptotagmin 1 C_2B-domain: synaptotagmin 1 as a phospholipid binding machine. Neuron 32:1057–1069

Fernández-Chacón R, Königstorfer A, Gerber SH, Garcia J, Matos MF, Stevens CF, Brose N, Rizo J, Rosenmund C, Südhof TC (2001) Synaptotagmin I functions as a calcium regulator of release probability. Nature 410:41–49

Fox MA, Sanes JR (2007) Synaptotagmin I and II are present in distinct subsets of central synapses. J Comp Neurol 503:280–296

Fukuda M (2003) Molecular cloning, expression, and characterization of a novel class of synaptotagmin (Syt XIV) conserved from *Drosophila* to humans. J Biochem 133:641–649

Fukuda M, Aruga J, Niinobe M, Aimoto S, Mikoshiba K (1994) Inositol-1,3,4,5-tetrakisphosphate binding to C2B domain of IP4BP/synaptotagmin II. J Biol Chem 269:29206–29211

Fukuda M, Moreira JE, Lewis FM, Sugimori M, Niinobe M, Mikoshiba K, Llinás R (1995) Role of the C2B domain of synaptotagmin in vesicular release and recycling as determined by specific antibody injection into the squid giant synapse preterminal. Proc Natl Acad Sci USA 92:10708–10712

Fukuda M, Moreira JE, Liu V, Sugimori M, Mikoshiba K, Llinas RR (2000) Role of the conserved WHXL motif in the C terminus of synaptotagmin in synaptic vesicle docking. Proc Natl Acad Sci USA 97:14715–14719

Fuson KL, Montes M, Robert JJ, Sutton RB (2007) Structure of human synaptotagmin 1 C2AB in the absence of Ca^{2+} reveals a novel domain association. Biochemistry 46:13041–13048

Geppert M, Archer BT III, Südhof TC (1991) Synaptotagmin II. A novel differentially distributed form of synaptotagmin. J Biol Chem 266:13548–13552

Geppert M, Goda Y, Hammer RE, Li C, Rosahl TW, Stevens CF, Südhof TC (1994) Synaptotagmin I: a major Ca^{2+} sensor for transmitter release at a central synapse. Cell 79:717–727

Gerona RR, Larsen EC, Kowalchyk JA, Martin TF (2000) The C terminus of SNAP25 is essential for Ca^{2+}-dependent binding of synaptotagmin to SNARE complexes. J Biol Chem 275:6328–6336

Giraudo CG, Eng WS, Melia TJ, Rothman JE (2006) A clamping mechanism involved in SNARE-dependent exocytosis. Science 313:676–680

Goda Y, Stevens CF (1994) Two components of transmitter release at a central synapse. Proc Natl Acad Sci USA 91:12942–12946

Herrick DZ, Sterbling S, Rasch KA, Hinderliter A, Cafiso DS (2006) Position of synaptotagmin I at the membrane interface: cooperative interactions of tandem C2 domains. Biochemistry 45:9668–9674

Herrick DZ, Kuo W, Huang H, Schwieters CD, Ellena JF, Cafiso DS (2009) Solution and membrane-bound conformations of the tandem C2A and C2B domains of synaptotagmin 1: evidence for bilayer bridging. J Mol Biol 390:913–923

Holt M, Riedel D, Stein A, Schuette C, Jahn R (2008) Synaptic vesicles are constitutively active fusion machines that function independently of Ca^{2+}. Curr Biol 18:715–722

Hu K, Carroll J, Fedorovich S, Rickman C, Sukhodub A, Davletov B (2002) Vesicular restriction of synaptobrevin suggests a role for calcium in membrane fusion. Nature 415:646–650

Hudson AW, Birnbaum MJ (1995) Identification of a nonneuronal isoform of synaptotagmin. Proc Natl Acad Sci USA 92:5895–5899

Hui E, Bai J, Chapman ER (2006) Ca^{2+}-triggered simultaneous membrane penetration of the tandem C2-domains of synaptotagmin I. Biophys J 91:1767–1777

Hui E, Johnson CP, Yao J, Dunning FM, Chapman ER (2009) Synaptotagmin-mediated bending of the target membrane is a critical step in Ca^{2+}-regulated fusion. Cell 138:709–721

Hui E, Gaffaney JD, Wang Z, Johnson CP, Evans CS, Chapman ER (2011) Mechanism and function of synaptotagmin-mediated membrane apposition. Nat Struct Mol Biol 18:813–821

Iezzi M, Eliasson L, Fukuda M, Wollheim CB (2005) Adenovirus-mediated silencing of synaptotagmin 9 inhibits Ca^{2+}-dependent insulin secretion in islets. FEBS Lett 579:5241–5246

Jorgensen EM, Hartwieg E, Schuske K, Nonet ML, Jin Y, Horvitz HR (1995) Defective recycling of synaptic vesicles in synaptotagmin mutants of *Caenorhabditis elegans*. Nature 378:196–199

Joung M-J, Mohan SK, Yu C (2012) Molecular level interaction of inositol hexaphosphate with the C2B domain of human synaptotagmin I. Biochemistry 51:3675–3683

Kee Y, Scheller RH (1996) Localization of synaptotagmin-binding domains on syntaxin. J Neurosci 16:1975–1981

Kerr AM, Reisinger E, Jonas P (2008) Differential dependence of phasic transmitter release on synaptotagmin 1 at GABAergic and glutamatergic hippocampal synapses. Proc Natl Acad Sci USA 105:15581–15586

Kim JY, Choi BK, Choi MG, Kim SA, Lai Y, Shin YK, Lee NK (2012) Solution single-vesicle assay reveals PIP_2-mediated sequential actions of synaptotagmin-1 on SNAREs. EMBO J 31:2144–2155

Kochubey O, Schneggenburger R (2011) Synaptotagmin increases the dynamic range of synapses by driving Ca^{2+}-evoked release and by clamping a near-linear remaining Ca^{2+} sensor. Neuron 69:736–748

Krishnakumar SS, Radoff DT, Kummel D, Giraudo CG, Li F, Khandan L, Baguley SW, Coleman J, Reinisch KM, Pincet F, Rothman JE (2011) A conformational switch in complexin is required for synaptotagmin to trigger synaptic fusion. Nat Struct Mol Biol 18:934–940

Kyoung M, Srivastava A, Zhang Y, Diao J, Vrljic M, Grob P, Nogales E, Chu S, Brunger AT (2011) In vitro system capable of differentiating fast Ca^{2+}-triggered content mixing from lipid exchange for mechanistic studies of neurotransmitter release. Proc Natl Acad Sci USA 108: E304–E313

Lai AL, Huang H, Herrick DZ, Epp N, Cafiso DS (2011) Synaptotagmin 1 and SNAREs form a complex that is structurally heterogeneous. J Mol Biol 405:696–706

Lai Y, Diao J, Liu Y, Ishitsuka Y, Su Z, Schulten K, Ha T, Shin Y-K (2013) Fusion pore formation and expansion induced by Ca^{2+} and synaptotagmin 1. Proc Natl Acad Sci USA 110:1333–1338

Lee HK, Yang Y, Su Z, Hyeon C, Lee TS, Lee HW, Kweon DH, Shin YK, Yoon TY (2010) Dynamic Ca^{2+}-dependent stimulation of vesicle fusion by membrane-anchored synaptotagmin 1. Science 328:760–763

Lee J, Guan Z, Akbergenova Y, Littleton JT (2013) Genetic analysis of synaptotagmin C2 domain specificity in regulating spontaneous and evoked neurotransmitter release. J Neurosci 33:187–200

Leveque C, Hoshino T, David P, Shoji-Kasai Y, Leys K, Omori A, Lang B, el Far O, Sato K, Martin-Moutot N, Newsom-Davis J, Takahashi M, Seagar MJ (1992) The synaptic vesicle protein synaptotagmin associates with calcium channels and is a putative Lambert-Eaton myasthenic syndrome antigen. Proc Natl Acad Sci USA 89:3625–3629

Lévêque C, el Far O, Martin-Moutot N, Sato K, Kato R, Takahashi M, Seagar MJ (1994) Purification of the N-type calcium channel associated with syntaxin and synaptotagmin. A complex implicated in synaptic vesicle exocytosis. J Biol Chem 269:6306–6312

Leveque C, Boudier JA, Takahashi M, Seagar M (2000) Calcium-dependent dissociation of synaptotagmin from synaptic SNARE complexes. J Neurochem 74:367–374

Li C, Ullrich B, Zhang JZ, Anderson RG, Brose N, Südhof TC (1995) Ca^{2+}-dependent and -independent activities of neural and non-neural synaptotagmins. Nature 375:594–599

Li L, Shin OH, Rhee JS, Araç D, Rah JC, Rizo J, Südhof T, Rosenmund C (2006) Phosphatidylinositol phosphates as co-activators of Ca^{2+} binding to C_2 domains of synaptotagmin 1. J Biol Chem 281:15845–15852

Littleton JT, Stern M, Schulze K, Perin M, Bellen HJ (1993) Mutational analysis of Drosophila *synaptotagmin* demonstrates its essential role in Ca^{2+}-activated neurotransmitter release. Cell 74:1125–1134

Littleton JT, Stern M, Perin M, Bellen HJ (1994) Calcium dependence of neurotransmitter release and rate of spontaneous vesicle fusions are altered in *Drosophila* synaptotagmin mutants. Proc Natl Acad Sci USA 91:10888–10892

Littleton JT, Bai J, Vyas B, Desai R, Baltus AE, Garment MB, Carlson SD, Ganetzky B, Chapman ER (2001) *Synaptotagmin* mutants reveal essential functions for the C2B domain in Ca^{2+}-triggered fusion and recycling of synaptic vesicles *in vivo*. J Neurosci 21:1421–1433

Llinás R, Sugimori M, Lang EJ, Morita M, Fukuda M, Niinobe M, Mikoshiba K (1994) The inositol high-polyphosphate series blocks synaptic transmission by preventing vesicular fusion: a squid giant synapse study. Proc Natl Acad Sci USA 91:12990–12993

Loewen CA, Lee SM, Shin YK, Reist NE (2006) C_2B polylysine motif of synaptotagmin facilitates a Ca^{2+}-independent stage of synaptic vesicle priming in vivo. Mol Biol Cell 17:5211–5226

Lynch KL, Martin TF (2007) Synaptotagmins I and IX function redundantly in regulated exocytosis but not endocytosis in PC12 cells. J Cell Sci 120:617–627

Lynch KL, Gerona RR, Larsen EC, Marcia RF, Mitchell JC, Martin TF (2007) Synaptotagmin C2A loop 2 mediates Ca^{2+}-dependent SNARE interactions essential for Ca^{2+}-triggered vesicle exocytosis. Mol Biol Cell 18:4957–4968

Mace KE, Biela LM, Sares AG, Reist NE (2009) Synaptotagmin I stabilizes synaptic vesicles via its C_2A polylysine motif. Genesis 47:337–345

Mackler JM, Reist NE (2001) Mutations in the second C_2 domain of synaptotagmin disrupt synaptic transmission at *Drosophila* neuromuscular junctions. J Comp Neurol 436:4–16

Mackler JM, Drummond JA, Loewen CA, Robinson IM, Reist NE (2002) The C_2B Ca^{2+}-binding motif of synaptotagmin is required for synaptic transmission *in vivo*. Nature 418:340–344

Mahal LK, Sequeira SM, Gureasko JM, Söllner TH (2002) Calcium-independent stimulation of membrane fusion and SNAREpin formation by synaptotagmin I. J Cell Biol 158:273–282

Marquèze B, Boudier JA, Mizuta M, Inagaki N, Seino S, Seagar M (1995) Cellular localization of synaptotagmin I, II, and III mRNAs in the central nervous system and pituitary and adrenal glands of the rat. J Neurosci 15:4906–4917

Martens S, Kozlov MM, McMahon HT (2007) How synaptotagmin promotes membrane fusion. Science 316:1205–1208

Martina JA, Bonangelino CJ, Aguilar RC, Bonifacino JS (2001) Stonin 2: an adaptor-like protein that interacts with components of the endocytic machinery. J Cell Biol 153:1111–1120

Masumoto T, Suzuki K, Ohmori I, Michiue H, Tomizawa K, Fujimura A, Nishiki T, Matsui H (2012) Ca^{2+}-independent syntaxin binding to the C_2B effector region of synaptotagmin. Mol Cell Neurosci 49:1–8

Matsuoka H, Harada K, Nakamura J, Fukuda M, Inoue M (2011) Differential distribution of synaptotagmin-1, -4, -7, and -9 in rat adrenal chromaffin cells. Cell Tissue Res 344:41–50

Matthew WD, Tsavaler L, Reichardt LF (1981) Identification of a synaptic vesicle-specific membrane protein with a wide distribution in neuronal and neurosecretory tissue. J Cell Biol 91:257–269

Mehrotra B, Elliott JT, Chen J, Olszewski JD, Profit AA, Chaudhary A, Fukuda M, Mikoshiba K, Prestwich GD (1997) Selective photoaffinity labeling of the inositol polyphosphate binding C_2B domains of synaptotagmins. J Biol Chem 272:4237–4244

Mikoshiba K, Fukuda M, Ibata K, Kabayama H, Mizutani A (1999) Role of synaptotagmin, a Ca^{2+} and inositol polyphosphate binding protein, in neurotransmitter release and neurite outgrowth. Chem Phys Lipids 98:59–67

Mittelsteadt T, Seifert G, Alvarez-Baron E, Steinhauser C, Becker AJ, Schoch S (2009) Differential mRNA expression patterns of the synaptotagmin gene family in the rodent brain. J Comp Neurol 512:514–528

Mizutani A, Fukuda M, Niinobe M, Mikoshiba K (1997) Regulation of AP-2-synaptotagmin interaction by inositol high polyphosphates. Biochem Biophys Res Commun 240:128–131

Mochida S, Fukuda M, Niinobe M, Kobayashi H, Mikoshiba K (1997) Roles of synaptotagmin C2 domains in neurotransmitter secretion and inositol high-polyphosphate binding at mammalian cholinergic synapses. Neuroscience 77:937–943

Mohrmann R, de Wit H, Connell E, Pinheiro PS, Leese C, Bruns D, Davletov B, Verhage M, Sørensen JB (2013) Synaptotagmin interaction with SNAP-25 governs vesicle docking, priming, and fusion triggering. J Neurosci 33:14417–14430

Morimoto T, Popov S, Buckley KM, Poo MM (1995) Calcium-dependent transmitter secretion from fibroblasts: modulation by synaptotagmin I. Neuron 15:689–696

Nagy G, Kim JH, Pang ZP, Matti U, Rettig J, Südhof TC, Sørensen JB (2006) Different effects on fast exocytosis induced by synaptotagmin 1 and 2 isoforms and abundance but not by phosphorylation. J Neurosci 26:632–643

Nicholson-Tomishima K, Ryan TA (2004) Kinetic efficiency of endocytosis at mammalian CNS synapses requires synaptotagmin I. Proc Natl Acad Sci USA 101:16648–16652

Niinobe M, Yamaguchi Y, Fukuda M, Mikoshiba K (1994) Synaptotagmin is an inositol polyphosphate binding protein: isolation and characterization as an Ins 1,3,4,5-P4 binding protein. Biochem Biophys Res Commun 205:1036–1042

Nishiki T, Augustine GJ (2004a) Synaptotagmin I synchronizes transmitter release in mouse hippocampal neurons. J Neurosci 24:6127–6132

Nishiki T, Augustine GJ (2004b) Dual roles of the C_2B domain of synaptotagmin I in synchronizing Ca^{2+}-dependent neurotransmitter release. J Neurosci 24:8542–8550

Nishizuka Y (1988) The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature 334:661–665

Nonet ML, Grundahl K, Meyer BJ, Rand JB (1993) Synaptic function is impaired but not eliminated in C. elegans mutants lacking synaptotagmin. Cell 73:1291–1305

Ohara-Imaizumi M, Fukuda M, Niinobe M, Misonou H, Ikeda K, Murakami T, Kawasaki M, Mikoshiba K, Kumakura K (1997) Distinct roles of C2A and C2B domains of synaptotagmin in the regulation of exocytosis in adrenal chromaffin cells. Proc Natl Acad Sci USA 94:287–291

Osborne SL, Herreros J, Bastiaens PI, Schiavo G (1999) Calcium-dependent oligomerization of synaptotagmins I and II. Synaptotagmins I and II are localized on the same synaptic vesicle and heterodimerize in the presence of calcium. J Biol Chem 274:59–66

Paddock BE, Wang Z, Biela LM, Chen K, Getzy MD, Striegel A, Richmond JE, Chapman ER, Featherstone DE, Reist NE (2011) Membrane penetration by synaptotagmin is required for coupling calcium binding to vesicle fusion *in vivo*. J Neurosci 31:2248–2257

Pang ZP, Melicoff E, Padgett D, Liu Y, Teich AF, Dickey BF, Lin W, Adachi R, Südhof TC (2006a) Synaptotagmin-2 is essential for survival and contributes to Ca^{2+} triggering of neurotransmitter release in central and neuromuscular synapses. J Neurosci 26:13493–13504

Pang ZP, Sun J, Rizo J, Maximov A, Südhof TC (2006b) Genetic analysis of synaptotagmin 2 in spontaneous and Ca^{2+}-triggered neurotransmitter release. EMBO J 25:2039–2050

Park Y, Hernandez JM, van den Bogaart G, Ahmed S, Holt M, Riedel D, Jahn R (2012) Controlling synaptotagmin activity by electrostatic screening. Nat Struct Mol Biol 19:991–997

Perin MS, Fried VA, Mignery GA, Jahn R, Südhof TC (1990) Phospholipid binding by a synaptic vesicle protein homologous to the regulatory region of protein kinase C. Nature 345:260–263

Perin MS, Brose N, Jahn R, Südhof TC (1991) Domain structure of synaptotagmin (p65). J Biol Chem 266:623–629

Popov SV, Poo MM (1993) Synaptotagmin: a calcium-sensitive inhibitor of exocytosis? Cell 73:1247–1249

Poskanzer KE, Marek KW, Sweeney ST, Davis GW (2003) Synaptotagmin I is necessary for compensatory synaptic vesicle endocytosis in vivo. Nature 426:559–563

Radhakrishnan A, Stein A, Jahn R, Fasshauer D (2009) The Ca^{2+} affinity of synaptotagmin 1 is markedly increased by a specific interaction of its C2B domain with phosphatidylinositol 4,5-bisphosphate. J Biol Chem 284:25749–25760

Reist NE, Buchanan J, Li J, DiAntonio A, Buxton EM, Schwarz TL (1998) Morphologically docked synaptic vesicles are reduced in synaptotagmin mutants of *Drosophila*. J Neurosci 18:7662–7673

Rhee JS, Li LY, Shin OH, Rah JC, Rizo J, Südhof TC, Rosenmund C (2005) Augmenting neurotransmitter release by enhancing the apparent Ca^{2+} affinity of synaptotagmin 1. Proc Natl Acad Sci USA 102:18664–18669

Rickman C, Davletov B (2003) Mechanism of calcium-independent synaptotagmin binding to target SNAREs. J Biol Chem 278:5501–5504

Rickman C, Archer DA, Meunier FA, Craxton M, Fukuda M, Burgoyne RD, Davletov B (2004) Synaptotagmin interaction with the syntaxin/SNAP-25 dimer is mediated by an evolutionarily conserved motif and is sensitive to inositol hexakisphosphate. J Biol Chem 279:12574–12579

Rickman C, Jiménez JL, Graham ME, Archer DA, Soloviev M, Burgoyne RD, Davletov B (2006) Conserved prefusion protein assembly in regulated exocytosis. Mol Biol Cell 17:283–294

Robinson IM, Ranjan R, Schwarz TL (2002) Synaptotagmins I and IV promote transmitter release independently of Ca^{2+} binding in the C_2A domain. Nature 418:336–340

Sabatini BL, Regehr WG (1996) Timing of neurotransmission at fast synapses in the mammalian brain. Nature 384:170–172

Schiavo G, Gu QM, Prestwich GD, Söllner TH, Rothman JE (1996) Calcium-dependent switching of the specificity of phosphoinositide binding to synaptotagmin. Proc Natl Acad Sci USA 93:13327–13332

Seven AB, Brewer KD, Shi L, Jiang Q-X, Rizo J (2013) Prevalent mechanism of membrane bridging by synaptotagmin-1. Proc Natl Acad Sci USA 110:E3243–E3252

Shahin V, Datta D, Hui E, Henderson RM, Chapman ER, Edwardson JM (2008) Synaptotagmin perturbs the structure of phospholipid bilayers. Biochemistry 47:2143–2152

Shao X, Davletov BA, Sutton RB, Südhof TC, Rizo J (1996) Bipartite Ca^{2+}-binding motif in C_2 domains of synaptotagmin and protein kinase C. Science 273:248–251

Shao X, Li C, Fernandez I, Zhang X, Südhof TC, Rizo J (1997) Synaptotagmin-syntaxin interaction: the C_2 domain as a Ca^{2+}-dependent electrostatic switch. Neuron 18:133–142

Shao X, Fernandez I, Südhof TC, Rizo J (1998) Solution structures of the Ca^{2+}-free and Ca^{2+}-bound C_2A domain of synaptotagmin I: does Ca^{2+} induce a conformational change? Biochemistry 37:16106–16115

Sheng ZH, Yokoyama CT, Catterall WA (1997) Interaction of the synprint site of N-type Ca^{2+} channels with the C2B domain of synaptotagmin I. Proc Natl Acad Sci USA 94:5405–5410

Shin OH, Rhee JS, Tang J, Sugita S, Rosenmund C, Südhof TC (2003) Sr^{2+} binding to the Ca^{2+} binding site of the synaptotagmin 1 C_2B domain triggers fast exocytosis without stimulating SNARE interactions. Neuron 37:99–108

Shin OH, Xu J, Rizo J, Südhof TC (2009) Differential but convergent functions of Ca^{2+} binding to synaptotagmin-1 C2 domains mediate neurotransmitter release. Proc Natl Acad Sci USA 106:16469–16474

Shoji-Kasai Y, Yoshida A, Sato K, Hoshino T, Ogura A, Kondo S, Fujimoto Y, Kuwahara R, Kato R, Takahashi M (1992) Neurotransmitter release from synaptotagmin-deficient clonal variants of PC12 cells. Science 256:1821–1823

Söllner T, Bennett MK, Whiteheart SW, Scheller RH, Rothman JE (1993) A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. Cell 75:409–418

Sørensen JB, Matti U, Wei SH, Nehring RB, Voets T, Ashery U, Binz T, Neher E, Rettig J (2002) The SNARE protein SNAP-25 is linked to fast calcium triggering of exocytosis. Proc Natl Acad Sci USA 99:1627–1632

Stein A, Radhakrishnan A, Riedel D, Fasshauer D, Jahn R (2007) Synaptotagmin activates membrane fusion through a Ca^{2+}-dependent *trans* interaction with phospholipids. Nat Struct Mol Biol 14:904–911

Stevens CF, Sullivan JM (2003) The synaptotagmin C2A domain is part of the calcium sensor controlling fast synaptic transmission. Neuron 39:299–308

Striegel AR, Biela LM, Evans CS, Wang Z, Delehoy JB, Sutton RB, Chapman ER, Reist NE (2012) Calcium binding by synaptotagmin's C2A domain is an essential element of the electrostatic switch that triggers synchronous synaptic transmission. J Neurosci 32:1253–1260

Südhof TC, Rothman JE (2009) Membrane fusion: grappling with SNARE and SM proteins. Science 323:474–477

Sun J, Pang ZP, Qin D, Fahim AT, Adachi R, Südhof TC (2007) A dual-Ca^{2+}-sensor model for neurotransmitter release in a central synapse. Nature 450:676–682

Sutton RB, Davletov BA, Berghuis AM, Südhof TC, Sprang SR (1995) Structure of the first C_2 domain of synaptotagmin I: a novel Ca^{2+}/phospholipid-binding fold. Cell 80:929–938

Sutton RB, Ernst JA, Brunger AT (1999) Crystal structure of the cytosolic C2A-C2B domains of synaptotagmin III. Implications for Ca^{+2}-independent snare complex interaction. J Cell Biol 147:589–598

Takahashi M, Arimatsu Y, Fujita S, Fujimoto Y, Kondo S, Hama T, Miyamoto E (1991) Protein kinase C and Ca^{2+}/calmodulin-dependent protein kinase II phosphorylate a novel 58-kDa protein in synaptic vesicles. Brain Res 551:279–292

Takamori S, Holt M, Stenius K, Lemke EA, Grønborg M, Riedel D, Urlaub H, Schenck S, Brügger B, Ringler P, Müller SA, Rammner B, Gräter F, Hub JS, De Groot BL, Mieskes G, Moriyama Y, Klingauf J, Grubmüller H, Heuser J, Wieland F, Jahn R (2006) Molecular anatomy of a trafficking organelle. Cell 127:831–846

Tang J, Maximov A, Shin OH, Dai H, Rizo J, Südhof TC (2006) A complexin/synaptotagmin 1 switch controls fast synaptic vesicle exocytosis. Cell 126:1175–1187

Tokumaru H, Shimizu-Okabe C, Abe T (2008) Direct interaction of SNARE complex binding protein synaphin/complexin with calcium sensor synaptotagmin 1. Brain Cell Biol 36:173–189

Tucker WC, Weber T, Chapman ER (2004) Reconstitution of Ca^{2+}-regulated membrane fusion by synaptotagmin and SNAREs. Science 304:435–438

Ubach J, Zhang X, Shao X, Südhof TC, Rizo J (1998) Ca^{2+} binding to synaptotagmin: how many Ca^{2+} ions bind to the tip of a C_2-domain? EMBO J 17:3921–3930

Ubach J, Lao Y, Fernandez I, Arac D, Südhof TC, Rizo J (2001) The C_2B domain of synaptotagmin I is a Ca^{2+}-binding module. Biochemistry 40:5854–5860

van den Bogaart G, Thutupalli S, Risselada JH, Meyenberg K, Holt M, Riedel D, Diederichsen U, Herminghaus S, Grubmüller H, Jahn R (2011) Synaptotagmin-1 may be a distance regulator acting upstream of SNARE nucleation. Nat Struct Mol Biol 18:805–812

van den Bogaart G, Meyenberg K, Diederichsen U, Jahn R (2012) Phosphatidylinositol 4,5-bisphosphate increases Ca^{2+} affinity of synaptotagmin-1 by 40-fold. J Biol Chem 287:16447–16453

Vennekate W, Schröder S, Lin CC, van den Bogaart G, Grunwald M, Jahn R, Walla PJ (2012) *Cis*- and *trans*-membrane interactions of synaptotagmin-1. Proc Natl Acad Sci USA 109:11037–11042

Voets T, Moser T, Lund PE, Chow RH, Geppert M, Südhof TC, Neher E (2001) Intracellular calcium dependence of large dense-core vesicle exocytosis in the absence of synaptotagmin I. Proc Natl Acad Sci USA 98:11680–11685

Vrljic M, Strop P, Hill RC, Hansen KC, Chu S, Brunger AT (2011) Post-translational modifications and lipid binding profile of insect cell-expressed full-length mammalian synaptotagmin 1. Biochemistry 50:9998–10012

Walther K, Krauss M, Diril MK, Lemke S, Ricotta D, Honing S, Kaiser S, Haucke V (2001) Human stoned B interacts with AP-2 and synaptotagmin and facilitates clathrin-coated vesicle uncoating. EMBO Rep 2:634–640

Wang CT, Grishanin R, Earles CA, Chang PY, Martin TF, Chapman ER, Jackson MB (2001) Synaptotagmin modulation of fusion pore kinetics in regulated exocytosis of dense-core vesicles. Science 294:1111–1115

Wang CT, Lu JC, Bai J, Chang PY, Martin TF, Chapman ER, Jackson MB (2003a) Different domains of synaptotagmin control the choice between kiss-and-run and full fusion. Nature 424:943–947

Wang P, Wang CT, Bai J, Jackson MB, Chapman ER (2003b) Mutations in the effector binding loops in the C2A and C2B domains of synaptotagmin I disrupt exocytosis in a nonadditive manner. J Biol Chem 278:47030–47037

Wang CT, Bai J, Chang PY, Chapman ER, Jackson MB (2006) Synaptotagmin-Ca^{2+} triggers two sequential steps in regulated exocytosis in rat PC12 cells: fusion pore opening and fusion pore dilation. J Physiol 570:295–307

Wang Z, Liu H, Gu Y, Chapman ER (2011) Reconstituted synaptotagmin I mediates vesicle docking, priming, and fusion. J Cell Biol 195:1159–1170

Weninger K, Bowen ME, Choi UB, Chu S, Brunger AT (2008) Accessory proteins stabilize the acceptor complex for synaptobrevin, the 1:1 syntaxin/SNAP-25 complex. Structure 16:308–320

Xu J, Mashimo T, Südhof TC (2007) Synaptotagmin-1, -2, and -9: Ca^{2+} sensors for fast release that specify distinct presynaptic properties in subsets of neurons. Neuron 54:567–581

Xu J, Pang ZP, Shin OH, Südhof TC (2009) Synaptotagmin-1 functions as a Ca^{2+} sensor for spontaneous release. Nat Neurosci 12:759–766

Xu W, Morishita W, Buckmaster PS, Pang ZP, Malenka RC, Südhof TC (2012) Distinct neuronal coding schemes in memory revealed by selective erasure of fast synchronous synaptic transmission. Neuron 73:990–1001

Xue M, Ma C, Craig TK, Rosenmund C, Rizo J (2008) The Janus-faced nature of the C_2B domain is fundamental for synaptotagmin-1 function. Nat Struct Mol Biol 15:1160–1168

Yang X, Kaeser-Woo YJ, Pang ZP, Xu W, Südhof TC (2010) Complexin clamps asynchronous release by blocking a secondary Ca^{2+} sensor via its accessory α helix. Neuron 68:907–920

Yang SN, Shi Y, Yang G, Li Y, Yu L, Shin O-H, Bacaj T, Südhof TC, Yu J, Berggren P-O (2012) Inositol hexakisphosphate suppresses excitatory neurotransmission via synaptotagmin-1 C2B domain in the hippocampal neuron. Proc Natl Acad Sci USA 109:12183–12188

Yao J, Gaffaney JD, Kwon SE, Chapman ER (2011) Doc2 is a Ca^{2+} sensor required for asynchronous neurotransmitter release. Cell 147:666–677

Yao J, Kwon SE, Gaffaney JD, Dunning FM, Chapman ER (2012a) Uncoupling the roles of synaptotagmin I during endo- and exocytosis of synaptic vesicles. Nat Neurosci 15:243–249

Yao LH, Rao Y, Varga K, Wang CY, Xiao P, Lindau M, Gong LW (2012b) Synaptotagmin 1 is necessary for the Ca^{2+} dependence of clathrin-mediated endocytosis. J Neurosci 32:3778–3785

Yoshida A, Oho C, Omori A, Kuwahara R, Ito T, Takahashi M (1992) HPC-1 is associated with synaptotagmin and omega-conotoxin receptor. J Biol Chem 267:24925–24928

Yoshihara M, Littleton JT (2002) Synaptotagmin I functions as a calcium sensor to synchronize neurotransmitter release. Neuron 36:897–908

Yoshihara M, Guan Z, Littleton JT (2010) Differential regulation of synchronous versus asynchronous neurotransmitter release by the C2 domains of synaptotagmin 1. Proc Natl Acad Sci USA 107:14869–14874

Young SM Jr, Neher E (2009) Synaptotagmin has an essential function in synaptic vesicle positioning for synchronous release in addition to its role as a calcium sensor. Neuron 63:482–496

Zhang JZ, Davletov BA, Südhof TC, Anderson RG (1994) Synaptotagmin I is a high affinity receptor for clathrin AP-2: implications for membrane recycling. Cell 78:751–760

Zhang X, Kim-Miller MJ, Fukuda M, Kowalchyk JA, Martin TF (2002) Ca^{2+}-dependent synaptotagmin binding to SNAP-25 is essential for Ca^{2+}-triggered exocytosis. Neuron 34:599–611

Zhou A, Brewer KD, Rizo J (2013) Analysis of SNARE complex/synaptotagmin-1 interactions by one-dimensional NMR spectroscopy. Biochemistry 52:3446–3456

Zhu D, Zhou W, Liang T, Yang F, Zhang RY, Wu ZX, Xu T (2007) Synaptotagmin I and IX function redundantly in controlling fusion pore of large dense core vesicles. Biochem Biophys Res Commun 361:922–927

Chapter 9
Regulation of Active Zone Ca^{2+} Channels

Karina Leal and Sumiko Mochida

Abstract At the presynaptic active zone, Ca^{2+} influx through voltage-gated Ca_V2 channels triggers fast, synchronous neurotransmitter release from synaptic vesicles. Synaptic vesicles localized to release sites are tightly coupled with presynaptic Ca_V2 channels whereby neurotransmitter release is proportional to the Ca^{2+} current, or the Ca^{2+} concentration, with the third or fourth power. Ca_V2 channel activity is regulated directly or indirectly by multiple mechanisms through protein-protein interactions, before and after synaptic vesicle exocytosis, resulting in fine-tuning of Ca^{2+} entry that effectively modulates basal neurotransmitter release and underlies presynaptic short-term plasticity. Presynaptic active zone proteins form a large complex, which tether Ca_V2 channels, dock and prime synaptic vesicles at release sites, and possess regulatory function. Ca_V2 channel modulation, which is upstream of synaptic vesicle exocytosis, that leads to changes in Ca^{2+} influx provides a powerful and efficient way to regulate synaptic transmission. In this chapter, we review progress toward understanding the cellular and molecular mechanisms that modulate the activity of Ca^{2+} channels at the presynaptic active zone. A remaining challenge is to understand how these processes work together to shape synaptic transmission and synaptic plasticity.

Keywords Calcium channel • $Ca_V2.1$ • $Ca_V2.2$ • SNARE • G protein • Neurotransmitter • Synaptic transmission

9.1 Introduction

It is well established that an increase in active zone Ca^{2+} concentration following influx through voltage-gated Ca_V2 channels triggers fast and synchronous neurotransmitter release. Through effects on ion channel activity and neurotransmitter release, Ca^{2+} signals can lead to a range of alterations in synaptic function,

K. Leal (✉)
von Gersdorff Lab, Oregon Health and Science University, Portland, OR, USA
e-mail: leal@ohsu.edu

S. Mochida
Department of Physiology, Tokyo Medical University, 6-1-1 Shinjuku, Shinjuku-ku, Tokyo 160-8402, Japan
e-mail: mochida@tokyo-med.ac.jp

S. Mochida (ed.), *Presynaptic Terminals*, DOI 10.1007/978-4-431-55166-9_9

including synaptic plasticity. After a brief overview of Ca_V2 channel structure and function, this chapter reviews advancement toward understanding the molecular mechanisms that modulate the activity of Ca_V2 channels at the active zone and highlights the importance of the Ca_V2 channel regulation in synaptic plasticity.

Ca^{2+} entry through a single (Stanley 1993) or multiple Ca_V2 channel (Holderith et al. 2012; Sheng et al. 2012) can trigger neurotransmitter release, using the SNARE complex (soluble NSF (*N*-ethylmaleimide-sensitive fusion protein) attachment protein receptor), a key component of the vesicle fusion machinery (Hanson et al. 1997; Otto et al. 1997). The SNARE complex consists of vesicle-associated v-SNARE protein synaptobrevin (VAMP) and two plasma membrane-associated t-SNARE proteins, SNAP-25 and syntaxin-1A (Bajjalieh and Scheller 1995; Sollner et al. 1993; Sudhof 1995, 2004) (see Chap. 4). Synaptotagmin, a Ca^{2+} sensor of the synaptic vesicle membrane, is required to drive fusion of a mature release-ready SNARE complex. Upon Ca^{2+} entry, Ca^{2+} binds to synaptotagmin, resulting in a conformational change in the SNARE complex, which causes fusion of synaptic vesicles with plasma membrane and release of neurotransmitter into the synaptic cleft (see Chap. 8).

The active zone protein complex, which includes RIM, Munc-13, RIM-BP, α-liprin, and ELKS, cooperates to provide molecular scaffolds to tether synaptic vesicles and render vesicles fusion ready. Additionally, the active zone protein complex interacts with the synaptic vesicle protein rab3 and couples Ca_V2 channels via the C-terminal tail of the pore-forming subunit to release sites (Sudhof 2012). Ca_V2 channels also interact with SNARE proteins; biochemical studies have shown Ca_V2 channels to be isolated in a complex with SNARE proteins (Bennett et al. 1992; Leveque et al. 1994; Yoshida et al. 1992) and colocalize with syntaxin-1A at presynaptic nerve terminals (Westenbroek et al. 1992; Westenbroek et al. 1995). The interaction of SNARE proteins and Ca_V2 channels relies on direct binding to a *syn*aptic *pr*otein *int*eraction (*synprint*) site located within the intracellular loops of the pore-forming subunit of Ca_V channels (Rettig et al. 1996; Sheng et al. 1994). The *synprint* site serves as a major regulatory domain of Ca_V2 channels by multiple presynaptic proteins beyond SNAREs, as discussed later in this chapter.

Furthermore, we will review Ca_V2 channel regulation by G-protein-coupled receptors. Once neurotransmitter is released, it initiates a feedback regulation of Ca_V2 channel activity through G-protein-coupled receptors. Gβγ produced by the receptors activation inhibits Ca_V2 channel activity (Brown and Sihra 2008). We also discuss PIP_2 regulation on Ca_V2 channel activity. PIP_2 has a similar function to modulation of Ca_V2 channel activity by Gβγ, suggesting that integrating second messenger signaling offers a mechanism for regulation of Ca_V2 channels, although the role of PIP_2 regulation of Ca_V2 channels in synaptic transmission has yet to be studied directly.

Finally, we will review fine-tuning of Ca_V2 channel activity by Ca^{2+} sensor (CaS) proteins, including calmodulin (CaM) and Ca^{2+}-calmodulin-dependent kinase II (CaMKII). Neuronal signals induce Ca^{2+} elevation at the active zone. During the decline of Ca^{2+} concentration to basal level, Ca^{2+} signal activates several CaS proteins that modulate Ca_V2 channel activity. Thus, neuronal activity

determines Ca^{2+} entry through Ca_V2 channels under Ca^{2+}-dependent regulation of key CaS proteins and thereby efficacy of synaptic transmission. We summarize the latest work in Ca_V2 channel regulation at neuronal synapses in two parts, corresponding to the Ca^{2+}-dependent regulation. First, we discuss CaMKII regulation of Ca_V2 channels. Second, we describe progress in Ca^{2+} channel regulation by neuronal CaS proteins, CaM, and related CaM-like proteins. Together, regulation of Ca_V channels comprises an emerging field in understanding the cellular and molecular mechanisms of synaptic transmission and plasticity.

9.2 Presynaptic Calcium Channels

9.2.1 Structure and Function

The family of voltage-gated Ca^{2+} channels is comprised of 10 members that serve distinct roles in cellular signaling. The voltage-gated Ca^{2+} channel currents recorded in different cell types have diverse physiological and pharmacological properties (Tsien et al. 1988). The Ca_V2 subfamily requires strong depolarization for activation; therefore, Ca_V2 channels are defined as high-voltage-activated (HVA) Ca^{2+} channels (Tsien et al. 1991). The Ca_V2 subfamily is specifically blocked with high affinity by peptide toxins from spider ($Ca_V2.1$: ω-agatoxin IVA; $Ca_V2.3$: SNX-482) and snail venom ($Ca_V2.2$: ω-conotoxin GVIA) (Miljanich and Ramachandran 1995). The Ca_V2 subfamily is relatively insensitive to dihydropyridines which primarily blocks the Ca_V1 subfamily of channels. Predominantly found at presynaptic nerve terminals, Ca^{2+} influx through Ca_V2 channels at the active zone triggers fast and synchronous release of neurotransmitter from synaptic vesicles (Fig. 9.1a).

Voltage-gated Ca^{2+} channels are composed of four or five distinct subunits encoded by multiple genes (Catterall 2000). The Ca_V2 channels are made up of an α1 subunit and distinct auxiliary subunits β and $\alpha_2\delta$, sometimes γ (Fig. 9.1a; Hofmann et al. 1999; Catterall 2000). The α1 subunit is the largest subunit (190–250 kDa) and incorporates the conduction pore, the voltage sensors and gating apparatus, and documented sites of regulation by second messengers, drugs, and toxins. The α1 subunit is organized in four homologous domains (I–IV), each containing six transmembrane α helices (S1–S6) and a membrane-associated P loop between S5 and S6. The voltage sensor module is identified as S1 through S4 segments, while segments S5 and S6 make up the pore of the channel (Fig. 9.1b). The large intracellular loops of the α1 subunit, including the N- and C-termini, serve as sites of interaction by different regulatory proteins that influence Ca^{2+} signals and channel function (Fig. 9.1b; Catterall 2000; Catterall and Few 2008).

The auxiliary subunits significantly affect the function of the channel (Hofmann et al. 1999; Dolphin 2003). The intracellular β subunit (50–65 kDa) is a hydrophilic protein associated with the α1 subunit. $Ca_V\beta$ subunits greatly enhance cell surface

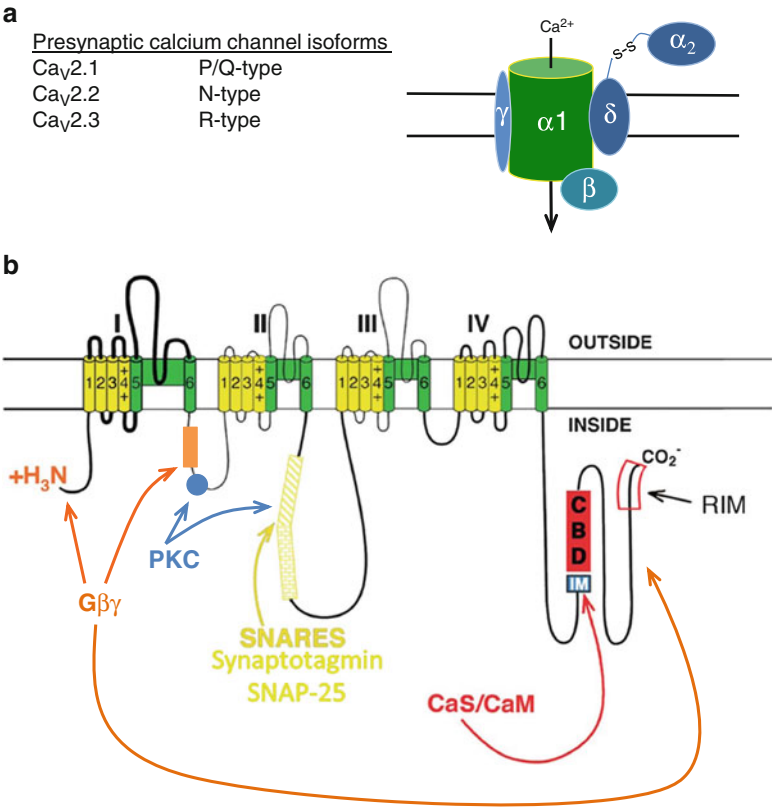

Fig. 9.1 Ca^{2+} channel structure and organization. (**a**) Overview of presynaptic voltage-gated Ca^{2+} channel isoforms and conducted currents. Representation of subunit composition of Ca^{2+} channels and auxiliary subunits. (**b**) Assumed membrane topology of Ca^{2+} channel α1 subunit composition (Adapted from Catterall and Few 2008). The α1 subunit consists of four homologous domains (I–IV), each consisting of six transmembrane segments (S1–S6). Cylinders represent predicted helices. The length of lines linking segments, including the N- and C-termini, corresponds approximately to the length of the polypeptide segments represented. S1–S4, shown in yellow, represents the voltage-sensing module. S5–S6, shown in green, represents the pore-forming unit. The large intracellular loops linking the different domains of the α1 subunit serve as sites of interaction of different regulatory proteins important for channel regulation, including SNARE proteins, CaM, and Ca^{2+} sensor (CaS) proteins

membrane expression of the α1 subunits. Additionally, $Ca_V\beta$ subunits alter the kinetics and voltage dependence of activation and inactivation of Ca_V channels. The transmembrane $\alpha_2\delta$ subunit is encoded by a single gene, posttranslationally cleaved, disulfide-bonded, and lipid anchored to yield the mature α_2 and γ subunits (Catterall 2000; Davies et al. 2007; Dolphin 2003). The $\alpha_2\delta$ subunit, like the β subunit, also enhances cell surface expression of the α1 subunit. Although the $\alpha_2\delta$ subunit has less effect on kinetics and voltage dependence of gating, $\alpha_2\delta$ does play a role in efficient coupling of Ca^{2+} entry to neurotransmitter release at presynaptic active zones (Hoppa et al. 2012). The γ subunit is a component of skeletal muscle

Ca_V1 channels and is unlikely a component of Ca_V2 channels in the brain. Further research is needed to elucidate the function and physiological role of the γ subunit of Ca_V channels.

9.2.2 Ca^{2+} Channels in Synaptic Transmission

In the central nervous system (CNS), Ca_V2 channels are the major sources of Ca^{2+} entry into the presynaptic compartment (Fig. 9.1a; Dunlap et al. 1995). $Ca_V2.1$ channels, which mediate P/Q-type currents, are mainly found at the mammalian neuromuscular junction and at most fast conventional synapses in the CNS (Cohen et al. 1991). In contrast, $Ca_V2.2$ channels, which conduct N-type currents, play a predominant role in neurotransmitter release at synapses in the peripheral nervous system (Olivera et al. 1994) and in certain synapses in the CNS (Poncer et al. 1997). Although the role of $Ca_V2.3$ channels in synaptic transmission is less well understood, $Ca_V2.3$ channels, which mediate R-type currents, are also localized to nerve terminals and have been shown to contribute in a much smaller extent to neurotransmitter release in some CNS synapses (Gasparini et al. 2001). At a given synapse, Ca^{2+} entry through a single Ca^{2+} channel is sufficient to trigger neurotransmitter release with low probability (e.g., chick ciliary ganglia) (Stanley 1993). However, more than one Ca^{2+} channel resides at the synaptic active zone and is thought to cooperate in triggering neurotransmitter release (e.g., rat hippocampal glutamatergic axonal terminals) (Holderith et al. 2012; Sheng et al. 2012). In both cases, Ca^{2+} entry determines the release probability, or synaptic efficacy; thus, modulation of the Ca^{2+} signal entering the presynaptic terminal is a critical factor for regulation of synaptic transmission. The release probability of a single synaptic vesicle has been shown to increase with the number of Ca^{2+} channels at the active zone and has been correlated with changes in short-term plasticity (Sheng et al. 2012). As mentioned above, at the presynaptic active zone, nerve impulse-triggered vesicle fusion is mediated by both assembly of the SNARE complex (Bajjalieh and Scheller 1995; Sollner et al. 1993; Sudhof 1995, 2004) and Ca^{2+} binding to synaptotagmin. Thus, the Ca^{2+} channel and SNARE complex coupling determines vesicle fusion efficiency (Mochida et al. 1996; Wadel et al. 2007).

Neurotransmitter release happens in two phases: a fast synchronous (or phasic) component and a slow asynchronous (tonic) component (Atluri and Regehr 1998; Barrett and Stevens 1972; Goda and Stevens 1994; Hubbard 1963; Rahamimoff and Yaari 1973). Both forms of neurotransmitter release are highly Ca^{2+} dependent and mediated by low affinity Ca^{2+}-sensor protein, such as synaptotagmin-1 (Yoshihara and Littleton 2002; Nishiki and Augustine 2004) and by higher affinity Ca^{2+}-sensor protein, such as Doc2 (Yao et al. 2011), respectively. Fast postsynaptic responses are the result of synchronous release driven by the strictly timed presynaptic Ca^{2+} current onset (Llinas et al. 1981; Sabatini and Regehr 1996), whereas residual Ca^{2+} remaining in the presynaptic terminal after an action potential sustains the slower asynchronous release, which provides a level of tonic neurotransmitter release at a number of synapses (Atluri and Regehr 1998; Hagler and Goda 2001; Lu and

Trussell 2000). The fast synchronous neurotransmitter release is proportional to the third or fourth power of Ca^{2+} influx (Dodge and Rahamimoff 1967; Katz and Miledi 1970; Zucker and Regehr 2002; Augustine et al. 1987); thus, modulation of presynaptic Ca^{2+} channels that leads to changes in Ca^{2+} influx provides an efficient way to modulate neurotransmitter release.

9.2.3 *Ca^{2+} Channels in Presynaptic Short-Term Synaptic Plasticity*

Ca^{2+} entry accompanied with neuronal action potentials determines the release probability, or synaptic efficacy. Thus, the strength of synaptic transmission can vary during repetitive presynaptic action potential firing and depends heavily on the previous firing pattern at a synapse. Neurons fire at frequencies ranging from less than once per second (1 Hz) to several hundred Hz at specialized synapses in the brain (von Gersdorff and Borst 2002). Ca^{2+} accumulates at the presynaptic active zone during high frequency action potential firing and prolonged firing, thus inducing short-term plasticity which represents an increase or decrease of synaptic strength that lasts from hundreds of milliseconds to seconds (Abbott and Regehr 2004; Zucker and Regehr 2002). The same pattern of action potential firing does not necessarily induce the same form of synaptic plasticity at different synapses (Ding et al. 2008; Dittman et al. 2000). All synapses in the brain and the peripheral nervous system show some form of short-term plasticity, either facilitation, depression, or a combination of both facilitation and depression. The input–output relationship between the pre- and postsynaptic neurons can be dynamically modulated by short-term synaptic plasticity, which determines how the signal is transformed from one neuron to the other (Zucker and Regehr 2002). Therefore, short-term plasticity is thought to regulate the activity of neural networks and information processing in the nervous system (Abbott and Regehr 2004; Katz and Miledi 1968; Zucker and Regehr 2002). Although postsynaptic changes, for example, receptor desensitization, have been observed at some synapses, short-term plasticity typically reflects a presynaptic change in neurotransmitter release (Del Castillo and Katz 1954; Katz and Miledi 1968; Zucker and Regehr 2002). However, the cellular and molecular mechanisms underlying short-term plasticity are not well understood. One common factor is that these forms of short-term plasticity are all Ca^{2+} dependent (Xu et al. 2007). Thus, Ca^{2+} channels are poised to play an important role in synaptic transmission and short-term synaptic plasticity.

9.3 Interaction with Active Zone Proteins, RIM and RIM-BP

The active zone is located at the presynaptic plasma membrane precisely opposite the synaptic cleft. The opening of the active zone Ca^{2+} channel creates a steep gradient of increased intracellular Ca^{2+} concentration, a Ca^{2+} nanodomain, at the

Ca^{2+} source (e.g., the voltage-gated Ca_V2. pore). If open channels are sufficiently close, their nanodomains will overlap and create a microdomain of elevated Ca^{2+}. The location of Ca^{2+} channels and the docking of synaptic vesicles to the release site are regulated by Rab-interacting molecule (RIM), a presynaptic active zone protein (Han et al. 2011). RIM interacts with the C-terminal sequences of $Ca_V2.1$ and $Ca_V2.2$ channels (Coppola et al. 2001; Hibino et al. 2002; Kaeser et al. 2011) and plays a role in determining the Ca^{2+} channel density at the active zone. Associated RIM-binding proteins, RIM-BPs, are SH3-domain proteins that also bind to RIMs and interact with $Ca_V2.2$ channels and possibly to $Ca_V2.1$ channels (Hibino et al. 2002; Sudhof 2004). Together, the molecular complex consisting of RIM, RIM-BPs, and C-terminal tails of the Ca_V2 channels regulate the recruitment of Ca_V2 channels to active zones.

RIM also binds directly to the synaptic protein-interaction (*synprint*) site of $Ca_V2.2$ channels (Coppola et al. 2001) where syntaxin-1A, SNAP-25, and synaptotagmin-1 interact (Zhong et al. 1999; Catterall 2000) (see below Sect. 9.4). RIM binding to the α1 subunit of $Ca_V2.2$ channels is Ca^{2+} independent, whereas Ca^{2+} can modulate the association of SNAP-25 and synaptotagmin-1 with RIM (Coppola et al. 2001). Yet it is not known whether binding of RIM to either SNAP-25 or synaptotagmin-1 affects RIM- or syntaxin-1A-dependent regulation of Ca^{2+} channel activity (see below Sect. 9.4.1). In heterologous expression systems, RIM interacts with the β-subunit of Ca_V2 channels and slows inactivation of channel gating (Kiyonaka et al. 2007).

Furthermore, RIMs have been implicated in synaptic plasticity (Castillo et al. 2002; Schoch et al. 2002) as the coupling between Ca^{2+} influx and transmitter release is reduced with removal of RIMs. Knockout of RIM1α showed a significant decrease in synaptic strength and alterations in short-term synaptic plasticity at hippocampal synapses (Schoch et al. 2002). Regulation of presynaptic Ca^{2+} channel function and vesicle docking by RIM provides an additional potential pathway to increase the release probability of synaptic vesicles docked close to Ca_V2 channels.

9.4 Interaction with Vesicle Fusion Proteins

9.4.1 SNAREs, Syntaxin-1 and SNAP-25

Both $Ca_V2.1$ and $Ca_V2.2$ channels contain a particular synaptic protein-interaction (*synprint*) site that binds synaptic proteins such as syntaxin-1A, SNAP-25, RIM, and synaptotagmin (Fig. 9.1b; Spafford et al. 2003; Zhong et al. 1999). The *synprint* site is localized in the intracellular loop that links domains II and III of Ca_V2 channels (Catterall 1999) and is a key motif for targeting synaptic proteins to $Ca_V2.1$ and $Ca_V2.2$ channels (Mochida et al. 2003; Szabo et al. 2006). The *synprint* site is not present in $Ca_V2.3$ channels and the Ca_V1 channel subfamily (Spafford and Zamponi 2003). As well as being a critical site for targeting, the *synprint* site is

also an important modulatory region that enables synaptic proteins like syntaxin-1A to regulate Ca_V channel activity. In nonneuronal cells, binding of syntaxin-1A shifts the voltage dependence of inactivation of $Ca_V2.1$ and $Ca_V2.2$ channels to more hyperpolarized potentials, thus reducing channel availability (Stanley 2003). Further work has shown that cleavage of syntaxin-1A by botulinum toxin C1 also shifts the voltage dependence of inactivation to more depolarized potentials, indicating a relief of inhibition and increased channel availability (Stanley 2003; Bergsman and Tsien 2000). However, the question remains, how does syntaxin-1A modulate Ca^{2+} channel gating? So far, structure function studies have identified two adjacent cysteines within the transmembrane domain of syntaxin-1A that are crucial for Ca^{2+} channel regulation and coordinating Ca^{2+} entry (Trus et al. 2001). Truncation of syntaxin-1A that includes the two adjacent cysteines does not mimic the reduction in current amplitude, suggesting an intact full-length syntaxin-1A is necessary to mediate its affect (Trus et al. 2001). Additionally, the 10 N-terminal residues of syntaxin-1A are able to interact with $Ca_V2.2$ channels and inhibit their activity (Davies et al. 2011). These results point to two different forms of interactions by syntaxin-1A, a modulatory interaction via the *synprint* site and perhaps a yet identified regulatory association site on Ca_V2 channels. The interaction with syntaxin-1A, a target-SNARE, suggests a negative regulation of basal neurotransmitter release, preventing vesicle exocytosis by controlling Ca_V2 channel activity.

In heterologous expression systems, SNAP-25 when coexpressed with $Ca_V2.1$ or $Ca_V2.2$ channels also shifts the voltage dependence of inactivation to more hyperpolarized potentials (Stanley 2003). This was confirmed in hippocampal neurons, where silencing of SNAP-25 leads to an increase in $Ca_V2.1$ channel currents due to a large depolarizing shift in voltage dependence of inactivation, thus increasing the availability of Ca_V channels (Condliffe et al. 2010). Similarly, SNAP-25 was found to bind to the *synprint* site of $Ca_V2.1$ and $Ca_V2.2$ channels and is capable of preventing syntaxin-1A-mediated inhibition (Wiser et al. 1996; Zhong et al. 1999; Jarvis and Zamponi 2001). Thus, association of SNAP-25, another target-SNARE, with Ca^{2+} channels through the *synprint* site may provide a feedback mechanism to modulate Ca^{2+} entry at the presynaptic active zone and conceivably affect neurotransmitter release (Catterall 1999; Pozzi et al. 2008).

The *synprint* site also interacts with the SNARE complex that consists of the vesicle and the target SNAREs. In contrast to the negative regulation of transmitter release by a target SNARE syntaxin-1A, the *synprint* site and the SNARE complex interaction relieves inhibition of Ca_V2 channel activity and is crucial for synchronous neurotransmitter release. Disruption of the interaction between Ca^{2+} channel and the SNARE complex with peptides derived from the *synprint* domain, or deletion of the *synprint* site, reduces synaptic transmission (Rettig et al. 1997; Mochida et al. 1996; Keith et al. 2007; Harkins et al. 2004). Injection of *synprint* peptides derived from $Ca_v2.2$ channels into presynaptic superior cervical ganglion neurons (SCGN) in culture significantly decreased excitatory postsynaptic responses (Mochida et al. 1996). The rapid, synchronous component of synaptic transmission was selectively inhibited following peptide injection. These results provide substantial evidence that the *synprint* region, in addition to the C-terminal RIM-binding site, is also a chief regulatory site of neurotransmitter release.

9.4.2 Synaptotagmin-1

Synaptotagmin-1 has been observed to play a modulatory role in Ca^{2+} channel activity; with an increase in Ca^{2+} concentration, synaptotagmin-1 displaces syntaxin-1A from the *synprint* site (Sheng et al. 1997). Synaptotagmin-1 is the putative calcium sensor for fast, synchronous neurotransmitter release coupling Ca^{2+} influx to vesicle fusion (Sudhof 2004). Synaptotagmin-1 binds Ca^{2+} to initiate synchronous neurotransmitter release via two rich negatively charged domains, C2A and C2B, which have distinct targets (Sudhof 2004). Once bound with Ca^{2+}, the C2A domain allows for insertion of synaptotagmin-1 into the plasma membrane, whereas the C2B domain binds to the *synprint* site of $Ca_V2.1$ and $Ca_V2.2$ channels (Fernandez-Chacon et al. 2001; Sheng et al. 1997). Direct binding of synaptotagmin-1 to the *synprint* site does not directly affect Ca^{2+} channel gating kinetics; instead, it lessens syntaxin-1A-dependent inhibition of $Ca_V2.2$ channels (Wiser et al. 1997; Atlas 2001). Binding of syntaxin-1A to the *synprint* site is Ca^{2+} dependent, low Ca^{2+} concentration favors binding of syntaxin-1A to the *synprint* site, whereas higher Ca^{2+} concentration promotes association with synaptotagmin-1 (Sheng et al. 1996). This sequential Ca^{2+}-dependent binding to the *synprint* site may be a central regulatory mechanism of neurotransmitter release. At low Ca^{2+} concentration, Ca^{2+} channel activity is inhibited by bound syntaxin-1A. Upon formation of a complete synaptotagmin/SNARE complex ready for exocytosis, high Ca^{2+} concentration at the mouth of the Ca^{2+} channel relieves this inhibition, allowing for neurotransmitter release.

In nonneuronal cells, synaptotagmin-1 was also shown to reverse the inhibitory effect of SNAP- 25 on $Ca_V2.1$ channel currents. Thus, competitive binding to the *synprint* site of $Ca_V2.1$ and $Ca_V2.2$ channels provides a potential mechanism to increase the release probability of synaptic vesicles docked near Ca^{2+} channels at the presynaptic active zone (Catterall and Few 2008). Furthermore, synaptotagmin-1 has also been shown to bind directly, in a Ca^{2+}-dependent manner, to auxiliary β_{4a} subunit of Ca_V2 channels (Vendel et al. 2006). The splice variant β_{4a} subunit may confer functions other than modulation of channel gating and cell surface expression. This interaction between the β_{4a} subunit of Ca^{2+} channels and synaptotagmin-1 may act as an additional scaffolding element to facilitate synaptic protein–Ca^{2+} channel coupling for neurotransmitter release (Weiss 2006).

9.4.3 Phosphorylation of Synprint Site

Activation of PKC has been shown to enhance $Ca_V2.2$ channel current directly, by phosphorylation of the domain I–II linker region with only limited effect on $Ca_V2.1$ channels. In contrast, phosphorylation of the *synprint* site by PKC and CaMKII strongly inhibits its binding to syntaxin-1A and SNAP-25 (Yokoyama et al. 1997), thus blocking the negative shift in the voltage dependence of inactivation of $Ca_V2.2$

channels (Jarvis and Zamponi 2001; Yokoyama et al. 2005). Phosphorylation of the $Ca_V2.2$ *synprint* site regulates the ability of the *synprint* site to interact with syntaxin-1A and SNAP-25. However, phosphorylation of syntaxin-1A and SNAP-25 with PKC or CaMKII does not affect the interaction with the *synprint* site (Mochida et al. 1998). Thus, these data demonstrate that phosphorylation of the *synprint* site by PKC or CaMKII may serve as a biochemical switch for interactions linking $Ca_V2.2$ channels and SNARE proteins. Additionally, phosphorylation of the *synprint* site of $Ca_V2.1$ channels by a different kinase, glycogen synthase kinase-3 (GSK-3), has also been shown to prevent binding of syntaxin-1A, SNAP-25, and synaptotagmin-1. Phosphorylation by GSK-3 inhibits neurotransmitter release, perhaps hindering SNARE-dependent regulation of $Ca_V2.1$ channel properties and therefore limiting Ca^{2+} entry (Zhu et al. 2010).

9.5 Interaction with Intracellular Messengers

9.5.1 G Proteins

G-protein inhibition of Ca_V2 channels supports a well-known component of presynaptic inhibition (Wu and Saggau 1997). Voltage-dependent G-protein ($G\beta\gamma$) inhibition of Ca_V2 channels represents a fundamental example of Ca^{2+} channel modulation. G-protein-coupled receptors in the nerve terminal bind neurotransmitters and provide negative feedback to inhibit presynaptic $Ca_V2.1$ and $Ca_V2.2$ channel currents resulting in a decrease in neurotransmitter release (Hille 1994; Ikeda and Dunlap 1999). Acetylcholine, norepinephrine, GABA, glutamate, biogenic amines, and many neuropeptides inhibit Ca_V2 channels in this manner. As mentioned previously, inhibition of Ca^{2+} currents is a very potent mechanism in negative regulation of neurotransmitters because of the steep dependence of Ca^{2+} influx on neurotransmitter release. This form of Ca_V2 channel inhibition is due to $G\beta\gamma$ subunits released from pertussis toxin-sensitive, heterotrimeric G proteins of the G_i/G_o class (Hille 1994; Ikeda and Dunlap 1999). Functionally, G-protein inhibition causes a positive shift in the voltage dependence of activation of the Ca^{2+} current, but not statically. Instead, inhibition can be reversed by strong positive depolarization (Fig. 9.2a; Bean 1989; Marchetti et al. 1986; Tsunoo et al. 1986) or repetitive physiological channel activation (Artim and Meriney 2000; Brody et al. 1997), in a manner that may support short-term plasticity (Fig. 9.2b, c) (Brody and Yue 2000). Relief of G-protein inhibition by paired stimulation results in facilitation in Ca^{2+} current and synaptic response (Fig. 9.2). Interestingly, reversal of inhibition by depolarization at the presynaptic terminal may provide a novel form of short-term plasticity that does not rely on residual Ca^{2+} for synaptic facilitation (see Catterall and Few 2008).

Studies revealed Ca_V2 channel inhibition is caused by direct binding of the G-protein $\beta\gamma$ subunit (Herlitze et al. 1996; Ikeda 1996; Zamponi and Snutch 1998),

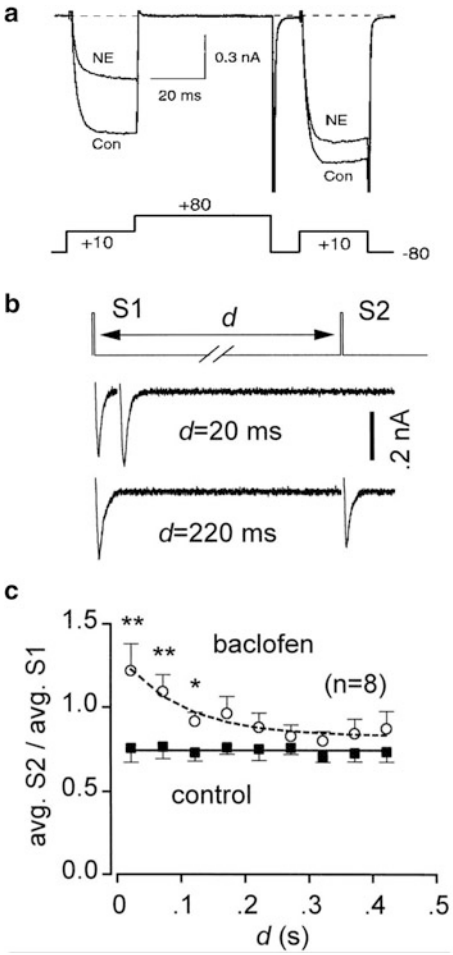

Fig. 9.2 G-protein-mediated regulation of Ca^{2+} channels and synaptic transmission. (**a**) Activation of G proteins by 10 μM norepinephrine (NE) inhibits Ca^{2+} current in SCGNs. In response to paired-pulse voltage stimulation, relief of G-protein inhibition induced facilitation of Ca^{2+} currents conducted by endogenous $Ca_V2.2$ channels (Adapted from Ruiz-Velasco and Ikeda 2000). (**b** and **c**) G-protein activation relieves paired-pulse synaptic depression in cultured hippocampal neurons (Adapted from Brody and Yue 2000). (**b**) Example of response traces of paired stimulus protocol (*top*, S1, S2) and synaptic response (*bottom*) in baclofen, a GABA-B receptor agonist. (**c**) In control, synaptic depression is present at all paired-pulse stimulus intervals tested (*solid squares*). In the presence of baclofen, at short time intervals facilitation of synaptic responses is observed (*open circles*)

making the channel difficult to open. Further studies have identified three sites of Gβγ interaction with the α1 subunit of Ca_V2 channels, the N-terminus (Canti et al. 1999), the intracellular loop connecting domains I and II ($L_{I–II}$) (Herlitze et al. 1997; Zamponi et al. 1997), and the C-terminus (Delmas et al. 2005;

Furukawa et al. 1998; Li et al. 2004; Qin et al. 1997). The binding of the Gβγ subunit to the N-terminus and $L_{I\text{-}II}$ is most effective in initiating inhibition. The transient reversal of inhibition by strong depolarization probably reflects temporary unbinding of Gβγ from the channel as the channel nears its open state (Bean 1989; Boland and Bean 1993; Elmslie et al. 1990). However, the structural mechanisms underlying G-protein modulation of Ca_V2 channels remain poorly understood (Dolphin 2003).

Voltage-independent inhibition of Ca_V2 channels is observed in many neurons and is dependent on intracellular signaling pathways (Dunlap et al. 1995; Hille 1994; Strock and Diverse-Pierluissi 2004). Voltage-independent inhibition of Ca_V2 channels often involves G proteins of the G_q family, which modulate levels of phosphatidylinositide lipids by inducing hydrolysis of phosphatidylinositol bisphosphate via activation of phospholipase C enzymes (Delmas et al. 2005). Although regulation of Ca_V2 channels by voltage-independent inhibition of G proteins has not yet been observed to affect synaptic transmission. Inhibition of $Ca_V2.2$ channels specifically involves interplay between *synprint* site interactions and G-protein Gβγ subunits. A requirement of G-protein inhibition on presynaptic Ca_V2 channels is the presence of intact syntaxin-1A in nerve terminals (Stanley and Mirotznik 1997). Cleavage of syntaxin-1A by botulinum toxin 1C prevents G-protein regulation of presynaptic Ca^{2+} channels. Further research showed that expression of syntaxin-1A is a prerequisite for tonic G-protein inhibition of $Ca_V2.2$ channels (Jarvis et al. 2000).

In the CNS, G-protein modulation of presynaptic Ca_V2 channels is a potent negative regulator of synaptic transmission. At the calyx of Held, modulation by GABA and glutamate acting on GABA-B receptors and metabotropic glutamate receptors, respectively, has been shown to directly affect Ca^{2+} currents and neurotransmitter release (Takahashi et al. 1996; Kajikawa et al. 2001). In hippocampal autapses, a train of action potential-like stimuli relieves synaptic inhibition caused by activation of GABA-B receptors (Brody and Yue 2000), resulting in synaptic facilitation. Furthermore, in SCGNs, injection of purified Gβγ significantly reduced neurotransmitter release by direct modulation of presynaptic $Ca_V2.2$ channels (Stephens and Mochida 2005).

9.5.2 PIP_2

Phosphatidylinositol-4,5-bisphosphate (PIP_2) has recently been reported to modulate presynaptic $Ca_V2.1$ and $Ca_V2.2$ channels. PIP_2 is a known regulator of exocytosis in some cell types and may affect synaptic transmission and plasticity (Koch and Holt 2012). In addition, recent studies have shown PIP_2 to directly influence the synaptic vesicle release machinery and thereby potentially affect Ca^{2+}-triggered exocytosis (Park et al. 2012). Perfusion of PIP_2 has a similar function to modulation by Gβγ subunit in excised membrane patches; PIP_2 shifts the voltage dependence of inactivation strongly to more hyperpolarized

potentials, thus reducing channel activity (Wu et al. 2002). To add to the complexity of presynaptic regulators, activation of PKA blocks PIP_2-mediated inhibition (Wu et al. 2002). Similarly, a reduction in PIP_2, by rapamycin-induced translocation of an inositol lipid 5-phosphatase or by a voltage-sensitive 5-phosphatase, causes a decrease in Ca_V2 channel currents with different sensitivities (Byung-Chang et al. 2010). In heterologous expression systems and SCGNs, $Ca_V2.2$ channels were inhibited to a greater extent than $Ca_V2.1$ channels, presumably due to inactivation of Ca^{2+} channels (Byung-Chang et al. 2010). Such regulation by PIP_2 might therefore be a potential mechanism to regulate Ca^{2+} channel activity and thereby neurotransmitter release. Thus, integrating second messenger signaling pathways offers another mechanism in which cells may access for regulation of Ca_V2 channels. However, the role of PIP_2 regulation of $Ca_V2.1$ or $Ca_V2.2$ channels in synaptic transmission has yet to be studied directly.

9.6 Interaction with CaMKII

Regulation of neurotransmitter release by second messengers through modulation of SNARE proteins may play a critical role in synaptic transmission (Sudhof 1995). As already discussed, SNARE protein interaction with Ca^{2+} channels controls multiply the channel properties that govern Ca^{2+} influx and vesicle exocytosis. Several protein kinases are expressed in the presynaptic terminals including CaMKII, protein kinase C (PKC), and protein kinase A (PKA), which have been shown to phosphorylate $Ca_V2.2$ channels and SNARE proteins (Hirling and Scheller 1996). In the postsynaptic compartment, CaMKII is the most prominent Ca^{2+} sensor-dependent regulator and a key component of long-term potentiation (Kennedy et al. 1990; Luscher et al. 2000; Shepherd and Huganir 2007). CaMKII also modulates presynaptic function (Llinas et al. 1985, 1991) and seems to influence synaptic plasticity at some synapses (Chapman et al. 1995; Lu and Hawkins 2006). CaMKII binds to presynaptic $Ca_V2.1$ channels and slows inactivation and positively shifts the voltage dependence of inactivation, therefore enhancing channel activity (Jiang et al. 2008). Once activated, CaMKII can autophosphorylate, leading to autonomous activity, which is required for modulation of $Ca_V2.1$ channel properties (Magupalli et al. 2013) and which does not require the catalytic activity of CaMKII (Jiang et al. 2008). Moreover, autophosphorylated CaMKII can also bind simultaneously to a neuronal substrate synapsin-1A. The α1 subunit of the $Ca_V2.1$ channel serves as a platform for association of CaMKII at the site of Ca^{2+} influx, where it is persistently activated and poised to phosphorylate synapsin-1A and other nearby substrates to regulate synaptic vesicle dynamics and synaptic plasticity.

To test the role of CaMKII-mediated regulation of $Ca_V2.1$ channels in synaptic transmission, $Ca_V2.1$ channels were expressed in SCGNs and injected with a competing peptide that blocks the interaction of CaMKII with the channel. Perfusion of the competing peptide ($Ca_V2.1_{1897–1912}$) prevented both synaptic

facilitation and depression, suggesting that binding of CaMKII is required for short-term presynaptic plasticity (Magupalli et al. 2013). Additionally, expression of a CaMKII inhibitor, CaMKIIN (Chang et al. 1998), which prevents CaMKII from binding to $Ca_V2.1$ channels (Jiang et al. 2008), also blocks synaptic facilitation and depression (Magupalli et al. 2013). Facilitation and inactivation of $Ca_V2.1$ channels by binding of Ca^{2+}/CaM mediate the short-term plasticity (Mochida et al. 2008) (see Sect. 9.7.1). The evidence defines the functional properties of a signaling complex of CaMKII, $Ca_V2.1$ channels, and the binding partner CaM and suggests an effector checkpoint model to enhance local Ca^{2+} signal. CaMKII binding to $Ca_V2.1$ channels is required to increase the activity of CaM which can effectively generate a cellular response via local Ca^{2+} signal (Magupalli et al. 2013).

9.7 Interaction with Ca^{2+} Sensor Proteins

CaM is the founding member of a large family of related Ca^{2+} sensor (CaS) proteins that are primarily expressed in neurons in the brain and retina (Braunewell and Gundelfinger 1999; Burgoyne and Weiss 2001; Haeseleer et al. 2002; Haeseleer and Palczewski 2002). CaS proteins and CaM share similar structural motifs. CaM has four functional EF-hand motifs, N-terminal EF hands (1 and 2) and C-terminal hands (3 and 4) that respond to the coordination of Ca^{2+}, joined by central α-helix (Burgoyne and Weiss 2001; DeMaria et al. 2001; Lee et al. 2003). Like CaM, all CaS proteins contain 4 putative EF-hand motifs although the first or second EF hand is often not able to bind Ca^{2+} due to changes in amino acid sequence (Burgoyne 2007). Many share a consensus sequence for N-terminal myristoylation (Burgoyne 2007; Burgoyne and Weiss 2001; Haeseleer et al. 2002; Mikhaylova et al. 2011). CaS proteins are similar in structure and displace CaM from shared binding sites in the α_1 subunits of Ca_V channels, but they contain different molecular determinants that confer distinct forms of regulation.

9.7.1 Calmodulin

Repetitive Ca^{2+} current recording showed Ca^{2+}-influx-dependent facilitation and inactivation of presynaptic $Ca_V2.1$ channels at the calyx of Held synapse in the rat brainstem (Forsythe et al. 1998; Inchauspe et al. 2004) or in transfected nonneuronal cells expressing $Ca_V2.1$ channels (Lee et al. 1999, 2000; DeMaria et al. 2001). In addition, during high-frequency presynaptic activity of consecutive action potentials, $Ca_v2.1$ channel currents show both Ca^{2+}-dependent facilitation and inactivation, which contribute to facilitation and depression of excitatory postsynaptic responses (Borst and Sakmann 1998; Cuttle et al. 1998; Forsythe et al. 1998). The degree of Ca^{2+} current facilitation and inactivation observed

strongly correlates to the amount of synaptic facilitation and depression according to the power law (Inchauspe et al. 2004; Ishikawa et al. 2005). Ca^{2+} influx through $Ca_V2.1$ channels is necessary for synaptic facilitation as it is absent in $Ca_V2.1$ channel knockout mice (Inchauspe et al. 2004; Ishikawa et al. 2005). Collectively, these data suggest that $Ca_V2.1$ channels preferentially support increases in neurotransmitter release compared to $Ca_V2.2$ channels, which show little facilitation and greater G-protein inhibition (Inchauspe et al. 2007).

The mechanism for Ca^{2+}-dependent facilitation of $Ca_v2.1$ channel current (CDF) and Ca^{2+}-dependent inactivation of $Ca_v2.1$ channel currents (CDI) as tested in nonneuronal cells involves the Ca^{2+} sensor protein, CaM, binding to two adjacent sites on the C-terminus of the channel—the calmodulin-binding domain (CBD) and the upstream IQ-like motif (IM) (Figs. 9.1b and 9.3a; DeMaria et al. 2001; Lee et al. 1999, 2003). Both of these binding sites are involved in Ca^{2+}-dependent feedback regulation (Lee et al. 1999, 2003; DeMaria et al. 2001). The IM site is required for CDF, whereas the CBD is required for CDI. Mutation of the IQ-like domain, IM-AA, completely prevents CDF, whereas deletion of the CBD inhibits CDI (Lee et al. 1999, 2003). Similarly, regulation of $Ca_v2.1$ channels by CaM is lobe specific. Mutation of the two EF hands in the carboxyl-terminal lobe of CaM primarily prevents CDF, whereas mutation of the EF hands in the amino-terminal lobe primarily prevents CDI (DeMaria et al. 2001; Erickson et al. 2001; Lee et al. 2003).

A molecular model has emerged based on these experiments that describe Ca^{2+}/CaM-dependent facilitation and inactivation of $Ca_v2.1$ channels (Fig. 9.3a). When $Ca_v2.1$ channels open, a local rise in intracellular Ca^{2+} activates the two high-affinity C-terminal Ca^{2+}-binding EF hands of CaM, which initiates or strengthens an interaction with the IQ-like motif causing CDF. Following prolonged Ca^{2+} entry, an increase in global intracellular Ca^{2+} leads to binding of Ca^{2+} to the lower-affinity N-terminal EF hands of CaM, where it interacts with both the IQ-like domain and CBD to produce CDI (DeMaria et al. 2001; Lee et al. 1999, 2003). This biphasic regulation of $Ca_v2.1$ channels by CaM depends on $Ca_v2.1$ channel density expressed in neurons (Xu et al. 2007; Mochida et al. 2008; Catterall and Few 2008). For example, $Ca_v2.1$ currents recorded at the cell body of SCGN show CDF (Mochida et al. 2008), while at the presynaptic terminal of calyx of Held synapse, CDI follows CDF (Forsythe et al. 1998). Both CDF and CDI are blocked by coexpression of a CaM inhibitor peptide (Lee et al. 1999; Xu and Wu 2005), suggesting that Ca^{2+}-dependent modulation of $Ca_v2.1$ channels is caused by two sequential interactions with CaM or a related Ca^{2+}-sensing protein. $Ca_V2.2$ and $Ca_V2.3$ channels have inactivation that depends on CaM, but neither shows evidence of Ca^{2+}/CaS-dependent facilitation of Ca^{2+} channel activity (Liang et al. 2003). These data suggest $Ca_V2.1$ channels are unique in that regulatory CaS proteins may modulate CDF where these channels are specifically expressed.

Direct evidence supporting the CaS protein regulation of $Ca_V2.1$ channels in synaptic transmission has emerged from studies of transfected SCGNs. Mutation of CaS-binding sites, IM and CBD, on the C-terminus of $Ca_V2.1$ channels prevents CaM binding and consequently prevents synaptic facilitation and depression in

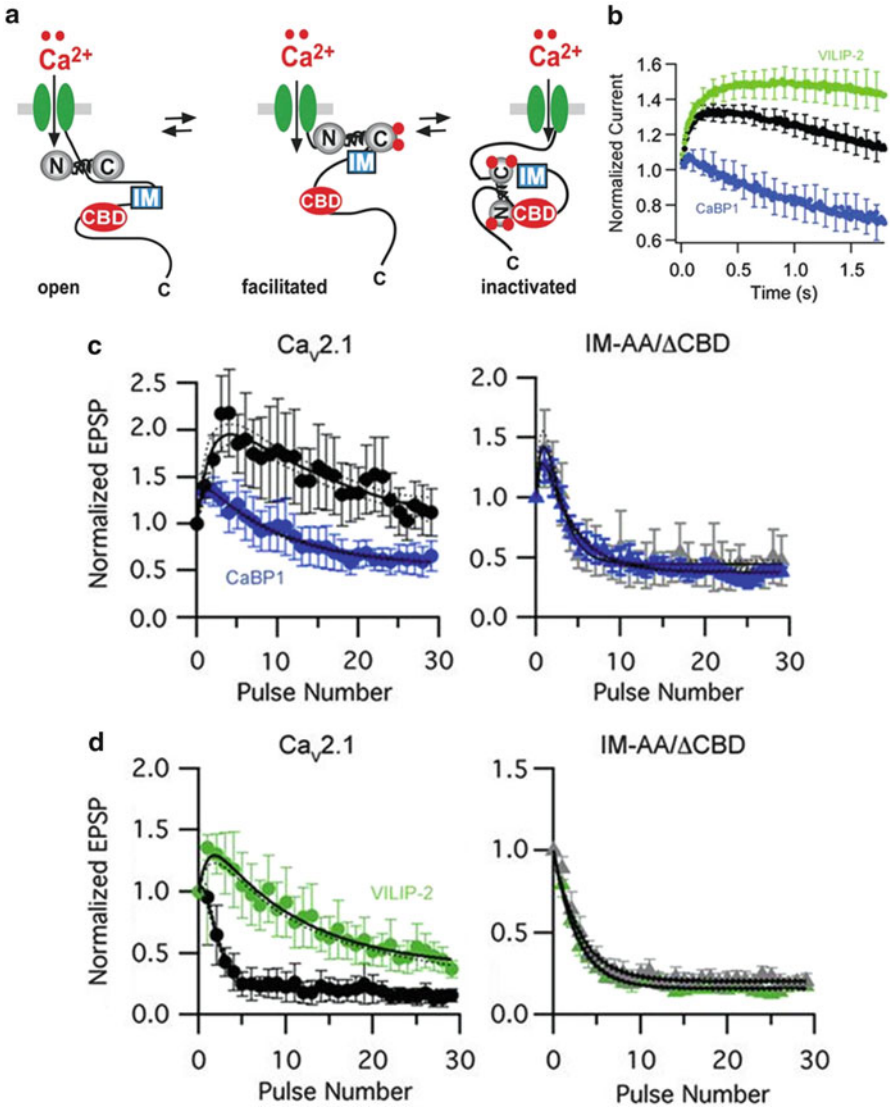

Fig. 9.3 Mechanism of Ca^{2+} sensor regulation of $Ca_V2.1$ channel currents and synaptic transmission. (**a**) Model for Ca^{2+}/CaM-dependent facilitation and inactivation of Ca^{2+} channel currents. The IQ-like domain (IM, *blue*) and the CaM-binding domain (CBD, *red*) are sites of interaction for CaM and CaS proteins. Upon Ca^{2+} channel opening, a local rise in intracellular Ca^{2+} activates the C-terminal lobe of CaM, strengthening the interaction with the IM site, which leads to facilitation of the Ca^{2+} current. Following prolonged Ca^{2+} influx, CaM becomes fully liganded and binds to both the IM site and CBD to produce inactivation of the Ca^{2+} current (Adapted from Catterall and Few 2008). (**b**) Ca^{2+} channel currents recorded from transfected nonneuronal cells expressing $Ca_V2.1$ channels in response to a train of depolarizations at 100 Hz. $Ca_V2.1$ channel currents modulated by endogenous CaM (*black*) show initial Ca^{2+}-dependent facilitation followed by Ca^{2+}-dependent inactivation. Overexpression of CaBP1 (*blue*) blocks Ca^{2+}-dependent facilitation, while coexpression of VILIP-2 (*green*) blocks Ca^{2+}-dependent inactivation (Adapted from Catterall and Few 2008). (**c** and **d**) Synaptic transmission mediated by $Ca_V2.1$ channels or mutant $Ca_V2.1$

transfected SCGNs (Mochida et al. 2008). Furthermore, injection of peptides into synaptic terminals that disrupt CaM binding reduced both Ca^{2+} current inactivation and synaptic depression (Xu and Wu 2005).

9.7.2 CaBP1 and VILIP-2

Included in this superfamily are the neuronal CaS proteins calcium-binding protein 1 (CaBP1) and visinin-like protein 2 (VILIP-2) that are similar in structure to CaM. Unlike CaM localized primarily in neurons (Haeseleer et al. 2000; Paterlini et al. 2000), CaBP1 is expressed in the cerebral cortex, in the hippocampus, and in the retina (Haeseleer et al. 2000) and VILIP-2 is highly expressed in the neocortex and hippocampus (Burgoyne and Weiss 2001).

CaBP1 colocalizes with presynaptic $Ca_V2.1$ channels and binds to the CaM-binding domain in the C-terminus of α1 subunit of $Ca_V2.1$ channels similar to CaM (Lautermilch et al. 2005; Lee et al. 2002). CaBP1 has distinct regulatory effects on $Ca_V2.1$ channel properties. CaBP1 causes rapid inactivation of $Ca_V2.1$ channel currents in a Ca^{2+}-independent manner (Lee et al. 2002). During trains of depolarization pulses, CaBP1 causes enhanced inactivation and prevents facilitation of $Ca_V2.1$ channel currents (Fig. 9.3b; Lee et al. 2002; Few et al. 2011). Thus, the changes in amino acid sequence of CaBP1 have great effects on its regulatory properties. VILIP-2 is another neuronal CaS protein that modulates $Ca_V2.1$ channels but with regulatory effects different from those of CaM and CaBP1. VILIP-2 increases $Ca_V2.1$ channel facilitation in response to trains of depolarization pulses, but inhibits $Ca_V2.1$ channel inactivation (Fig. 9.3b; Lautermilch et al. 2005; Nanou et al. 2012). Thus, CaBP1 and VILIP-2 bind to the same site as CaM but have opposite effects on $Ca_V2.1$ channel activity. This differential effect by CaM, CaBP1, and VILIP-2 in regulating $Ca_v2.1$ channels is a potentially important determinant of Ca^{2+} entry in neurotransmission. Chimeric analysis of CaS protein domains transferred to CaM has shed light on the regulatory domains necessary for CaBP1 and VILIP-2 to mediate their distinct effect (Nanou et al. 2012; Few et al. 2011).

Using transfected SCGNs, CaBP1 and VILIP-2 have been shown to alter synaptic transmission by direct modulation of $Ca_V2.1$ channels (Fig. 9.3c, d; Leal et al. 2012). Coexpression of $Ca_V2.1$ channels with CaBP1, which enhances inactivation of $Ca_V2.1$ channel currents, significantly reduces synaptic facilitation and enhances synaptic depression (Fig. 9.3c; Lee et al. 2002; Leal et al. 2012). In contrast, expression of $Ca_V2.1$ with VILIP-2, which enhances facilitation of $Ca_V2.1$

Fig. 9.3 (continued) channels lacking sites of CaM/CaS-dependent regulation (IM-AA/ΔCBD) expressed alone or with CaBP1 (*blue*) or VILIP-2 (*green*) in transfected SCGNs. Mean normalized EPSP amplitude with SEM induced by presynaptic action potentials at 20 Hz in 1 mM Ca^{2+} (**c**) and 2 mM Ca^{2+} (**d**)

channel currents (Lautermilch et al. 2005), reduces synaptic depression and enhances synaptic facilitation when synaptic depression dominates (Fig. 9.3d; Leal et al. 2012). CaS-dependent modulation by CaBP1 and VILIP-2 is absent when $Ca_V2.1$ channels with impaired IM motif and CBD are coexpressed (Fig. 9.3c, d; Leal et al. 2012). These data indicate that CaBP1 and VILIP-2 by directly binding to the C-terminal regulatory site of $Ca_V2.1$ channels can have opposing effects on short-term synaptic plasticity, either favoring synaptic depression or facilitation. CaS proteins via regulation of presynaptic channels may play a critical role in determining the diversity of short-term synaptic plasticity at CNS synapses.

Additionally, alternative splicing of Ca_V2 channel mRNAs has recently been shown to impact the diversity of Ca_V2 channel regulation. Alternative splicing of exons encoding the C-terminal domain of $Ca_V2.1$ channels affects modulation by Ca^{2+} and CaM (Chaudhuri et al. 2004). For $Ca_V2.2$ channels, alternative splicing of exons encoding the intracellular linker between domains I and II affects the regulation by opiates and tyrosine phosphorylation (Altier et al. 2007; Raingo et al. 2007). Thus, presynaptic Ca_V2 channel regulation by multiple mechanisms is important for fine-tuning Ca^{2+} entry and supporting diversity of synaptic plasticity.

9.8 Conclusion

Regulation of active zone Ca^{2+} channels is an important determinant of Ca^{2+} influx for synchronous synaptic transmission. Interactions of Ca_V2 channels with synaptic proteins function in two ways, localizing vesicles and Ca^{2+} channels at the active zone for exocytosis and modulating Ca^{2+} channel activity and thereby neurotransmitter release. The regulation by synaptic proteins and second messenger pathways is complex. At rest, Ca_V2 channels are inhibited by the interaction of active zone proteins and a t-SNARE. Neuronal firing relieves this inhibition, allowing the SNARE complex and synaptotagmin to interact with Ca_V2 channels and initiate Ca^{2+}-triggered exocytosis of synaptic vesicles. However, the precise molecular and structural mechanism of these protein interactions to regulate Ca_V2 channels remains unknown. The intracellular *synprint* site is necessary for Ca_V2 channel activation to trigger neurotransmitter release, while other intracellular sites function to inhibit the response such as intracellular messenger Gβγ. Additionally, local Ca^{2+} signal at the active zone acts on CaM and neuronal CaS proteins that modulate Ca_V2 channels inducing short-term plasticity. CaS proteins, CaM, CaBP1, and VILIP-2, sensing different Ca^{2+} signal level with neuronal activity, lend to diversity of synaptic modulation via direct temporal interactions with $Ca_V2.1$ channels. Future work to understand the structural basis of CaS regulation will bring understanding to the selective modulation of Ca_V2 channels and short-term plasticity.

Recent studies in synaptic preparations where presynaptic terminals may be molecularly modified and physiologically recorded will provide future insight in the emerging field of presynaptic Ca^{2+} channel regulation in synaptic transmission

and synaptic plasticity. Overall, modifying the function and regulatory properties of presynaptic Ca_V2 currents allows for a greater range of regulatory diversity upstream of neurotransmitter release.

References

Abbott LF, Regehr WG (2004) Synaptic computation. Nature 431:796–803

Altier C, Dale CS, Kisilevsky AE, Chapman K, Castiglioni AJ, Matthews EA, Evans RM, Dickenson AH, Lipscombe D, Vergnolle N, Zamponi GW (2007) Differential role of N-type calcium channel splice isoforms in pain. J Neurosci 27:6363–6373

Artim DE, Meriney SD (2000) G-protein-modulated Ca^{2+} current with slowed activation does not alter the kinetics of action potential-evoked Ca^{2+} current. J Neurophysiol 84:2417–2425

Atlas D (2001) Functional and physical coupling of voltage-sensitive calcium channels with exocytotic proteins: ramifications for the secretion mechanism. J Neurochem 77:972–985

Atluri PP, Regehr WG (1998) Delayed release of neurotransmitter from cerebellar granule cells. J Neurosci 18:8214–8227

Augustine GJ, Charlton MP, Smith SJ (1987) Calcium action in synaptic transmitter release. Annu Rev Neurosci 10:633–693

Bajjalieh SM, Scheller RH (1995) The biochemistry of neurotransmitter secretion. J Biol Chem 270:1971–1974

Barrett EF, Stevens CF (1972) The kinetics of transmitter release at the frog neuromuscular junction. J Physiol 227:691–708

Bean BP (1989) Neurotransmitter inhibition of neuronal calcium currents by changes in channel voltage dependence. Nature 340:153–156

Bennett MK, Calakos N, Scheller RH (1992) Syntaxin: a synaptic protein implicated in docking of synaptic vesicles at presynaptic active zones. Science 257:255–259

Bergsman JB, Tsien RW (2000) Syntaxin modulation of calcium channels in cortical synaptosomes as revealed by botulinum toxin C1. J Neurosci 20:4368–4378

Boland LM, Bean BP (1993) Modulation of N-type calcium channels in bullfrog sympathetic neurons by luteinizing hormone- releasing hormone: kinetics and voltage dependence. J Neurosci 13:516–533

Borst JG, Sakmann B (1998) Facilitation of presynaptic calcium currents in the rat brainstem. J Physiol 513(Pt 1):149–155

Braunewell KH, Gundelfinger ED (1999) Intracellular neuronal calcium sensor proteins: a family of EF-hand calcium-binding proteins in search of a function. Cell Tissue Res 295:1–12

Brody D, Yue D (2000) Relief of G-protein inhibition of calcium channels and short-term synaptic facilitation in cultured hippocampal neurons. J Neurosci 20:889–898

Brody DL, Patil PG, Mulle JG, Snutch TP, Yue DT (1997) Bursts of action potential waveforms relieve G-protein inhibition of recombinant P/Q-type Ca^{2+} channels in HEK 293 cells. J Physiol 499:637–644

Brown DA, Sihra TS (2008) Presynaptic signaling by heterotrimeric G-proteins. Handb Exp Pharmacol 184:207–260

Burgoyne RD (2007) Neuronal calcium sensor proteins: generating diversity in neuronal Ca^{2+} signalling. Nat Rev Neurosci 8:182–193

Burgoyne RD, Weiss JL (2001) The neuronal calcium sensor family of Ca^{2+}-binding proteins. Biochem J 353:1–12

Byung-Chang S, Leal K, Hille B (2010) Modulation of high-voltage activated Ca^{2+} channels by membrane phosphatidylinositol 4,5-biphosphate. Neuron 67:224–238

Canti C, Page KM, Stephens GJ, Dolphin AC (1999) Identification of residues in the N terminus of alpha 1B critical for inhibition of the voltage-dependent calcium channel by Gβγ. J Neurosci 19:6855–6864

Castillo PE, Schoch S, Schmitz F, Sudhof TC, Malenka RC (2002) RIM1 is required for presynaptic long-term potentiation. Nature 415:327–330

Catterall WA (1999) Interactions of presynaptic Ca^{2+} channels and snare proteins in neurotransmitter release. Ann N Y Acad Sci 868:144–159

Catterall WA (2000) Structure and regulation of voltage-gated calcium channels. Annu Rev Cell Dev Biol 16:521–555

Catterall WA, Few AP (2008) Calcium channel regulation and presynaptic plasticity. Neuron 59:882–901

Chang BH, Mukherji S, Soderling TR (1998) Characterization of a calmodulin kinase II inhibitor protein in brain. Proc Natl Acad Sci U S A 95:10890–10895

Chapman PF, Frenguelli BG, Smith A, Chen CM, Silva AJ (1995) The alpha-Ca^{2+}/calmodulin kinase II: a bidirectional modulator of presynaptic plasticity. Neuron 14:591–597

Chaudhuri D, Chang SY, DeMaria CD, Alvania RS, Soong TW, Yue DT (2004) Alternative splicing as a molecular switch for Ca^{2+}/calmodulin-dependent facilitation of P/Q-type Ca^{2+} channels. J Neurosci 24:6334–6342

Cohen MW, Jones OT, Angelides KJ (1991) Distribution of Ca^{2+} channels on frog motor nerve terminals revealed by fluorescent omega-conotoxin. J Neurosci 11:1032–1039

Condliffe SB, Corradini I, Pozzi D, Verderio C, Matteoli M (2010) Endogenous SNAP-25 regulates native voltage-gated calcium channels in glutamatergic neurons. J Biol Chem 285:24968–24976

Coppola T, Magnin-Luthi S, Perret-Menoud V, Gattesco S, Schiavo G, Regazzi R (2001) Direct interaction of the Rab3 effector RIM with Ca^{2+} channels, SNAP-25, and synaptotagmin. J Biol Chem 276:32756–32762

Cuttle MF, Tsujimoto T, Forsythe ID, Takahashi T (1998) Facilitation of the presynaptic calcium current at an auditory synapse in rat brainstem. J Physiol 512:723–729

Davies A, Hendrich J, Van Minh AT, Wratten J, Douglas L, Dolphin AC (2007) Functional biology of the α2δ subunits of voltage-gated calcium channels. Trends Pharmacol Sci 28:220–228

Davies JN, Jarvis SE, Zamponi GW (2011) Bipartite syntaxin 1A interactions mediate $Ca_V2.2$ calcium channel regulation. Biochem Biophys Res Commun 411:562–568

Del Castillo J, Katz B (1954) Changes in end-plate activity produced by presynaptic polarization. J Physiol 124:586–604

Delmas P, Coste B, Gamper N, Shapiro MS (2005) Phosphoinositide lipid second messengers: new paradigms for calcium channel modulation. Neuron 47:179–182

DeMaria CD, Soong TW, Alseikhan BA, Alvania RS, Yue DT (2001) Calmodulin bifurcates the local Ca^{2+} signal that modulates P/Q-type Ca^{2+} channels. Nature 411:484–489

Ding J, Peterson JD, Surmeier DJ (2008) Corticostriatal and thalamostriatal synapses have distinctive properties. J Neurosci 28:6483–6492

Dittman JS, Kreitzer AC, Regehr WG (2000) Interplay between facilitation, depression, and residual calcium at three presynaptic terminals. J Neurosci 20:1374–1385

Dodge FA Jr, Rahamimoff R (1967) Cooperative action a calcium ions in transmitter release at the neuromuscular junction. J Physiol 193:419–432

Dolphin AC (2003) Beta subunits of voltage-gated calcium channels. J Bioenerg Biomembr 35:599–620

Dunlap K, Luebke JI, Turner TJ (1995) Exocytotic Ca^{2+} channels in mammalian central neurons. Trends Neurosci 18:89–98

Elmslie KS, Zhou W, Jones SW (1990) LHRH and GTP-gamma-S modify calcium current activation in bullfrog sympathetic neurons. Neuron 5:75–80

Erickson MG, Badr AA, Peterson BZ, Yue DT (2001) Preassociation of calmodulin with voltage-gated Ca^{2+} channels revealed by FRET in single living cells. Neuron 31:973–985

Fernandez-Chacon R, Konigstorfer A, Gerber SH et al (2001) Synaptotagmin I functions as a calcium regulator of release probability. Nature 410:41–49

Few AP, Nanou E, Scheuer T, Catterall WA (2011) Molecular determinants of $Ca_V2.1$ channel regulation by calcium-binding protein-1. J Biol Chem 286:41917–41923

Forsythe ID, Tsujimoto T, Barnes-Davies M, Cuttle MF, Takahashi T (1998) Inactivation of presynaptic calcium current contributes to synaptic depression at a fast central synapse. Neuron 20:797–807

Furukawa T, Nukada T, Mori Y, Wakamori M, Fujita Y, Ishida H, Fukuda K, Kato S, Yoshii M (1998) Differential interactions of the C terminus and the cytoplasmic I-II loop of neuronal $Ca2^+$ channels with G-protein alpha and beta gamma subunits. I. Molecular determination. J Biol Chem 273:17585–17594

Gasparini S, Kasyanov AM, Pietrobon D, Voronin LL, Cherubini E (2001) Presynaptic R-type calcium channels contribute to fast excitatory synaptic transmission in the rat hippocampus. J Neurosci 21:8715–8721

Goda Y, Stevens CF (1994) Two components of transmitter release at a central synapse. Proc Natl Acad Sci U S A 91:12942–12946

Haeseleer F, Palczewski K (2002) Calmodulin and Ca^{2+}-binding proteins (CaBPs): variations on a theme. Adv Exp Med Biol 514:303–317

Haeseleer F, Sokal I, Verlinde CL, Erdjument-Bromage H, Tempst P, Pronin AN, Benovic JL, Fariss RN, Palczewski K (2000) Five members of a novel Ca^{2+}-binding protein (CaBP) subfamily with similarity to calmodulin. J Biol Chem 275:1247–1260

Haeseleer F, Imanishi Y, Sokal I, Filipek S, Palczewski K (2002) Calcium-binding proteins: intracellular sensors from the calmodulin superfamily. Biochem Biophys Res Commun 290:615–623

Hagler DJ Jr, Goda Y (2001) Properties of synchronous and asynchronous release during pulse train depression in cultured hippocampal neurons. J Neurophysiol 85:2324–2334

Han Y, Kaeser PS, Sudhof TC, Schneggenburger R (2011) RIM determines Ca^{2+} channel density and vesicle docking at the presynaptic active zone. Neuron 69:304–316

Hanson PI, Heuser JE, Jahn R (1997) Neurotransmitter release – four years of SNARE complexes. Curr Opin Neurobiol 7:310–315

Harkins AB, Cahill AL, Powers JF, Tischler AS, Fox AP (2004) Deletion of the synaptic protein interaction site of the N-type ($Ca_V2.2$) calcium channel inhibits secretion in mouse pheochromocytoma cells. Proc Natl Acad Sci U S A 101:15219–15224

Herlitze S, Garcia DE, Mackie K, Hille B, Scheuer T, Catterall WA (1996) Modulation of Ca^{2+} channels by G protein $\beta\gamma$ subunits. Nature 380:258–262

Herlitze S, Hockerman GH, Scheuer T, Catterall WA (1997) Molecular determinants of inactivation and G protein modulation in the intracellular loop connecting domains I and II of the calcium channel a_{1A} subunit. Proc Natl Acad Sci U S A 94:1512–1516

Hibino H, Pironkova R, Onwumere O, Vologodskaia M, Hudspeth AJ, Lesage F (2002) RIM binding proteins (RBPs) couple Rab3-interacting molecules (RIMs) to voltage-gated Ca^{2+} channels. Neuron 34:411–423

Hille B (1994) Modulation of ion-channel function by G-protein-coupled receptors. Trends Neurosci 17:531–536

Hirling H, Scheller RH (1996) Phosphorylation of synaptic vesicle proteins: modulation of the alpha SNAP interaction with the core complex. Proc Natl Acad Sci U S A 21:11945–11949

Hofmann F, Lacinova L, Klugbauer N (1999) Voltage-dependent calcium channels: from structure to function. Rev Physiol Biochem Pharmacol 139:33–87

Holderith N, Lorincz A, Katona G, Rozsa B, Kulik A, Watanabe M, Nusser Z (2012) Release probability of hippocampal glutamatergic terminals scales with the size of the active zone. Nat Neurosci 15:988–997

Hoppa MB, Lana B, Margas W, Dolphin AC, Ryan TA (2012) $\alpha_2\delta$ expression sets presynaptic calcium channel abundance and release probability. Nature 486:122–125

Hubbard JI (1963) Repetitive stimulation at the mammalian neuromuscular junction, and the mobilization of transmitter. J Physiol 169:641–662

Ikeda SR (1996) Voltage-dependent modulation of N-type calcium channels by G-protein βγ subunits. Nature 380:255–258

Ikeda SR, Dunlap K (1999) Voltage-dependent modulation of N-type calcium channels: role of G protein subunits. Adv Second Messenger Phosphoprotein Res 33:131–151

Inchauspe CCG, Martini FJ, Forsythe ID, Uchitel OD (2004) Functional compensation of P/Q by N-type channels blocks short-term plasticity at the calyx of Held presynaptic terminal. J Neurosci 24:10379–10383

Inchauspe CG, Forsythe ID, Uchitel OD (2007) Changes in synaptic transmission properties due to the expression of N-type calcium channels at the calyx of Held synapse of mice lacking P/Q-type calcium channels. J Physiol 584:835–851

Ishikawa T, Kaneko M, Shin HS, Takahashi T (2005) Presynaptic N-type and P/Q-type Ca^{2+} channels mediating synaptic transmission at the calyx of Held of mice. J Physiol 568:199–209

Jarvis SE, Zamponi GW (2001) Distinct molecular determinants govern syntaxin 1A-mediated inactivation and G-protein inhibition of N-type calcium channels. J Neurosci 21:2939–2948

Jarvis SE, Magga JM, Beedle AM, Braun JE, Zamponi GW (2000) G protein modulation of N-type calcium channels is facilitated by physical interactions between syntaxin 1A and Gbetagamma. J Biol Chem 275:6388–6394

Jiang X, Lautermilch NJ, Watari H, Westenbroek RE, Scheuer T, Catterall WA (2008) Modulation of $Ca_V2.1$ channels by Ca^{2+}/calmodulin-dependent protein kinase II bound to the C-terminal domain. Proc Natl Acad Sci U S A 105:341–346

Kaeser PS, Deng L, Wang Y, Dulubova I, Liu X, Rizo J, Sudhof TC (2011) RIM proteins tether Ca^{2+} channels to presynaptic active zones via a direct PDZ-domain interaction. Cell 144:282–295

Kajikawa Y, Saitoh N, Takahashi T (2001) GTP-binding protein beta gamma subunits mediate presynaptic calcium current inhibition by $GABA_B$ receptor. Proc Natl Acad Sci U S A 98:8054–8058

Katz B, Miledi R (1968) The role of calcium in neuromuscular facilitation. J Physiol 195:481–492

Katz B, Miledi R (1970) Further study of the role of calcium in synaptic transmission. J Physiol 207:789–801

Keith RK, Poage RE, Yokoyama CT, Catterall WA, Meriney SD (2007) Bidirectional modulation of transmitter release by calcium channel/syntaxin interactions in vivo. J Neurosci 27:265–269

Kennedy MB, Bennett MK, Bulleit RF, Erondu NE, Jennings VR, Miller SG, Molloy SS, Patton BL, Schenker LJ (1990) Structure and regulation of type II calcium/calmodulin-dependent protein kinase in central nervous system neurons. Cold Spring Harb Symp Quant Biol 55:101–110

Kiyonaka S, Wakamori M, Miki T, Uriu Y, Nonaka M, Bito H, Beedle AM, Mori E, Hara Y, De Waard M et al (2007) RIM1 confers sustained activity and neurotransmitter vesicle anchoring to presynaptic Ca^{2+} channels. Nat Neurosci 10:691–701

Koch M, Holt M (2012) Coupling exo- and endocytosis: an essential role for PIP_2 at the synapse. Biochim Biophys Acta 1821:1114–1132

Lautermilch NJ, Few AP, Scheuer T, Catterall WA (2005) Modulation of $Ca_V2.1$ channels by the neuronal calcium-binding protein visinin-like protein-2. J Neurosci 25:7062–7070

Leal K, Mochida S, Scheuer T, Catterall WA (2012) Fine-tuning synaptic plasticity by modulation of CaV2.1 channels with calcium sensor proteins. Proc Natl Acad Sci U S A 109(42):17069–17074

Lee A, Wong ST, Gallagher D, Li B, Storm DR, Scheuer T, Catterall WA (1999) Calcium/calmodulin binds to and modulates P/Q-type calcium channels. Nature 399:155–159

Lee A, Scheuer T, Catterall WA (2000) Ca^{2+}/calmodulin-dependent facilitation and inactivation of P/Q-type Ca^{2+} channels. J Neurosci 20:6830–6838

Lee A, Westenbroek RE, Haeseleer F, Palczewski K, Scheuer T, Catterall WA (2002) Differential modulation of $Ca_V2.1$ channels by calmodulin and Ca^{2+}-binding protein 1. Nat Neurosci 5:210–217

Lee A, Zhou H, Scheuer T, Catterall WA (2003) Molecular determinants of Ca^{2+}/calmodulin-dependent regulation of $Ca_V2.1$ channels. Proc Natl Acad Sci U S A 100:16059–16064

Leveque C, el Far O, Martin-Moutot N et al (1994) Purification of the N-type calcium channel associated with syntaxin and synaptotagmin. A complex implicated in synaptic vesicle exocytosis. J Biol Chem 269:6306–6312

Liang H, DeMaria CD, Erickson MG, Mori MX, Alseikhan BA, Yue DT (2003) Unified mechanisms of Ca^{2+} regulation across the Ca^{2+} channel family. Neuron 39:951–960

Li B, Zhong H, Scheuer T, Catterall WA (2004) Functional role of a C-terminal Gbetagamma-binding domain of $Ca_V2.2$ channels. Mol Pharmacol 66:761–769

Llinas R, Steinberg IZ, Walton K (1981) Relationship between presynaptic calcium current and postsynaptic potential in squid giant synapse. Biophys J 33:323–351

Llinas R, McGuinness TL, Leonard CS, Sugimori M, Greengard P (1985) Intraterminal injection of synapsin I or calcium/calmodulin-dependent protein kinase II alters neurotransmitter release at the squid giant synapse. Proc Natl Acad Sci U S A 82:3035–3039

Llinas R, Gruner JA, Sugimori M, McGuinness TL, Greengard P (1991) Regulation by synapsin I and Ca^{2+}-calmodulin-dependent protein kinase II of the transmitter release in squid giant synapse. J Physiol 436:257–282

Lu FM, Hawkins RD (2006) Presynaptic and postsynaptic Ca^{2+} and CamKII contribute to long-term potentiation at synapses between individual CA3 neurons. Proc Natl Acad Sci U S A 103:4264–4269

Lu T, Trussell LO (2000) Inhibitory transmission mediated by asynchronous transmitter release. Neuron 26:683–694

Luscher C, Nicoll RA, Malenka RC, Muller D (2000) Synaptic plasticity and dynamic modulation of the postsynaptic membrane. Nat Neurosci 3:545–550

Magupalli VG, Mochida S, Jiang X, Westenbroek RE, Nairn AC, Scheuer T, Catterall WA (2013) Ca^{2+}-independent activation of Ca^{2+}/calmodulin-dependent protein kinase II bound to the C-terminal domain of $Ca_V2.1$ channels. J Biol Chem 288:4637–4648

Marchetti C, Carbone E, Lux HD (1986) Effects of dopamine and noradrenaline on Ca^{2+} channels of cultured sensory and sympathetic neurons of chick. Pflugers Arch 406:104–111

Mikhaylova M, Hradsky J, Kreutz MR (2011) Between promiscuity and specificity: novel roles of EF-hand calcium sensors in neuronal Ca^{2+} signalling. J Neurochem 118:695–713

Miljanich GP, Ramachandran J (1995) Antagonists of neuronal calcium channels: structure, function, and therapeutic implications. Annu Rev Pharmacol Toxicol 35:707–734

Mochida S, Sheng ZH, Baker C, Kobayashi H, Catterall WA (1996) Inhibition of neurotransmission by peptides containing the synaptic protein interaction site of N-type Ca^{2+} channels. Neuron 17:781–788

Mochida S, Yokoyama CT, Kim DK, Itoh K, Catterall WA (1998) Evidence for a voltage-dependent enhancement of neurotransmitter release mediated via the synaptic protein interaction site of N-type Ca^{2+} channels. Proc Natl Acad Sci U S A 24:14523–14528

Mochida S, Westenbroek RE, Yokoyama CT, Zhong H, Myers SJ, Scheuer T, Itoh K, Catterall WA (2003) Requirement for the synaptic protein interaction site for reconstitution of synaptic transmission by P/Q-type calcium channels. Proc Natl Acad Sci U S A 100:2819–2824

Mochida S, Few AP, Scheuer T, Catterall WA (2008) Regulation of presynaptic $Ca_V2.1$ channels by Ca^{2+} sensor proteins mediates short-term synaptic plasticity. Neuron 57:210–216

Nanou E, Martinez GQ, Scheuer T, Catterall WA (2012) Molecular determinants of modulation of $Ca_V2.1$ channels by visinin-like protein 2. J Biol Chem 287:504–513

Nishiki T, Augustine GJ (2004) Synaptotagmin I synchronizes transmitter release in mouse hippocampal neurons. J Neurosci 24:6127–6132

Olivera BM, Miljanich GP, Ramachandran J, Adams ME (1994) Calcium channel diversity and neurotransmitter release: the omega-conotoxins and omega-agatoxins. Annu Rev Biochem 63:823–867

Otto H, Hanson PI, Jahn R (1997) Assembly and disassembly of a ternary complex of synaptobrevin, syntaxin, and SNAP-25 in the membrane of synaptic vesicles. Proc Natl Acad Sci U S A 94:6197–6201

Park Y et al (2012) Controlling synaptotagmin activity by electrostatic screening. Nat Struct Mol Biol 19:991–997

Paterlini M, Revilla V, Grant AL, Wisden W (2000) Expression of the neuronal calcium sensor protein family in the rat brain. Neuroscience 99:205–216

Poncer JC, McKinney RA, Gahwiler BH, Thompson SM (1997) Either N- or P-type calcium channels mediate GABA release at distinct hippocampal inhibitory synapses. Neuron 18:463–472

Pozzi D, Condliffe S, Bozzi Y et al (2008) Activity-dependent phosphorylation of Ser187 is required for SNAP-25-negative modulation of neuronal voltage-gated calcium channels. Proc Natl Acad Sci U S A 105:323–328

Qin N, Platano D, Olcese R, Stefani E, Birnbaumer L (1997) Direct interaction of Gβγ with a C-terminal Gβγ-binding domain of the Ca^{2+} channel α1 subunit is responsible for channel inhibition by G protein-coupled receptors. Proc Natl Acad Sci U S A 94:8866–8871

Rahamimoff R, Yaari Y (1973) Delayed release of transmitter at the frog neuromuscular junction. J Physiol 228:241–257

Raingo J, Castiglioni AJ, Lipscombe D (2007) Alternative splicing controls G protein-dependent inhibition of N-type calcium channels in nociceptors. Nat Neurosci 10:285–292

Rettig J, Sheng ZH, Kim DK, Hodson CD, Snutch TP, Catterall WA (1996) Isoform specific interaction of the alpha1A subunits of brain Ca^{2+} channels with the presynaptic proteins syntaxin and SNAP-25. Proc Natl Acad Sci U S A 93:7363–7368

Rettig J, Heinemann C, Ashery U et al (1997) Alteration of Ca^{2+} dependence of neurotransmitter release by disruption of Ca^{2+} channel/syntaxin interaction. J Neurosci 17:6647–6656

Ruiz-Velasco V, Ikeda SR (2000) Multiple G-protein βγ combinations produce voltage-dependent inhibition of N-type calcium channels in rat superior cervical ganglion neurons. J Neurosci 20:2183–2191

Sabatini BL, Regehr WG (1996) Timing of neurotransmission at fast synapses in the mammalian brain. Nature 384:170–172

Schoch S, Castillo PE, Jo T, Mukherjee K, Geppert M, Wang Y, Schmitz F, Malenka RC, Sudhof TC (2002) RIM1alpha forms a protein scaffold for regulating neurotransmitter release at the active zone. Nature 415:321–326

Sheng ZH, Rettig J, Takahashi M, Catterall WA (1994) Identification of a syntaxin-binding site on N-type calcium channels. Neuron 13:1303–1313

Sheng ZH, Rettig J, Cook T, Catterall WA (1996) Calcium-dependent interaction of N-type calcium channels with the synaptic core complex. Nature 379:451–454

Sheng ZH, Yokoyama CT, Catterall WA (1997) Interaction of the synprint site of N-type Ca^{2+} channels with the C2B domain of synaptotagmin I. Proc Natl Acad Sci U S A 94:5405–5410

Sheng J, He L, Zheng H, Xue L, Luo F, Shin W, Sun T, Kuner T, Yue DT, Wu LG (2012) Calcium-channel number critically influences synaptic strength and plasticity at the active zone. Nat Neurosci 15:998–1008

Shepherd JD, Huganir RL (2007) The cell biology of synaptic plasticity: AMPA receptor trafficking. Annu Rev Cell Dev Biol 23:613–643

Sollner T, Whiteheart SW, Brunner M, Erdjument-Bromage H, Geromanos S, Tempst P, Rothman JE (1993) SNAP receptors implicated in vesicle targeting and fusion. Nature 362:297–298

Spafford JD, Zamponi GW (2003) Functional interactions between presynaptic calcium channels and the neurotransmitter release machinery. Curr Opin Neurobiol 13:308–314

Spafford JD, Munno DW, Van Nierop P, Feng ZP, Jarvis SE, Gallin WJ, Smit AB, Zamponi GW, Syed NI (2003) Calcium channel structural determinants of synaptic transmission between identified invertebrate neurons. J Biol Chem 278:4258–4267

Stanley EF (1993) Single calcium channels and acetylcholine release at a presynaptic nerve terminal. Neuron 11:1007–1011

Stanley EF (2003) Syntaxin I modulation of presynaptic calcium channel inactivation revealed by botulinum toxin C1. Eur J Neurosci 17:1303–1305

Stanley EF, Mirotznik RR (1997) Cleavage of syntaxin prevents G-protein regulation of presynaptic calcium channels. Nature 385:340–343

Stephens GJ, Mochida S (2005) G protein βγ subunits mediate presynaptic inhibition of transmitter release from rat superior cervical ganglion neurones in culture. J Physiol 563:765–776
Strock J, Diverse-Pierluissi MA (2004) Ca^{2+} channels as integrators of G protein-mediated signaling in neurons. Mol Pharmacol 66:1071–1076
Sudhof TC (1995) The synaptic vesicle cycle: a cascade of protein-protein interactions. Nature 375:645–653
Sudhof TC (2004) The synaptic vesicle cycle. Annu Rev Neurosci 27:509–547
Sudhof TC (2012) The presynaptic active zone. Neuron 75:11–25
Szabo Z, Obermair GJ, Cooper CB, Zamponi GW, Flucher BE (2006) Role of the synprint site in presynaptic targeting of the calcium channel $Ca_V2.2$ in hippocampal neurons. Eur J Neurosci 24:709–718
Takahashi T, Forsythe ID, Tsujimoto T, Barnes-Davies M, Onodera K (1996) Presynaptic calcium current modulation by a metabotropic glutamate receptor. Science 274:594–597
Trus M, Wiser O, Goodnough MC, Atlas D (2001) The transmembrane domain of syntaxin 1A negatively regulates voltage-sensitive Ca^{2+} channels. Neuroscience 104:599–607
Tsien RW, Lipscombe D, Madison DV, Bley KR, Fox AP (1988) Multiple types of neuronal calcium channels and their selective modulation. Trends Neurosci 11:431–438
Tsien RW, Elinor PT, Horne WA (1991) Molecular diversity of voltage-dependent calcium channels. Trends Neurosci 12:349–354
Tsunoo A, Yoshii M, Narahashi T (1986) Block of calcium channels by enkephalin and somatostatin in neuroblastoma-glioma hybrid NG108–15 cells. Proc Natl Acad Sci U S A 83:9832–9836
Vendel AC, Terry MD, Striegel AR et al (2006) Alternative splicing of the voltage-gated Ca^{2+} channel β4 subunit creates a uniquely folded N-terminal protein binding domain with cell-specific expression in the cerebellar cortex. J Neurosci 26:2635–2644
von Gersdorff H, Borst JG (2002) Short-term plasticity at the calyx of Held. Nat Rev Neurosci 3:53–64
Wadel K, Neher E, Sakaba T (2007) The coupling between synaptic vesicles and Ca^{2+} channels determines fast neurotransmitter release. Neuron 53:563–575
Weiss N (2006) The calcium channel β_{4a} subunit: a scaffolding protein between voltage-gated calcium channel and presynaptic vesicle-release machinery? J Neurosci 26:6117–6118
Westenbroek RE, Hell JW, Warner C, Dubel SJ, Snutch TP, Catterall WA (1992) Biochemical properties and subcellular distribution of an N-type calcium channel alpha 1 subunit. Neuron 9:1099–1115
Westenbroek RE, Sakurai T, Elliott EM et al (1995) Immunochemical identification and subcellular distribution of the alpha 1A subunits of brain calcium channels. J Neurosci 15:6403–6418
Wiser O, Bennett MK, Atlas D (1996) Functional interaction of syntaxin and SNAP-25 with voltage-sensitive L- and N-type Ca^{2+} channels. EMBO J 15:4100–4110
Wiser O, Tobi D, Trus M, Atlas D (1997) Synaptotagmin restores kinetic properties of a syntaxin-associated N-type voltage sensitive calcium channel. FEBS Lett 404:203–207
Wu LG, Saggau P (1997) Presynaptic inhibition of elicited neurotransmitter release. Trends Neurosci 20:204–212
Wu L, Bauer CS, Zhen XG, Xie C, Yang J (2002) Dual regulation of voltage-gated calcium channels by PtdIns(4.5)P-2. Nature 419:947–952
Xu J, Wu LG (2005) The decrease in the presynaptic calcium current is a major cause of short-term depression at a calyx-type synapse. Neuron 46:633–645
Xu J, He L, Wu LG (2007) Role of Ca^{2+} channels in short-term synaptic plasticity. Curr Opin Neurobiol 17:352–359
Yao J, Gaffaney Jon D, Kwon Sung E, Chapman Edwin R (2011) Doc2 is a Ca^{2+} sensor required for asynchronous neurotransmitter release. Cell 147:666–677
Yokoyama CT, Sheng ZH, Catterall WA (1997) Phosphorylation of the synaptic protein interaction site on N-type calcium channels inhibits interactions with SNARE proteins. J Neurosci 17:6929–6938

Yokoyama CT, Myers SJ, Fu J, Mockus SM, Scheuer T, Catterall WA (2005) Mechanism of SNARE protein binding and regulation of Ca_V2 channels by phosphorylation of the synaptic protein interaction site. Mol Cell Neurosci 28:1–17

Yoshida A, Oho C, Omori A, Kuwahara R, Ito T, Takahashi M (1992) HPC-1 is associated with synaptotagmin and omega-conotoxin receptor. J Biol Chem 267:24925–24928

Yoshihara M, Littleton JT (2002) Synaptotagmin I functions as a calcium sensor to synchronize neurotransmitter release. Neuron 36:897–908

Zamponi GW, Snutch TP (1998) Decay of prepulse facilitation of N type calcium channels during G protein inhibition is consistent with binding of a single Gbeta subunit. Proc Natl Acad Sci U S A 95:4035–4039

Zamponi GW, Bourinet E, Nelson D, Nargeot J, Snutch TP (1997) Crosstalk between G proteins and protein kinase C mediated by the calcium channel alpha1 subunit. Nature 385:442–446

Zhong H, Yokoyama C, Scheuer T, Catterall WA (1999) Reciprocal regulation of P/Q-type Ca^{2+} channels by SNAP-25, syntaxin and synaptotagmin. Nat Neurosci 2:939–941

Zhu LQ, Liu D, Hu J et al (2010) GSK-3 beta inhibits presynaptic vesicle exocytosis by phosphorylating P/Q-type calcium channel and interrupting SNARE complex formation. J Neurosci 30:3624–3633

Zucker RS, Regehr WG (2002) Short-term synaptic plasticity. Annu Rev Physiol 64:355–405

Part IV
Synaptic Vesicle Endocytosis

Chapter 10
Endocytic Machinery at the Neuronal Synapse

Ira Milosevic

Abstract Neurotransmission, a quantal process that conveys signals from one nerve cell to another, forms the basis of intercellular communication in nervous and sensory systems. Even at modest levels of neuronal activity, the hundreds of synaptic vesicles typically present at each synapse would be rapidly used up in the absence of equally robust mechanisms that form new synaptic vesicles. Thus, normal physiology of neuronal synapses is critically dependent on synaptic vesicle recycling. However, 40 years after Heuser and Reese showed that the synaptic vesicles are locally formed and recycled at the presynaptic terminal, the precise mechanism(s) of synaptic vesicle recycling remain(s) elusive. A major role in this process is thought to be played by clathrin-mediated endocytosis, a form of endocytosis that utilizes the clathrin coat, the GTPase dynamin, and a variety of accessory factors. The contribution of two other types of endocytosis, kiss-and-run and bulk endocytosis, to the regeneration of new synaptic vesicles is still an open question. This chapter summarizes current knowledge on endocytic modes and their machinery at the presynaptic terminals, with a strong emphasis on clathrin-mediated endocytosis as the predominant pathway of synaptic vesicle recycling. Given the essential nature of this topic, future progress in this field will not only advance our understanding of synaptic transmission but also have wide implications for neurophysiology, pharmacology, and medicine.

Keywords Neuron • Synapse • Synaptic vesicle • Endocytosis • Synaptic vesicle recycling • Clathrin-mediated endocytosis • Bulk endocytosis • Kiss-and-run

10.1 Biogenesis and Recycling of Synaptic Vesicles at the Presynaptic Terminal

As a consequence of the membrane-based organization of life, cells developed processes by which they can shuttle molecules between compartments, as well as release them to the extracellular space. This latter process, referred to as exocytosis, is

I. Milosevic (✉)
European Neuroscience Institute (ENI), Grisebachstr. 5, 37077 Göttingen, Germany
e-mail: imilose@gwdg.de

S. Mochida (ed.), *Presynaptic Terminals*, DOI 10.1007/978-4-431-55166-9_10

of particular importance since it enables the cell to communicate with its surrounding. It can occur constitutively or in a regulated manner, triggered by specific signals. For neurons and neuroendocrine cells, the specialized cells whose main function is to secrete signaling molecules rapidly and on demand, this signal is an increase in the intracellular calcium (Ca^{2+}) concentration (Douglas 1968).

In neurons, Ca^{2+}-triggered exocytosis is a complex and highly controlled process (Sudhof 2013). It is based on secretory vesicles, individual modules that contain a defined amount (quantum) of signaling molecules (neurotransmitters or signaling peptides/amines) destined to be released. Neurotransmitters accumulate in the synaptic vesicles, while peptides and biogenic amines are stored in large dense-core vesicles (LDCVs). Both types of vesicles commonly accumulate at the contact points between neurons, called synapses, and in close proximity to the plasma membrane until directed to undergo exocytosis. Although synaptic vesicles and LDCVs differ in a number of aspects, they share the fundamental mechanism of release (De Camilli and Jahn 1990).

The existence of synaptic vesicles is essential for maintaining the properties of neuronal synapses, including directionality of the signal, quantal release, and modulation of synaptic signal. Presynaptic nerve terminals can store a limited number of synaptic vesicles; therefore, the used membranes and proteins must be rapidly recycled in order to prevent their depletion and consequent synaptic fatigue (Fig. 10.1). The conserved vesicle recycling mechanisms at the synapse, which

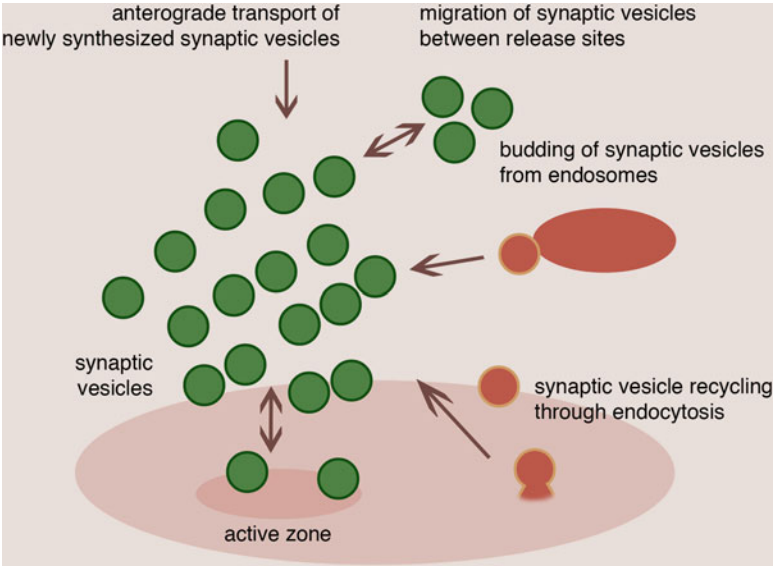

Fig. 10.1 Schematic representation of the synaptic vesicle dynamics at the presynaptic terminus. Synaptic vesicles that accumulate at the presynaptic terminus originate from various sources. Synaptic vesicles are either actively transported to the synapses or recycled locally, both in close proximity to the active zone (periactive zone) and by budding from endosomes. Furthermore, significant exchange of synaptic vesicles happens between adjacent synapses

result in the formation of new homogeneously sized synaptic vesicles through endocytosis, enable neuronal cells to sustain high rates of synaptic transmission. Consequently, nerve terminals must be capable of regenerating SVs locally with high efficiency and fidelity, and as a rule they should be able to meet any demands imposed by neuronal activity.

Neurotransmission starts with a short-lasting event in which the electrical membrane potential of a neuron rapidly rises and falls (action potential), allowing calcium entry in a presynaptic nerve terminal and thus triggering the fusion of synaptic vesicles with the presynaptic plasma membrane to release neurotransmitters. The exocytic process encompasses targeted translocation of vesicles to the release sites, the initial contact with the plasma membrane (docking), the preparation of vesicles for the fusion (priming), and the Ca^{2+}-triggered fusion of membranes that results in the release of vesicular content (Sudhof 2013). The hundreds of synaptic vesicles typically present at the synapse would be used up rapidly without an equally robust mechanism for forming new synaptic vesicles, even at a modest level of neuronal activity. Thus, to compensate for the added membrane during exocytosis, the cells maintain the net surface area by recycling the added proteins and lipids through a process of compensatory endocytosis (McMahon and Boucrot 2011; Saheki and De Camilli 2012).

Over 40 years after it was shown that the synaptic vesicles are locally formed and recycled at the presynaptic terminal (Heuser and Reese 1973), the precise mechanisms of SV recycling remain elusive and are presently heavily debated (McMahon and Boucrot 2011; Saheki and De Camilli 2012). The uniquely small and homogeneous size of synaptic vesicles, as well as their defined protein composition, suggests the existence of very precise mechanisms that are intimately linked with the endocytic machinery that shapes and fissions them. Three mechanisms for synaptic vesicle endocytosis have been proposed up to date: clathrin-mediated endocytosis, bulk endocytosis, and direct reformation of vesicles via fast closure of a transient fusion pore ("kiss-and-run"), as depicted in Fig. 10.2. In spite of the multiple endocytic pathways that exist, it is presently considered that the clathrin-mediated endocytosis is a major pathway for SV recycling (Dittman and Ryan 2009; Heuser and Reese 1973; Murthy and De Camilli 2003).

During the last three decades, exocytosis and endocytosis have been studied independently in great detail. Although still mechanistically unclear, it is currently believed that these processes are tightly coupled (Gundelfinger et al. 2003; Wu et al. 2014). Further, it has been proposed that the proteins and membrane recently added (after vesicle fusion) must be removed from sites of fusion so that the docking of new synaptic vesicles can take place (Hosoi et al. 2009). However, it is presently debated whether the added proteins remain associated with each other or diffuse throughout the plasma membrane (Fernandez-Alfonso et al. 2006; Wienisch and Klingauf 2006; Willig et al. 2006). In any case, at the neuronal synapses, the processes of regulated exocytosis and compensatory endocytosis are considered tightly coupled and evolutionary perfected.

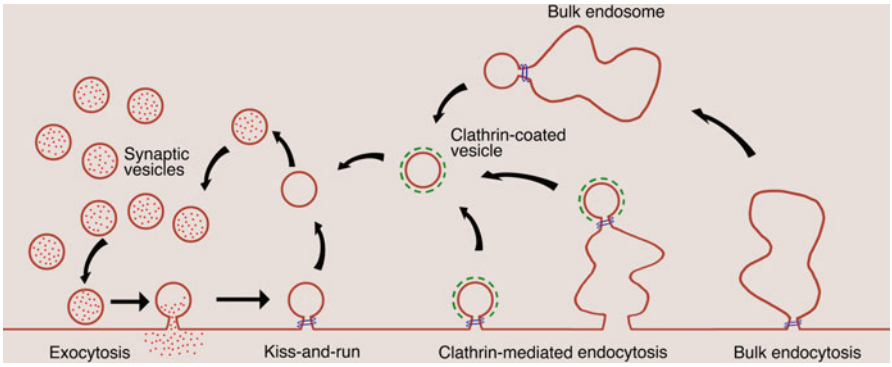

Fig. 10.2 Schematic representation of membrane traffic at the synapse illustrating endocytosis of synaptic vesicle membranes via clathrin-coated pits from the plasma membrane, "kiss-and-run," and bulk endocytosis followed by vesicle formation via unknown mechanisms from endocytic intermediates. This recycling traffic is interconnected with housekeeping membrane recycling involving clathrin-mediated endocytosis and canonical early endosomes

10.2 Clathrin-Mediated Endocytosis: A Major Pathway for Synaptic Vesicle Recycling at the Neuronal Synapse

Clathrin-mediated endocytosis is a classic example of vesicle formation mediated by coat assembly (Bonifacino and Glick 2004; Saheki and De Camilli 2012). Clathrin-coated profiles (pits and vesicles) are rarely observed in resting presynaptic terminals, and their number increases only after stimulation (Heuser and Reese 1973; Roth and Porter 1964). Therefore, most proteins involved in clathrin-coated endocytosis are present in the cytosol of the presynaptic terminal, and their engagement in the process is triggered during and after stimulation.

The basic aspects of clathrin-mediated endocytosis were described using electron microscopy by Roth and Porter (Roth and Porter 1964). Understanding of the process was greatly expanded in the 1970s by several biochemical and morphological studies performed on the coated vesicles. In 1975 and 1976, two major studies conducted by Barbara Pearse purified the coated vesicles and identified clathrin as the most abundant protein (Pearse 1975, 1976). Furthermore, it was shown that clathrin is able to self-assemble into a honeycomb-shaped lattice to form the clathrin coat at low pH (Van Jaarsveld et al. 1981; Woodward and Roth 1978). Additional work led to the discovery of a clathrin assembly factor, a heterotetrameric protein complex found in the coated vesicles, which possessed the ability to bind clathrin and assist in the assembly of coats at physiological pH (Keen et al. 1979; Van Jaarsveld et al. 1981). The pool of this protein complex that is distributed to the plasma membrane was later named adaptor protein 2 (AP2), and it was suggested that the AP2 is also responsible for cargo sorting.

The basic steps of clathrin-mediated endocytosis are well described (Fig. 10.3; (Kirchhausen 2009; McMahon and Boucrot 2011; Saheki and De Camilli 2012)). The creation of the clathrin-coated vesicle is a highly orchestrated, dynamic process that involves simultaneous activity of an extensive network of protein–protein

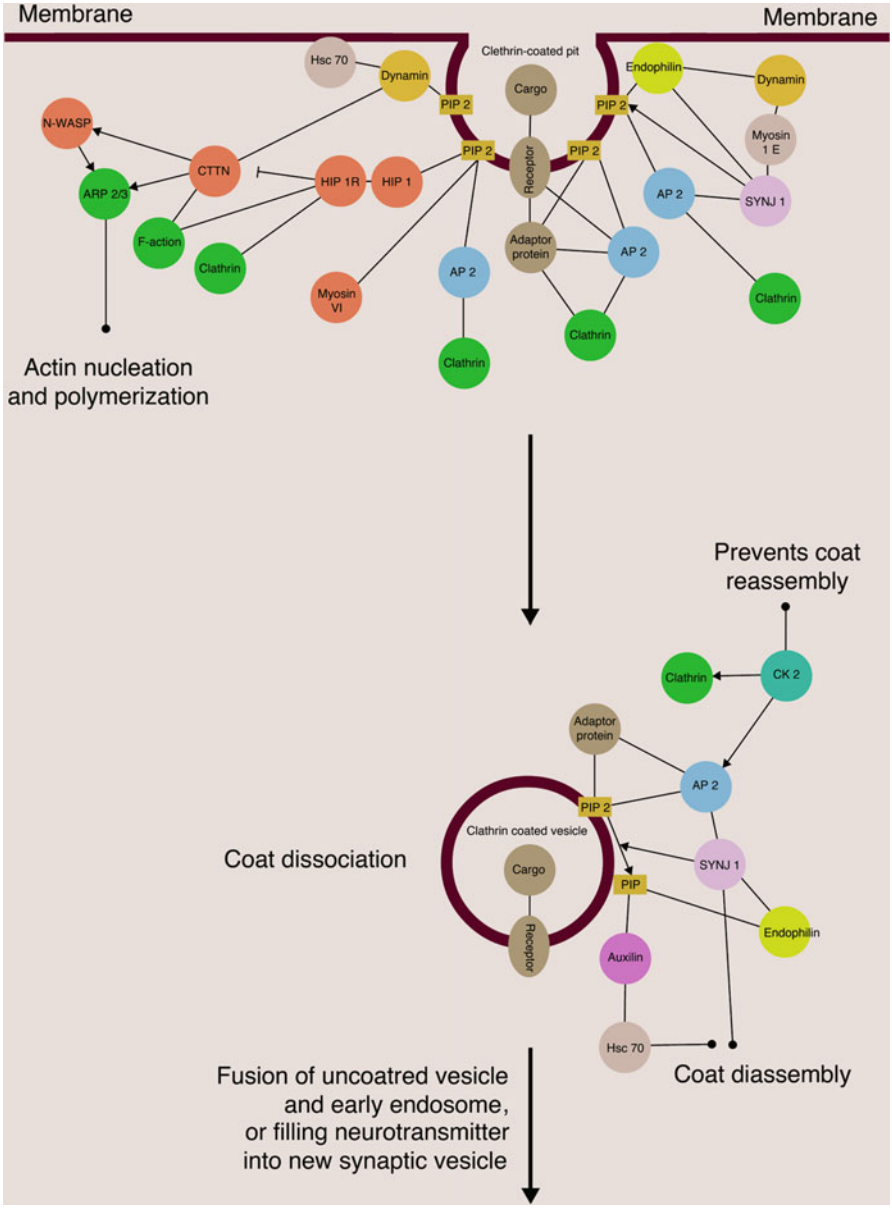

Fig. 10.3 Schematic of molecular machinery that drives clathrin-mediated endocytosis

interactions along with regulated phosphorylation and dephosphorylation events. A long-standing question in this field is how the formation of clathrin-coated pits starts (Antonescu et al. 2010; Cocucci et al. 2012; Ehrlich et al. 2004; Henne et al. 2010; Umasankar et al. 2012). The process is thought to be initiated by the local increase in the amount of plasmalemmal phosphatidylinositol-4,5-bisphosphate [$PI(4,5)P_2$] and the number of cytosolic domains of cargo/SV proteins in the inner layer of the plasma membrane. While $PI(4,5)P_2$ is likely increased through the action of recruited PI-4 and PI-5 kinases, the SV proteins (cargo) are added through the preceding exocytic step. Further, members of adaptor protein family with a high affinity for $PI(4,5)P_2$ and selective cargo proteins are being recruited, as described below in more details.

The binding of adaptor proteins determines the site of a coated pit formation by further initiating the recruitment of clathrin triskelia, which can polymerize in the characteristic lattice that adds to the invaginated coated pit formation. Accessory proteins, most prominently the Bin–Amphiphysin–Rvs (BAR) family members, which have structural and/or regulatory functions, are recruited to the forming pit as well. These proteins are primarily involved in the membrane curvature formation and remodeling of membrane and actin cytoskeleton. When clathrin-coated pit formation is completed, the fission of the pit is mediated by the GTPase dynamin, which assembles at the pit neck in a collar-like structure. After dynamin action, a freely moving coated vesicle will rapidly lose its coat through the action of uncoating factors. $PI(4,5)P_2$ phosphatases (predominantly synaptojanin 1, which is recruited to its site of action by BAR protein endophilin) convert $PI(4,5)P_2$ into PI and subsequently promote the dissociation of clathrin adaptors and accessory proteins. Finally, the coat is being disassembled by an ATPase Hsc70 and its cofactor auxilin, and the coat-free vesicle containing the cargo/SV proteins can either tether and fuse with the presynaptic endosomes or get loaded with neurotransmitters and prepared for a new round of exocytosis.

As outlined above, the endocytic proteins are recruited to the site of vesicle formation in a sequential manner, each protein having its specific times of arrival and departure. For easier understanding, the formation of a new vesicle through clathrin-mediated endocytosis can be considered a five-step process, as suggested by McMahon and Boucrot (2011):

1. *Nucleation of a coated pit*: FCHO proteins bind $PI(4,5)P_2$-enriched zones on the plasma membrane and recruit EPS15–EPS15R and intersectin, which subsequently initiates clathrin-coated pit formation by recruiting adaptor protein 2, AP2.
2. *Cargo selection*: AP2 can directly recruit several classes of cargo proteins through its μ- and σ-subunits. Other cargo-specific adaptors (e.g., stonin and epsin) can also bind AP2 and thus recruit their cargo to the AP2 hub.
3. *Coat assembly*: Self-polymerizing clathrin triskelia are recruited by the AP2 hub and form a pentagon- and hexagon-based clathrin coat around the nascent pit. Several accessory proteins support the coat formation.
4. *Vesicle scission*: The GTPase dynamin is recruited at the pit's neck by BAR domain-containing proteins, where it can self-polymerize and induce membrane

scission upon GTP hydrolysis. The actin polymerization at the neck of the pit also aids in vesicle production.

5. *Vesicle uncoating*: Auxilin recruits the ATPase Hsc70 to disassemble the clathrin coat and produce a coat-free vesicle that contains the cargo proteins. Synaptojanin facilitates this reaction by releasing adaptor proteins from the vesicle membrane through its $PI(4,5)P_2$ phosphatase activity. The components of the clathrin machinery are then freed and can become available for another round of clathrin-coated vesicle formation.

10.2.1 Molecular Machinery That Drives Clathrin-Mediated Endocytosis

The formation of new synaptic vesicles through clathrin-coated endocytic vesicles is driven by complex and highly dynamic molecular machinery. Over 50 different proteins are thought to have roles in this process (for review, see McMahon and Boucrot 2011). Although clathrin-mediated endocytosis has been studied for four decades and many details are well understood (as mentioned above), the complete molecular machinery needed for this process is likely not yet known. The difficulty of unraveling the complete set of involved proteins and lipids is not only a result of the large number of components needed and the small size of the presynaptic terminal but is also due to the dynamic nature of the endocytic machinery whose composition can change in a matter of seconds. Recent advancements in live imaging of fluorescently labeled proteins, using confocal microscopy, have started to provide good temporal resolution of these dynamic events (Cocucci et al. 2012; Taylor et al. 2011). When combined with morphological, physiological, molecular, and genetic studies, key insights into the main molecular players that govern the clathrin-mediated endocytosis and its regulation have been obtained (as recently reviewed by Saheki and De Camilli 2012). The short summary on main protein participants is presented first, followed by lipids in the next section. For an easier overview, the proteins have been listed in Table 10.1 and classified in five groups. This classification is somewhat arbitrary, as some proteins wear multiple hats (e.g., endophilin acts as a membrane curvature sensor/inducer as well as an adaptor protein for vesicular glutamate transporter 1, VGLUT1).

1. Core clathrin coat components: clathrin and clathrin adaptors (e.g., AP2, AP180, epsin, stonin)
2. Membrane curvature sensors and inducers (e.g., endophilins 1–3, amphiphysin, SNX9, FCHO, syndapin, Toca/CIP4/FBP17)
3. Scaffolding proteins (e.g., Tuba HIP1, HIP1R, actin, Eps15, Eps15R, intersectin)
4. Scission protein (dynamins 1–3)
5. Uncoating molecules (synaptojanin, endophilin, auxilin, Hsc70)

Table 10.1 Major proteins involved in clathrin-mediated endocytosis and synaptic vesicle recycling at the neuronal synapse

Molecule	Function
Proteins	
FChO	Major nucleator of clathrin-coated pit, interacts with intersectin, Esp15, and dynamin
Toca/CIP4/ FBP17	Initiates actin cytoskeleton nucleation and reorganization, binds N-WASP and dynamin
Syndapin	Coordinates endocytosis and actin nucleation, binds N-WASP, dynamin, and synaptojanin
Endophilin	BAR protein (membrane curvature sensor and inducer) that binds dynamin and synaptojanin, implicated in vesicle fission and uncoating
Amphiphysin	BAR protein (membrane curvature sensor and inducer) that binds AP2, clathrin, N-WASP, dynamin, and synaptojanin 1
Clathrin	Consists of heavy and light chains, builds clathrin lattice around endocytic vesicles, and interacts with dynamin, AP2, amphiphysin, and a number of other molecules
AP2	Heterotetramer that acts as clathrin adaptor for several synaptic vesicle membrane proteins; major interactor of clathrin, AP180/CALM, stonin, epsin, intersectin, Esp15, amphiphysin, auxilin, and PIPKIγ; binds $PI(4,5)P_2$
AP180/CALM	Clathrin adaptor for synaptobrevin-2/VAMP2, interacts with AP2 and clathrin, binds $PI(4,5)P_2$
Stonin	Clathrin adaptor for synaptotagmin; interacts with AP2, Eps15, and intersectin; binds $PI(4,5)P_2$
Epsin	Adaptor for clathrin and ubiquitinated membrane proteins that may also act in membrane deformation and fission; interacts with AP2, Eps15, and intersectin; binds $PI(4,5)P_2$
Intersectin	Scaffolding protein that coordinates and recruits many other proteins; interacts with AP2, FCHO, Eps15, epsin, stonin, N-WASP, Cdc42, dynamin, and synaptojanin 1
Eps15	Scaffolding protein that recruits other proteins; interacts with AP2, intersectin, epsin, and stonin
Actin	Makes 5 nm cytoskeletal filaments around endocytic pits
Dynamin	GTPase that executes membrane fission by pinches off endocytic vesicles/ clathrin-coated pits during endocytosis
PIPKIγ	$PI(4,5)P_2$ synthesizing enzyme that interacts with AP2 and talin
Synaptojanin 1	$PI(4,5)P_2$ phosphatase that acts on $PI(4,5)P_2$ and couples fission of clathrin-coated pits with subsequent vesicle uncoating
Auxilin	Recruits Hsc70 to clathrin-coated vesicle, interacts with clathrin, dynamin, and AP2
Hsc70	ATPase that disassembles clathrin cage, interacts with clathrin and auxilin

10.2.1.1 Core Clathrin Coat Components: Clathrin and Clathrin Adaptor Proteins

As mentioned above, adaptor proteins are able to simultaneously bind both the plasma membrane lipids and clathrin molecules, bringing the two together. Clathrin

is thought to have a central role in the clathrin-mediated endocytosis: as a core component of clathrin coat, it interacts with a number of proteins including adaptor proteins (e.g., AP2, AP180/CALM, epsin), membrane curvature inducing proteins (e.g., amphiphysin), as well as the clathrin coat disassembly machinery (synaptojanin, Hsc70, auxilin). The two most common adaptor proteins at the presynaptic terminals are considered to be AP2 and AP180.

10.2.1.1.1 Clathrin: Self-Polymerizing Lattice Protein

Clathrin represents a perfect case of a "form following function" protein: it forms a polyhedral lattice that surrounds and shapes the forming vesicle. Clathrin was named and isolated by Barbara Pearse in 1975/1976. In the cytosol, it adopts a characteristic triskelion (from the Greek for "three-legged structure") shape that is composed of three clathrin heavy chains and three clathrin light chains interacting at their C-termini. While the heavy chains mainly provide the structural backbone of the clathrin lattice, the light chains regulate the formation and disassembly of a clathrin lattice. The structure of clathrin triskelion and clathrin coat is thoughtfully investigated (Kirchhausen 2000, 2009; Kirchhausen and Harrison 1981). Clathrin heavy chain (192 kDa) has multiple subdomains, including the N-terminal, ankle, distal leg, knee, proximal leg, and trimerization domains. Only the N-terminal domain is comprised of a seven-bladed β-propeller structure; the other domains are shaped as a superhelix composed of short alpha helices. Clathrin light chains (23–25 kDa) primarily bind to the proximal leg of the heavy chains, with some interaction near the trimerization domain (for more information, see Kirchhausen 2000).

In the cytoplasm of the presynaptic terminal, where clathrin-coated endocytosis takes place continuously, clathrin triskelia are often already bound to an adaptor protein, whose function is to link clathrin to the membrane. A membrane-attached triskelion easily connects to other membrane-attached triskelia or recruits other triskelia from the cytosol. Clathrin triskelia can assemble into either 5-sided rings (pentagons) that are needed for curved lattice formation or 6-sided rings (hexagons) that yield a flatter lattice. Self-polymerizing properties of triskelia result first in a basket-like (bud) structure and eventually lead to a soccer ball-like (pit) structure that engulfs the membrane of the nascent vesicle to be endocytosed. By constructing different combinations of pentagons and hexagons, vesicles of different sizes can assemble. The smallest clathrin cage commonly imaged has 12 pentagons and only two hexagons.

The well-described clathrin triskelion structure has advanced the understanding of molecular mechanisms of clathrin-mediated endocytosis and formed the basis of a search for compounds that interfere with clathrin polymerization and coat structure assembly. So far, two chemical compounds, Pitstop 1 and Pitstop 2, have been characterized as the first-known clathrin inhibitors that can selectively block the endocytic ligand association with the clathrin terminal domain (von Kleist et al. 2011).

10.2.1.1.2 AP2: Adaptor for Clathrin and Several Synaptic Vesicle Proteins

AP2 is the main adaptor of clathrin-coated vesicles, and as such, it has been studied extensively over the years (as reviewed in McMahon and Boucrot 2011; Owen et al. 2004). The crystal structure of the individual AP2 subunits depicts well how the complex can, at the same time, collect cargo, assemble clathrin, and recruit endocytic accessory proteins (Collins et al. 2002; Owen et al. 1999; Traub et al. 1999). AP2 is a heterotetrameric protein complex that is composed of two large subunits (α and β2) and two smaller subunits (μ2 and σ2) (reviewed by Kirchhausen 1999; Owen et al. 2004). It consists of a core and two appendages. The core is made of two N-terminal domains of α and β2 large subunits (referred to as the trunks), the μ subunit and the σ subunit (Heldwein et al. 2004). The two large subunits also have a globular C-terminal domain (known as the ear or appendage) that is connected to the trunks by a flexible unstructured linker, allowing the ear to reach out and to interact with accessory proteins (Owen et al. 2004). Binding motifs for AP2 ears are being found in a growing number of proteins with accessory roles in clathrin-mediated endocytosis (Slepnev and De Camilli 2000). In particular, the β2 subunit contains two binding sites for clathrin: one (stronger binding site) in the hinge region and the other (weaker binding site) in the appendage domain (Owen et al. 2000; ter Haar et al. 2000).

In order for the transmembrane receptors to be incorporated into a newly forming clathrin-coated pit, these proteins require an appropriate sorting signal. The μ2 subunit of AP2 has the ability to bind to YXXØ and [DE]XXXL [LI] sequences (where single letters designate the amino acids, with X as any amino acid, and Ø as any bulky hydrophobic amino acid) (Bonifacino and Traub 2003). These motifs act as strong endocytic signals, and experimental data support the possible engagement of the motif by a complex of α/σ2 as well as cross-linking studies showing a β2 interaction (Bonifacino and Traub 2003). Next, the adaptor-associated kinase AAK1 and auxilin, members of the Ark/Prk family of kinases, can phosphorylate μ2 in vitro and have been implicated in μ2 activation, which allows for YXXØ cargo capture and exposes another AP2 PI(4,5)P_2-binding site that was sequestered in the unphosphorylated state (Edeling et al. 2006; Smythe 2002). Clathrin was reported to promote this activation by stimulation of kinase activity (Conner et al. 2003; Jackson et al. 2003). Some of the AP2 interacting proteins include synaptotagmin, synaptojanin, synaptic vesicle 2 (SV2), AP180/CALM, stonin, amphiphysin, PIPK1γ, intersectin, epsin, Eps15, and auxilin (as reviewed by Saheki and De Camilli 2012).

10.2.1.1.3 AP180: Specific Adaptor Protein for Synaptobrevin-2/VAMP2

Adaptor protein 180 (AP180) plays an important role in clathrin-mediated endocytosis at the presynaptic terminal, firstly by recruiting and promoting polymerization of clathrin triskelia to the plasma membrane's site of a newly forming bud and secondly by serving as a specific adaptor for SNARE protein synaptobrevin-2/

vesicle-associated membrane protein 2 (VAMP2) (for review, see Maritzen et al. 2012). Besides being able to simultaneously bind the plasma membrane PI(4,5)P_2 and lipids (via an ANTH domain) as well as clathrin, bringing the two together, AP180 can also bind AP2 and thus recruit VAMP2/synaptobrevin-2 to the endocytic pit (Maritzen et al. 2012). Deletion of the AP180 homologue in a fruit fly leads to fewer synaptic vesicles and an overall decrease in neurotransmission (Bao et al. 2005). In addition, the C-terminus of AP180 can be used as a specific inhibitor of clathrin-mediated endocytosis. The ubiquitous form of the AP180 protein in mammals is CALM (clathrin assembly lymphoid myeloid leukemia protein), named after its association with myeloid and lymphoid leukemias (Maritzen et al. 2012).

10.2.1.1.4 Epsin: Adaptor for Ubiquitinated Membrane Proteins with a Role in Membrane Deformation and Fission

Epsin functions as an adaptor by recognizing ubiquitinated cargo and as an endocytic accessory protein by contributing to endocytic network stability/regulation and membrane bending [(Chen and De Camilli 2005; Chen et al. 1998, 1999, 2009); reviewed by (Saheki and De Camilli 2012; Sen et al. 2012)]. Similar to AP180, epsin has a PI(4,5)P_2 binding domain and is able to recruit clathrin to PI(4,5)P_2-containing membranes. However, unlike AP180, epsin can itself deform the lipid membrane by inserting helix0, which results in membrane deformation/curvature. Thus, a dual role for epsin has been proposed: (1) it promotes clathrin-coated pit formation in conjunction with clathrin polymerization, and (2) it actively drives the deformation of the membrane, primarily at the edges of invagination sites, by binding PI(4,5)P_2 in the inner layer of plasma membrane and inserting helix0. Besides binding to clathrin and PI(4,5)P_2, epsin is a specific adaptor for ubiquitinated membrane proteins, and it interacts with AP2, intersectin, Eps15, EHD, and ubiquitin (for more information, see Saheki and De Camilli 2012; Sen et al. 2012).

10.2.1.1.5 Stonin: More than a Specific Adaptor for Synaptotagmin

Stonin (termed stonin 2 in mammals, stoned B in fruit flies) was initially considered as a specific adaptor for synaptotagmin-1 (Diril et al. 2006; Jung et al. 2007; Maritzen et al. 2010). In addition to binding synaptotagmin, stonin can also bind the plasma membrane lipid PI(4,5)P_2 and AP2, as well as Eps15 and intersectin (reviewed by Maritzen et al. 2010). Neurons with stonin loss-of-function mutation show defective neurotransmission and a reduced number of synaptic vesicles at the presynaptic terminal (Fergestad et al. 1999; Mullen et al. 2012; Phillips et al. 2010; Stimson et al. 2001). Interestingly, deletion of stonin in mice accelerated synaptic vesicle endocytosis and elevated the synaptic vesicle pool size, in addition to causing selective missorting of synaptotagmin-1 to the neuronal surface

(Kononenko et al. 2013). These data are consistent with synaptotagmin-1 being a facilitator of SV membrane retrieval in mice, flies, and worms.

10.2.1.2 Membrane Curvature Sensors and Inducers

A superfamily of BAR (Bin–Amphiphysin–Rvs) domain-containing proteins plays an important role in membrane dynamics in a cell by inducing membrane curvature de novo, detecting curvature of a particular shape or size in order to recruit cytosolic factors to membranes, and stabilizing already present curvature (Daumke et al. 2014; Frost et al. 2009). Each member of this family comprises a structurally conserved region of approximately 200 amino acids, based on long α-helices, which can sense and/or induce membrane curvature. The BAR superfamily is subdivided into BAR/N-BAR modules that bind to membranes of high positive curvature, F-BAR modules that bind to a different range of positive membrane curvatures, and I-BAR modules that bind to negatively curved membranes (reviewed by Frost et al. 2009; Itoh and De Camilli 2006). Here we review selected BAR proteins with proposed function in endocytosis and/or at the neuronal synapse.

10.2.1.2.1 FCHo: Sculpts the Initial Bud Site and Recruits the Clathrin Machinery

In parallel with the recruitment of the first AP2 molecules to the formation site of new clathrin-coated pit, FCHo 1 and FCHo 2 (Fer/Cip4 homology (FCH) domain-only proteins 1 and 2; FCHo 2 shares 94.6 % identity with mouse FCHo 1) are also recruited [reviewed by (McMahon and Boucrot 2011)]. These F-BAR domain-containing proteins are recruited to the plasma membrane and required for both clathrin-coated vesicle budding and to mark sites of clathrin-coated pit formation. More specifically, FCHo has been considered a key nucleator of endocytic clathrin-coated pits (Henne et al. 2010). Changes in FCHO 1/2 expression levels correlated directly with numbers of clathrin-coated budding events, ligand endocytosis, and synaptic vesicle marker recycling (Henne et al. 2010). In addition, FCHO 1/2 proteins recruit the scaffold proteins Eps15 and intersectin, which in turn engage AP2 (McMahon and Boucrot 2011).

10.2.1.2.2 Endophilin: A Multifunctional Endocytic Adaptor

Endophilin-A (henceforth endophilin) is a BAR protein that is required for clathrin-mediated endocytosis by acting as the hub of a protein network that coordinates cargo packing, bud constriction, actin assembly, and recruitment of factors needed for fission and uncoating (de Heuvel et al. 1997; Itoh and De Camilli 2006; Milosevic et al. 2011; Ringstad et al. 1997; Takei et al. 1999). The three mammalian endophilin proteins (A1, A2, and A3) are present in the brain and enriched at the synapses. Besides the characteristic BAR domain needed to sense and introduce

membrane curvature, endophilins have the Src homology 3 (SH3) domain needed for the protein interactions and, in particular, for the recruitment of dynamin and synaptojanin 1 at the neck of the clathrin-coated pits (de Heuvel et al. 1997; Ringstad et al. 1997). Cells lacking dynamin accumulate endophilin-rich tubes capped by a clathrin cage, suggesting that the endophilin polymerizes at the vesicle neck and its recruitment to clathrin-coated pits is upstream of dynamin function (Ferguson et al. 2009). However, genetic and targeted ablation studies at invertebrate and mammalian synapses demonstrated that the lack of functional endophilin results not only in an accumulation of clathrin-coated pits but also of clathrin-coated vesicles, revealing a phenotype that is similar to that seen with loss of synaptojanin function (Milosevic et al. 2011; Schuske et al. 2003; Verstreken et al. 2003). Other prominent endophilin binding partners include vesicular glutamate transporter 1 (VGLUT1), which is responsible for loading neurotransmitter into synaptic vesicles (Vinatier et al. 2006), and a number of proteins implicated in neurodegenerative disorders, including huntingtin and ataxin-2 [underlying Huntington's disease and spinocerebellar ataxia type 2, respectively (Ralser et al. 2005)] as well as parkin [a protein linked to Parkinson's disease (Trempe et al. 2009)]. Overall, endophilin acts as one of the central players in endocytosis and signaling by linking cargo loading with fission and uncoating.

10.2.1.2.3 Amphiphysin Coordinates Membrane Curvature Induction

Amphiphysin has an N-terminal BAR domain involved in dimerization, lipid interaction, and membrane bending, a middle clathrin- and adaptor protein-binding domain, and a C-terminal SH3 domain (most recently reviewed by Saheki and De Camilli 2012). Two mammalian amphiphysins with similar overall structure exist: amphiphysin 1 and amphiphysin 2/bridging integrator 1 (BIN1). Amphiphysin 1 is strongly expressed in the brain, where its primary function is thought to be the recruitment of dynamin to the sites of clathrin-mediated endocytosis (Saheki and De Camilli 2012). The ubiquitous amphiphysin 2, which is highly expressed in muscles and involved in the formation and stabilization of the T-tubule network, does not contain clathrin- or adaptor protein-binding sites and has been recently identified as a risk locus for late onset Alzheimer's disease (reviewed by Tan et al. 2013).

10.2.1.2.4 SNX9 Stimulates Dynamin's GTPase Activity

Sorting nexin 9 (SNX9) is a ubiquitously expressed BAR domain protein that has been classified as a member of the sorting nexin (SNX) protein family, given that it contains a variant of the PX domain characteristic of this family (Lundmark and Carlsson 2009). The PX domain is located in the middle of the primary structure and is followed by a linker and the C-terminal BAR domain. This particular domain organization is only shared by the sequentially similar SNX18. In addition, these

proteins are the only sorting nexins to have a Src homology 3 (SH3) domain and are thought to function in protein-sorting events in different compartments of the cell, including the plasma membrane and neuronal synapse [reviewed by (Lundmark and Carlsson 2009)]. SNX9 binds to dynamin and is considered partly responsible for the recruitment of dynamin to its site of action and stimulation of its GTPase activity to facilitate the scission reaction (Lundmark and Carlsson 2003; Soulet et al. 2005). In addition, SNX9 also has a high capacity for membrane reshaping, and it may participate in the formation of the narrow neck of endocytic vesicles before scission occurs (Lundmark and Carlsson 2009; Soulet et al. 2005). Last but not least, SNX9 can also activate the actin regulator N-WASP and coordinate actin polymerization with vesicle fusion (Shin et al. 2007).

10.2.1.2.5 Syndapin/Pascin Coordinates Curvature Acquisition with Vesicle Fission and Actin Cytoskeleton

Syndapin, also called pascin (protein kinase C and casein kinase II interacting protein), is a Fes/CIP4 homology Bin–Amphiphysin–Rvs 161/167 (F-BAR) and SH3 domain-containing protein. Three genes give rise to three main homologues in mammalian cells (Kessels and Qualmann 2004). They each function in different endocytic and vesicle trafficking pathways and provide critical links between the cytoskeletal network in different cellular processes, such as neuronal morphogenesis and cell migration (reviewed by Quan and Robinson 2013). The membrane remodeling activity of syndapin via its F-BAR domain and its interaction partners, dynamin and neural Wiskott–Aldrich syndrome protein (N-WASP), which bind to its SH3 domain, are central to its function. Signaling pathways leading to the regulation of syndapin function by phosphorylation are presently contributing to our understanding of the broader functions of this family of proteins (Quan and Robinson 2013).

10.2.1.2.6 Toca1/FBP17/CIP4: Nucleates and Organizes Actin Cytoskeleton

An important signaling pathway to the actin cytoskeleton links the Rho family GTPase Cdc42 to the actin-nucleating Arp2/3 complex through N-WASP. However, these components are not sufficient to mediate Cdc42-induced actin polymerization in a physiological context. Ho et al. (2004) identified Toca1 as an essential component of the Cdc42 pathway. TOCA1 binds both N-WASP and Cdc42 and promotes Cdc42-induced actin nucleation by activating the N-WASP–WIP complex (Ho et al. 2004). This 547-amino acid protein belongs to the evolutionarily conserved PCH-protein family, which also includes CIP4 and FBP17 (Lippincott and Li 2000), and, like the other members of this family, TOCA1 contains an N-terminal FCH domain, two coiled-coil regions, and a C-terminal SH3 domain (Ho et al. 2004).

10.2.1.3 Clustering/Scaffolding Molecules

Adaptor and scaffold proteins serve as platforms for the assembly of multiprotein complexes and regulate the efficiency of clathrin coat formation.

10.2.1.3.1 Eps15: A Scaffolding Protein That Recruits Other Endocytic Proteins

The Eps15 protein has 3 N-terminal Eps15 homology (EH) domains, a central coiled-coil region, an AP2-binding domain, and a proline-rich domain (Bakowska et al. 2005), while its C-terminal region contains two ubiquitin-interacting motifs (Fallon et al. 2006). Eps15 co-associates with FCHo, epsin, and intersectin: Eps15 and FCHo 1/2 localize to the rim of the growing clathrin-coated pit (Henne et al. 2010; Tebar et al. 1996). In their absence, clathrin coat assembly aborts (Cocucci et al. 2012). Similar to epsin, Eps15 binds ubiquitin and thus may help to recruit ubiquitinated cargo molecules into clathrin-coated pits (Polo et al. 2002).

10.2.1.3.2 Intersectin: Scaffolding Protein That Coordinates and Recruits Other Endocytic Proteins

Intersectin is an evolutionarily conserved adaptor/scaffold proteins that coordinate endocytic membrane traffic with the actin assembly machinery and signaling (Pechstein et al. 2010; Tsyba et al. 2011). It has been shown that intersectin interacts with a number of endocytic and cytoskeletal factors, namely, dynamin, Eps15, epsin, stonin 2, Cdc42, and even the SNARE proteins SNAP-25 and SNAP-23 that have a role in exocytosis (Pechstein et al. 2010; Tsyba et al. 2011). Furthermore, it has been recently proposed that intersectin, as a multimodular scaffolding and adaptor protein shuttling between the active and periactive zones, may also act as a regulator of synaptic vesicle recycling by aiding the assembly of macromolecular protein complexes that execute exocytic and endocytic processes (Pechstein et al. 2010).

10.2.1.3.3 Actin: Role in Endocytosis

As one of the most important building blocks of the cell cytoskeleton, monomeric actin has a potential to form filaments essential for a number of biological functions. At the neuronal presynaptic terminus, actin has a prominent role in vesicular trafficking, both in exocytic and endocytic pathways (for review, see Brodsky 2012; Kirchhausen 1999; Mooren et al. 2012; Saheki and De Camilli 2012; Schafer 2002; Stamnes 2002).

In addition, actin may play different roles in endocytosis through interactions with different regulatory proteins. Firstly, spatial organization through anchoring of

actin filaments is thought to generate hot spots for endocytosis by generating a shield to stop endocytic proteins from diffusion. Secondly, the force generated by actin polymerization and fiber formation can aid clathrin-coated pit or vesicle invagination, fission, and transport of vesicles.

Actin polymerization is not rate limiting for clathrin-coated pit dynamics or transferrin uptake in cultured cells under normal growth conditions (Saffarian et al. 2009), but clathrin coat pit dynamics is stalled when membrane tension is elevated (Boulant et al. 2011). Actin polymerization is known to be regulated by Rho GTPases: at the plasma membrane, the Rho GTPase Cdc42 together with PI $(4,5)P_2$ and SH3 domain-containing protein Grb2 activates the auto-inhibited WASP protein family (Saarikangas et al. 2010). These proteins initiate polymerization as well as branching of actin via the Arp2/3 complex. Furthermore, several proteins, including syndapin and intersectin, link dynamin and other clathrin-associated proteins to actin polymerization machinery (Merrifield et al. 2005; Pechstein et al. 2010; Quan and Robinson 2013). Last but not least, endocytic vesicles have been shown to be transported at the tips of actin tails, and an intensification of dynamin staining at vesicular scission is followed by an increased actin signal, suggesting that dynamin and actin have sequential roles at the late stages of endocytosis (Merrifield et al. 2002). After the clathrin coat is shed, free vesicles can recruit actin and move rapidly from the fission site; however, the details of this directed motion are not well understood (Brodsky 2012; Merrifield et al. 2002).

10.2.1.4 Vesicle Scission

10.2.1.4.1 Dynamin: Molecular Scissor for Vesicles

The endocytic vesicle is excised from the plasma membrane by membrane scission that is executed through the GTPase dynamin (Campelo and Malhotra 2012; Ferguson and De Camilli 2012). Dynamin is thus essential for synaptic vesicle recycling at the synapse. The molecular mechanism of dynamin-induced membrane scission has been studied in details over the last two decades (Campelo and Malhotra 2012; Ferguson and De Camilli 2012; Morlot and Roux 2013). Mechanistically, this mechanochemical enzyme forms a spiral around the neck of an invaginating vesicle. Once the spiral is formed, it extends lengthwise and compresses the membrane through GTP hydrolysis (Morlot and Roux 2013). The lengthening and tightening of the coil around the vesicle neck causes it to break, resulting in the pinching off of the vesicle from the plasma membrane. In addition, dynamin may affect the earlier stages of endocytic invagination, but the precise mechanisms are not yet clear (Mettlen et al. 2009). Mammals have 3 dynamins (dynamin 1, dynamin 2, and dynamin 3); dynamins1 and 3 are considered to be expressed primarily in the central nervous system, while dynamin 2 is ubiquitously expressed (Ferguson and De Camilli 2012). Synapses without dynamins 1 and 3 show a strong accumulation of clathrin-coated pits connected by long membrane

tubules to the plasma membrane (Ferguson et al. 2007; Raimondi et al. 2011). In fibroblasts without dynamins 1 and 2, these long tubules are coated with endophilin and actin (Ferguson et al. 2009), suggesting that invagination and actin polymerization still persist in the absence of dynamin and that a model in which dynamin is recruited after endophilin has formed a thin clathrin-coated pit neck (see also Milosevic et al. 2011). Recently, an additional mechanism of vesicle fission has been suggested, involving the generation of line tension in the bud neck due to the $PI(4,5)P_2$ enrichment in the tubular invagination and PI4P enrichment in the bud, presumably created through the action of phosphatase synaptojanin (Xiao et al. 2006). A compelling model now emerges that couples both dynamin-mediated membrane constriction and line tension instability in promoting more efficient membrane scission. Lastly, despite the large body of evidence that dynamin is required for an efficient fission of new vesicles from the plasma membrane, it is also known that vesicles can bud from membrane surfaces in a dynamin-independent manner (as reviewed by Campelo and Malhotra 2012).

10.2.1.5 Proteins That Uncoat Clathrin-Coated Vesicles

After scission, clathrin-coated vesicles rapidly shed their coat through a coordinated action of the phosphatase synaptojanin 1 (recruited by endophilins), the J domain kinase auxilin/GAK, and the ATP/chaperone Hsc70.

10.2.1.5.1 Synaptojanin 1: $PI(4,5)P_2$ Phosphatase with a Role in Clathrin-Coated Vesicle Uncoating

Synaptojanin 1 is a $PI(4,5)P_2$ phosphatase with both 4-phosphatase and 5-phosphatase (its N-terminal domain is homologous with yeast Sac1) activity and a proline-rich domain that interacts with the SH3 domains of endophilin and amphiphysin (Haffner et al. 1997; McPherson et al. 1996). Out of two synaptojanin 1 isoforms, brain-specific 145 kD and ubiquitously expressed 170 kD, only the 170-kD isoform has an asparagine–proline–phenylalanine (NPF) domain which binds Eps15 and a C-terminal region containing binding sites for the ear domains of AP2 and clathrin (Haffner et al. 1997; Perera et al. 2006). Using multicolor total internal reflection fluorescence microscopy, Perera et al. (2006) reported that the two synaptojanin 1 isoforms are differentially recruited to clathrin-coated pits: the 170-kD isoform was present at all stages of clathrin-coated pit formation, whereas the 145-kD isoform was recruited at a late stage of clathrin-coated pit formation, together with endophilin and dynamin. Thus, dynamic phosphoinositide metabolism occurs throughout the lifetime of a clathrin-coated pit.

Synaptojanin 1-deficient mice die shortly after birth (Cremona et al. 1999). Their neurons have increased levels of $PI(4,5)P_2$ and their presynaptic terminals accumulate clathrin-coated vesicles. Hippocampal neurons without synaptojanin 1 show enhanced synaptic depression during prolonged high-frequency stimulation, as well

as a delayed recovery (Cremona et al. 1999). Taken together, these results argue for a critical role of phosphoinositide metabolism in endocytosis and synaptic vesicle recycling, which is further addressed below.

10.2.1.5.2 Auxilin: A Trigger for Clathrin Cage Disassembly

Vertebrates have two auxilin isoforms: the brain-specific auxilin-1 and the ubiquitous auxilin-2/cyclin G-associated kinase (GAK) (Umeda et al. 2000; Ungewickell et al. 1995). Auxilin is recruited to the clathrin-coated vesicle immediately after it pinches off from its fission site, and it binds the clathrin lattice near C-terminal helical tripod and the crossing of two ankle segments and a terminal domain of clathrin leg (Lee et al. 2006; Massol et al. 2006; Milosevic et al. 2011). Through its J domain, auxilin binds and recruits the ATPase Hsc70 to the clathrin lattice and consequently initiates a set of critical interactions (described below in more detail; (Xiao et al. 2006)).

In addition to the J domain and clathrin-binding site, auxilin also contains a phosphoinositide phosphatase (PTEN)-like region, which may retain phosphoinositide-binding activity in spite of the fact that the critical residues in the catalytic domain are different. Auxilin binding produces a local change in clathrin heavy-chain contacts, creating a detectable global distortion of the clathrin coat; Fotin et al. (2004) thus proposed a mechanism by which local destabilization of the lattice promotes general uncoating. It was further found that mice without auxilin-1 have a high rate of early postnatal mortality (although surviving pups had a normal life span) and impaired synaptic vesicle recycling with increased number of clathrin-coated vesicles (Yim et al. 2010). Interestingly, similar to endophilin, auxilin-1 has also been connected to Parkinson's disease: two separate and likely pathogenic mutations in the auxilin-1 gene have been found in two independent case studies (Edvardson et al. 2012; Koroglu et al. 2013).

10.2.1.5.3 Hsc70 Triggers Disassembly of Clathrin Cages

The heat-shock cognate protein 70 (Hsc70) works together with auxilin to remove the clathrin coat from the newly formed vesicles (Rothman and Schmid 1986). Hsc70 belongs to the Hsp70 family that contains both the heat-inducible and constitutively expressed members (the latter are also called Hsc proteins). In addition to its role in endocytosis, Hsc70 functions as an ATP-dependent chaperone (protein that helps protein folding), and it associates transiently with nascent polypeptides to facilitate correct folding (Tavaria et al. 1995). When Hsc70 is in the ATP-bound form, its association with the polypeptide is weak, while the ADP-bound Hsc70 form acts a tight clamp. Importantly, Hsc70 and its substrate are brought together by a J domain-containing protein (auxilin in the case of clathrin coat), which strengthens the initial weak interaction (Xing et al. 2010). More specifically, auxilin interacts with the clathrin lattice in such way that Hsc70

can bind clathrin's hydrophobic C-terminal sequence, thus generating a local distortion of the clathrin lattice that is stabilized by ATP hydrolysis (Jiang et al. 2007; Xing et al. 2010). It is thus essential that auxilin is recruited to the clathrin-coated vesicle after the fission step is completed; otherwise, the action of Hsc70-driven uncoating will hamper the formation of the clathrin-coated pit. It is presently unclear how the lattice assembly–disassembly cycle is controlled or, more specifically, how the auxilin recruitment is timed: a possible mechanism points to lipid modification by synaptojanin 1 phosphatase action (Milosevic et al. 2011).

10.2.2 Vesicular Fate After Clathrin Coat Shedding

While the textbook models of clathrin-mediated endocytic recycling imply that the newly uncoated vesicles fuse with early endosomes (Fig. 10.2) (Heuser and Reese 1973), it has also been suggested that synaptic vesicles can be derived directly from the uncoating of endocytic clathrin-coated vesicles (Fig. 10.2; (Saheki and De Camilli 2012; Takei et al. 1996)). Indeed, live imaging studies have shown that an individual SV that has taken up an extracellular tracer by a single endocytic event can entirely release the tracer by a subsequent single fusion event and without dilution in the intermediate compartment (Murthy and Stevens 1998; Ryan et al. 1997). The characterization of clathrin coat machinery, together with the discovery of new vesicle coats evolutionarily related to clathrin coat (COPI and COPII), led to a dogma that curved proteinaceous scaffolds are essential for vesicle formation. This cornerstone of vesicular trafficking has been recently challenged by studies of synapses that still contain synaptic vesicles regardless of almost complete ablation of clathrin and the endocytic adaptor AP2 (Gu et al. 2008; Kim and Ryan 2009; Sato et al. 2009). While clathrin coat-independent SV recycling has been proposed for decades, to date, no molecular mechanism has been suggested. Following high rates of neurotransmitter release, transient endosome-like structures accumulate at the synapses, and they are generally considered to result from bulk endocytosis (Fig. 10.2) and not from gradual fusion of clathrin-coated vesicles with an existing endosome (Murthy and De Camilli 2003). Their formation is essential for membrane trafficking at the synapse, controlling both SV recycling and the availability of presynaptic membrane proteins and lipids (Saheki and De Camilli 2012).

10.2.3 Lipids Involved in Clathrin-Mediated Endocytosis and Synaptic Vesicle Recycling

Membrane lipids have an important regulatory function in exocytosis, endocytosis, and membrane traffic in general (Cremona and De Camilli 2001). It has been known since the 1950s that stimulation of cell secretion leads to increased phosphorylation

of phospholipids (Hokin and Hokin 1953). Ten years later, Larrabee and collaborators (Larrabee et al. 1963) revealed that this modification is limited to a minor fraction of phospholipids, the phosphatidylinositides (PIs). PIs are present in all eukaryotic cells where they generally constitute less than 10 % of total cellular phospholipids (Janssens 1988; Rana and Hokin 1990). The importance of these molecules is best illustrated by the long-standing research that has accompanied them. The intense research on (1) membrane structure (~1930–1950) coincided with the characterization of PIs, (2) cell signaling (~1950–1980) with phosphatidylinositol-4-phosphate [PI(4)P] and PI(4,5)P_2, and (3) membrane trafficking and the cytoskeleton (~1980–2005) with the remaining PIs. The inositol head group of PI can be reversibly phosphorylated at various positions, resulting in seven naturally occurring PIs. The molecules of the PI family are stable and versatile (Irvine 2005): all forms can be rapidly interconverted by specialized lipid kinases and phosphatases, which add or remove specific phosphate groups. Some forms are also broken down by phospholipases.

Hundreds of proteins have domains that recognize particular PIs, and the continual changes in the relative population of these lipids profoundly affect cellular activities. In general, PI-responsive proteins can be grouped into two classes (Suh and Hille 2005). The first class comprises cytoplasmic enzymes, vesicle trafficking factors, and proteins involved in cytoskeletal rearrangement. These proteins have recognition domains (e.g., phox homology (PX), PH, E/ANTH, FERM, FYVE, Tubby; reviewed by Lemmon 2003) which interact with lipid head groups facing the cytoplasm. They are attracted to target membranes through their lipid ligand and shuttle between membrane and cytoplasm as the PI composition changes. The second class of proteins comprises intrinsic membrane proteins and includes ion channels and transporters. Their activity is either dependent on or blocked by specific PIs. They presumably recognize the PI head group and interact laterally with their phospholipid ligand in the inner leaflet of the plasma membrane.

Since PIs are heterogeneously distributed in the cellular membranes, it was suggested that these lipids determine which proteins are recruited to each membrane and when they are active (Cremona and De Camilli 2001; De Matteis and Godi 2004). The different membrane-restricted PIs may serve to program/control vesicular trafficking, which in turn may regulate a multitude of cellular signaling events. PI(4)P is mainly detected on the Golgi apparatus, PI(3)P on early endosomes, PI(3,5)P_2 on late endosomes, and PI(3,4)P_2, PI(4,5)P_2, and PI(3,4,5)P_3 on the plasma membrane (Cremona and De Camilli 2001; De Matteis and Godi 2004). Enzymes of PI metabolism often serve as molecular switches; they are interconnected and precisely regulated. The PI phosphatases eliminate inappropriate PI synthesis products and terminate the signal, the act of which may in itself create a messenger for a different trafficking event. Because different PI kinases are localized to specific target sites, PI turnover regulates exocytosis, endocytosis, and intracellular membrane trafficking. To further understand the spatial and temporal regulation of membrane turnover, it is necessary to understand which of the numerous PI kinases and phosphatases are involved and how they are regulated.

The best characterized PI, $PI(4,5)P_2$, was originally isolated from the brain and described as a brain lipid (Dittmer and Dawson 1960; Ellis and Hawthorne 1961; Folch 1946; Tomlinson and Ballou 1961). A few decades later, $PI(4,5)P_2$ was recognized as a precursor of two major signaling molecules, $I(1,4,5)P_3$ and diacylglycerol (DAG) (Berridge and Irvine 1984), and for many years it was assumed that $PI(4,5)P_2$ was simply the source of these molecules. Over the last 20 years, a large body of evidence describing additional functions of this phospholipid has accumulated, and $PI(4,5)P_2$ is presently considered to be one of the most important signaling molecules and a key player in regulated exocytosis and endocytosis in neurons and neuroendocrine cells (Cremona and De Camilli 2001; Martin 2001; Wenk and De Camilli 2004). In addition, the dual roles of $PI(4,5)P_2$ in exocytosis and endocytosis led to the conclusion that this lipid controls the plasma membrane trafficking and a model in which a PI cycle is nestled within the secretory vesicle cycle (Cremona and De Camilli 2001).

$PI(4,5)P_2$ has been found to control the level of exocytosis (Gong et al. 2005; Milosevic et al. 2005) and endocytosis (Cremona et al. 1999; Di Paolo and De Camilli 2003; Ford et al. 2001; Krauss et al. 2003). Moreover, many exocytic and endocytic proteins have been shown to interact with $PI(4,5)P_2$ (reviewed by Saheki and De Camilli 2012; Wenk and De Camilli 2004). In exocytosis, $PI(4,5)P_2$ is bound by synaptotagmin and CAPS proteins (Loyet et al. 1998; Schiavo et al. 1996), Mints (Okamoto and Sudhof 1997), and rabphilin 3 (Chung et al. 1998). In endocytosis, $PI(4,5)P_2$ has a central role, as some of the $PI(4,5)P_2$ binding proteins include clathrin adaptor proteins (AP2, AP180, Hip1, and epsin) as well as dynamin (Traub 2003).

The importance of $PI(4,5)P_2$ in the recruitment of endocytic and endocytic proteins has been demonstrated by several functional studies. The clathrin coat assembles on $PI(4,5)P_2$ liposomes in vitro (Takei et al. 1998), and manipulations which stimulated clathrin coat nucleation were shown to act through $PI(4,5)P_2$ synthesis (Cremona et al. 1999; Ford et al. 2001). Dephosphorylation of $PI(4,5)P_2$ by overexpression of a membrane-tagged inositol 5′-phosphatase domain of synaptojanin 1 (IPP1-CAAX) or $PI(4,5)P_2$ masking by PH-PLCδ_1 and neomycin inhibited clathrin-mediated endocytosis (Jost et al. 1998; Krauss et al. 2003). Furthermore, neurons without synaptojanin 1 had elevated $PI(4,5)P_2$ levels and defective synaptic vesicle recycling, presumably due to a delay in clathrin coat disassembly (Cremona et al. 1999). In short, $PI(4,5)P_2$ seems to play an important role in all steps of endocytic process: clathrin coat recruitment (through clathrin adaptor proteins), fission of endocytic pits (through dynamin), and clathrin uncoating (through synaptojanin 1).

Most recently, another phosphatidylinositol besides $PI(4,5)P_2$, phosphatidylinositol-3,4-bisphosphate [$PI(3,4)P_2$], which is formed by class II phosphatidylinositol-3-kinase, PI3K C2α, has been implicated in a spatiotemporal control of clathrin-mediated endocytosis (Posor et al. 2013). Timed formation of $PI(3,4)P_2$ by PI3K C2α is needed for a selective enrichment of the BAR protein SNX9 at the late-stage endocytic intermediates. Consequently, reduction in $PI(3,4)P_2$ or PI(3)K C2α levels affected the maturation of late-stage clathrin-coated pits before fission.

10.3 Clathrin-Independent Pathways of Endocytosis at the Presynaptic Terminal

While clathrin-mediated endocytosis is presently considered the predominant pathway of synaptic vesicle recycling, the contribution of other endocytic types that play a role in the re-formation of new synaptic vesicles is still an open question. These pathways do not use clathrin and its attendant molecular machinery, and they differ in their kinetics and mechanism of endocytic vesicle formation and associated molecular machinery. Here, I will discuss in brief the characteristics of two endocytic pathways that are studied at the presynaptic terminals, kiss-and-run and bulk endocytosis.

10.3.1 Kiss-and-Run

Classical description of membrane fusion at the presynaptic terminal postulates that the fused synaptic vesicle fully collapses into the plasma membrane and is subsequently retrieved by a clathrin-mediated endocytosis (Fig. 10.2) (Rizzoli and Jahn 2007). In contrast, kiss-and-run is a type of synaptic vesicle release wherein the synaptic vesicle docks and transiently fuses with the plasma membrane enough to allow the neurotransmitter release, but the vesicle itself does not fully collapse into the plasma membrane, and its proteins do not mix with the plasma membrane proteins (see Fig. 10.2; for review Rizzoli and Jahn 2007; Saheki and De Camilli 2012).

The idea of a kiss-and-run exocytic/endocytic model was first proposed in 1955 by Katz and Castillo, but the first systematic studies and the actual term "kiss-and-run" originated from Ceccarelli and collaborators in 1973. These classical studies proposed that synaptic vesicle fusion occurs transiently *via* the opening of a small fusion pore followed by rapid closure, and without the full collapse of the synaptic vesicle membrane (Ceccarelli et al. 1972, 1973). This model suggests that the synaptic vesicle retains its biochemical identity, and it is largely based on the increased incidence of uncoated "omega" membrane profiles with a narrow neck attached to the plasma membrane (Ceccarelli et al. 1973; He and Wu 2007; Koenig et al. 1998). However, it remained unclear whether these synaptic vesicles fully fuse or reseal their necks.

Interestingly, this mode of synaptic vesicle fusion allows the synapse to restrict the release of transmitters through the narrow fusion pore, thus resulting in a sub-quantal release (He et al. 2006; Klyachko and Jackson 2002). If compared to full collapse synaptic vesicle fusion, this might also be a mechanism to regulate synaptic strength (Choi et al. 2000). At present, the presence of kiss-and-run mode at nerve terminals is controversial (Rizzoli and Jahn 2007). The strongest evidence in favor of this pathway was derived from optical assays based on partial destaining of single synaptic vesicle loaded with slowly dissociating (~3 s) styryl

FM dye FM1-43 (Aravanis et al. 2003; Klingauf et al. 1998; Pyle et al. 2000). The results indicated partial dye retention in synaptic vesicles, as was further supported using a hydrophilic FM1-43 quencher, bromophenol blue (Harata et al. 2006). In another imaging study, Gandhi and Stevens (2003) imaged single synaptic vesicle exocytosis–endocytosis cycle by overexpressing synapto-pHluorin in hippocampal neurons. A rapid fluorescent transient with a retrieval time of less than 1 s was observed, which was credited to the fast kiss-and-run mode (Gandhi and Stevens 2003). Subsequent studies based on pH-sensitive "quantum dots" have shown that synaptic vesicles loaded with these tiny inorganic particles exhibited distinct patterns of photoluminescence upon kiss-and-run versus full collapse fusion (Zhang et al. 2007, 2009).

In spite of the fact that kiss-and-run may provide more efficient and faster vesicle recycling, there is a long-standing debate over kiss-and-run and full fusion. One of the major issues with kiss-and-run supporting data, and subsequently, the basis for some counterarguments against kiss-and-run, is a short fusion event that is hard to capture on record (reviewed by Rizzoli and Jahn 2007). However, accumulation of partially empty vesicles following secretion favors the kiss-and-run mechanism, suggesting that during the secretory process, only a portion of the vesicular contents are able to exit the cell, which could only be possible if secretory vesicles were to temporarily establish continuity with the cell plasma membrane, release a portion of their contents, and then detach and reseal.

10.3.2 Bulk Endocytosis

While clathrin-mediated endocytosis is considered the most common endocytic form at the presynaptic terminals under physiological conditions, bulk endocytosis occurs most commonly upon intense stimulations and following the addition of a large quantity of vesicle membrane during a short time period (Clayton et al. 2008; Hayashi et al. 2008; Heuser and Reese 1973; Holt et al. 2003; Miller and Heuser 1984; Paillart et al. 2003; Wu and Wu 2007). Some examples of strong stimulations include high levels of neuronal activity in vivo and induced membrane depolarization by high extracellular concentrations of potassium or by a high-frequency train of action potentials in vitro. Given that bulk endocytosis occurs in a response to a quick, massive addition of membranes to the spatially limited presynaptic terminus, much larger areas of presynaptic plasma membrane are internalized in comparison to clathrin-mediated endocytosis. Furthermore, the excess plasma membrane is rapidly recaptured in a nonselective manner, through formation of deep plasma membrane infoldings, which subsequently undergo fission and generate intracellular cisternae (endosome-like intermediates) from which synaptic vesicles can subsequently bud off (for more information, please see Clayton et al. 2008; Heuser and Reese 1973; Teng et al. 2007; Torri-Tarelli et al. 1987).

At present, the molecular mechanisms of bulk endocytosis are not well understood. It has been proposed that high levels of neuronal activity and, subsequently,

high calcium levels in the presynaptic terminus activate the calcium-dependent serine–threonine phosphatase calcineurin, which quickly dephosphorylates dynamin (Liu et al. 1994). Dephosphorylated dynamin can interact with the BAR family members to induce the formation of deep membrane infoldings: this is likely done through the interaction of F-BAR protein syndapin with the actin cytoskeleton, Arp2/3, and N-WASP (Andersson et al. 2008; Clayton et al. 2009). The fission of these infoldings from the plasma membrane is an active process that was originally proposed to be executed by dynamin, although recent publications have shown that bulk endocytosis happens even in the absence of dynamins (Ferguson et al. 2007; Hayashi et al. 2008; Raimondi et al. 2011).

Even though the model has been proposed for a while, it is presently not known how the large cisternae (endocytic intermediates) that are formed by bulk endocytosis eventually convert to new synaptic vesicles. Several scenarios have been recently discussed (Saheki and De Camilli 2012). In short, synaptic vesicles may re-form from cisternae/endocytic intermediates by a coat-mediated mechanism, by a novel protein- or lipid-mediated mechanism independent of classical coats, and/or by clathrin-mediated endocytosis after the large cisternae back-fuse with the plasma membrane.

1. Synaptic vesicles re-form from cisternae/endocytic intermediates by a coat-dependent mechanism. It was originally proposed that such a mechanism would happen via the clathrin coat-mediated budding (Takei et al. 1996). However, the recent studies on presynaptic terminals performed by electron tomography have shown that the overwhelming majority of clathrin-coated pits are connected to the plasma membrane, even when they appear to be emerging from endosomes in the single electron microscope section (Ferguson et al. 2007; Hayashi et al. 2008; Raimondi et al. 2011). Thus, the coats may likely differ from the clathrin coat. Two potential candidates involve AP2-like complexes that can function independently of clathrin, AP1, and AP3 (Faundez et al. 1998; Glyvuk et al. 2010; Kim and Ryan 2009; Nakatsu et al. 2004; Newell-Litwa et al. 2009). However, despite AP1 and AP3 are involved in synaptic vesicle recycling (Glyvuk et al. 2010; Voglmaier et al. 2006), they are not considered to be a major component of the presynaptic terminus.
2. Synaptic vesicles re-form from cisternae/endocytic intermediates by a novel mechanism independent of classical coats that is either protein or lipid mediated (Graham 2004; Kirchhausen 2000). In vitro studies have shown that selective BAR proteins can tubulate lipids and even generate small vesicles (Gallop et al. 2006; Wang et al. 2009). It is likely that this mechanism cannot sort the membrane cargo efficiently; thus, the fidelity of synaptic vesicle reformation may be compromised, and a new cycle of exocytosis and clathrin-dependent endocytosis may be needed to re-form a vesicle with proper size and composition. Recent studies in worms have raised the possibility of clathrin-independent mechanisms supporting neurotransmission (Gu et al. 2008; Sato et al. 2009).
3. Synaptic vesicles re-form by clathrin-mediated endocytosis after the large cisternae/endocytic intermediates back-fuse with the plasma membrane. Thus,

clathrin-dependent endocytosis may be the only pathway needed for the generation of synaptic vesicles. However, robust conversion of the endocytic intermediates was observed at synapses lacking dynamin 1, in which clathrin-mediated endocytosis is severely impaired, thus calling this possibility into question (Ferguson et al. 2007).

Lastly, it is important to note that at highly active presynaptic terminals, clathrin-mediated endocytosis and bulk endocytosis can occur concurrently. The dephosphorylation of dynamin does not prevent its interactions with the BAR proteins amphiphysin and endophilin, and therefore the two processes are permitted to happen independently of each other.

10.4 Concluding Remarks

In conclusion, much has been learned about endocytosis and the mechanisms of synaptic vesicle recycling at the presynaptic terminals, but a great deal remains unknown. The core features of the basic mechanism of clathrin-mediated endocytosis have been established. However, as pointed out before, little is known about parallel endocytic pathways that re-form synaptic vesicles. In addition, the dynamics and fate of newly endocytosed vesicles and endosomal intermediates, including the mechanisms by which they give rise to new synaptic vesicles, have barely been investigated thus far. Furthermore, it remains a mystery how the pace of the synaptic vesicle recycling process is controlled and how its flexibility is maintained. Traditionally, the high speed of synaptic vesicle recycling, and small size of nerve terminals, has hampered studies of this sort. Recently, several mouse models with defective endocytosis were generated: their synapses accumulate recycling intermediates that are stable for minutes to hours, as opposed to milliseconds to seconds in wild-type synapses (Ferguson et al. 2007; Milosevic et al. 2011; Raimondi et al. 2011). Moreover, a higher level of understanding may be achieved through current advances in super-resolution microscopy, ultrafast sample stimulation, and high-pressure freezing techniques, followed by electron microscopy and tomography, making it possible to inspect synaptic organelles within milliseconds of their formation (Kittelmann et al. 2013; Watanabe et al. 2013). Given the essential nature of these questions, and the importance of efficient synaptic vesicle recycling, it is to be expected that further progress along these lines will not only advance the field of synaptic transmission but have wide-ranging implications for pharmacology, neurophysiology, and medicine.

Acknowledgments I would like to apologize to the authors of many important papers whose work I may have inadvertently failed to cite here due to space constraints. I thank Dr Ante Milosevic for his kind help with the figures and Dr Nuno Raimundo for discussion. My work is supported by the Emmy Noether Young Investigator Award from German Research Foundation (DFG).

References

Andersson F, Jakobsson J, Low P, Shupliakov O, Brodin L (2008) Perturbation of syndapin/PACSIN impairs synaptic vesicle recycling evoked by intense stimulation. J Neurosci 28:3925–3933

Antonescu CN, Danuser G, Schmid SL (2010) Phosphatidic acid plays a regulatory role in clathrin-mediated endocytosis. Mol Biol Cell 21:2944–2952

Aravanis AM, Pyle JL, Tsien RW (2003) Single synaptic vesicles fusing transiently and successively without loss of identity. Nature 423:643–647

Bakowska JC, Jenkins R, Pendleton J, Blackstone C (2005) The Troyer syndrome (SPG20) protein spartin interacts with Eps15. Biochem Biophys Res Commun 334:1042–1048

Bao H, Daniels RW, MacLeod GT, Charlton MP, Atwood HL, Zhang B (2005) AP180 maintains the distribution of synaptic and vesicle proteins in the nerve terminal and indirectly regulates the efficacy of Ca^{2+}-triggered exocytosis. J Neurophysiol 94:1888–1903

Berridge MJ, Irvine RF (1984) Inositol trisphosphate, a novel second messenger in cellular signal transduction. Nature 312:315–321

Bonifacino JS, Glick BS (2004) The mechanisms of vesicle budding and fusion. Cell 116:153–166

Bonifacino JS, Traub LM (2003) Signals for sorting of transmembrane proteins to endosomes and lysosomes. Annu Rev Biochem 72:395–447

Boulant S, Kural C, Zeeh JC, Ubelmann F, Kirchhausen T (2011) Actin dynamics counteract membrane tension during clathrin-mediated endocytosis. Nat Cell Biol 13:1124–1131

Brodsky FM (2012) Diversity of clathrin function: new tricks for an old protein. Annu Rev Cell Dev Biol 28:309–336

Campelo F, Malhotra V (2012) Membrane fission: the biogenesis of transport carriers. Annu Rev Biochem 81:407–427

Ceccarelli B, Hurlbut WP, Mauro A (1972) Depletion of vesicles from frog neuromuscular junctions by prolonged tetanic stimulation. J Cell Biol 54:30–38

Ceccarelli B, Hurlbut WP, Mauro A (1973) Turnover of transmitter and synaptic vesicles at the frog neuromuscular junction. J Cell Biol 57:499–524

Chen H, De Camilli P (2005) The association of epsin with ubiquitinated cargo along the endocytic pathway is negatively regulated by its interaction with clathrin. Proc Natl Acad Sci U S A 102:2766–2771

Chen H, Fre S, Slepnev VI, Capua MR, Takei K, Butler MH, Di Fiore PP, De Camilli P (1998) Epsin is an EH-domain-binding protein implicated in clathrin-mediated endocytosis. Nature 394:793–797

Chen H, Slepnev VI, Di Fiore PP, De Camilli P (1999) The interaction of epsin and Eps15 with the clathrin adaptor AP-2 is inhibited by mitotic phosphorylation and enhanced by stimulation-dependent dephosphorylation in nerve terminals. J Biol Chem 274:3257–3260

Chen H, Ko G, Zatti A, Di Giacomo G, Liu L, Raiteri E, Perucco E, Collesi C, Min W, Zeiss C et al (2009) Embryonic arrest at midgestation and disruption of Notch signaling produced by the absence of both epsin 1 and epsin 2 in mice. Proc Natl Acad Sci U S A 106:13838–13843

Choi S, Klingauf J, Tsien RW (2000) Postfusional regulation of cleft glutamate concentration during LTP at 'silent synapses'. Nat Neurosci 3:330–336

Chung SH, Song WJ, Kim K, Bednarski JJ, Chen J, Prestwich GD, Holz RW (1998) The C2 domains of Rabphilin3A specifically bind phosphatidylinositol 4,5-bisphosphate containing vesicles in a Ca^{2+}-dependent manner. In vitro characteristics and possible significance. J Biol Chem 273:10240–10248

Clayton EL, Evans GJ, Cousin MA (2008) Bulk synaptic vesicle endocytosis is rapidly triggered during strong stimulation. J Neurosci 28:6627–6632

Clayton EL, Anggono V, Smillie KJ, Chau N, Robinson PJ, Cousin MA (2009) The phosphodependent dynamin-syndapin interaction triggers activity-dependent bulk endocytosis of synaptic vesicles. J Neurosci 29:7706–7717

Cocucci E, Aguet F, Boulant S, Kirchhausen T (2012) The first five seconds in the life of a clathrin-coated pit. Cell 150:495–507
Collins BM, McCoy AJ, Kent HM, Evans PR, Owen DJ (2002) Molecular architecture and functional model of the endocytic AP2 complex. Cell 109:523–535
Conner SD, Schroter T, Schmid SL (2003) AAK1-mediated micro2 phosphorylation is stimulated by assembled clathrin. Traffic 4:885–890
Cremona O, De Camilli P (2001) Phosphoinositides in membrane traffic at the synapse. J Cell Sci 114:1041–1052
Cremona O, Di Paolo G, Wenk MR, Luthi A, Kim WT, Takei K, Daniell L, Nemoto Y, Shears SB, Flavell RA et al (1999) Essential role of phosphoinositide metabolism in synaptic vesicle recycling. Cell 99:179–188
Daumke O, Roux A, Haucke V (2014) BAR domain scaffolds in dynamin-mediated membrane fission. Cell 156:882–892
De Camilli P, Jahn R (1990) Pathways to regulated exocytosis in neurons. Annu Rev Physiol 52:625–645
de Heuvel E, Bell AW, Ramjaun AR, Wong K, Sossin WS, McPherson PS (1997) Identification of the major synaptojanin-binding proteins in brain. J Biol Chem 272:8710–8716
De Matteis MA, Godi A (2004) PI-loting membrane traffic. Nat Cell Biol 6:487–492
Di Paolo G, De Camilli P (2003) Does clathrin pull the fission trigger? Proc Natl Acad Sci U S A 100:4981–4983
Diril MK, Wienisch M, Jung N, Klingauf J, Haucke V (2006) Stonin 2 is an AP-2-dependent endocytic sorting adaptor for synaptotagmin internalization and recycling. Dev Cell 10:233–244
Dittman J, Ryan TA (2009) Molecular circuitry of endocytosis at nerve terminals. Annu Rev Cell Dev Biol 25:133–160
Dittmer JC, Dawson RM (1960) The isolation of a new complex lipid: triphosphoinostide from ox brain. Biochim Biophys Acta 40:379–380
Douglas WW (1968) Stimulus-secretion coupling: the concept and clues from chromaffin and other cells. Br J Pharmacol 34:451–474
Edeling MA, Smith C, Owen D (2006) Life of a clathrin coat: insights from clathrin and AP structures. Nat Rev Mol Cell Biol 7:32–44
Edvardson S, Cinnamon Y, Ta-Shma A, Shaag A, Yim YI, Zenvirt S, Jalas C, Lesage S, Brice A, Taraboulos A et al (2012) A deleterious mutation in DNAJC6 encoding the neuronal-specific clathrin-uncoating co-chaperone auxilin, is associated with juvenile parkinsonism. PLoS One 7:e36458
Ehrlich M, Boll W, Van Oijen A, Hariharan R, Chandran K, Nibert ML, Kirchhausen T (2004) Endocytosis by random initiation and stabilization of clathrin-coated pits. Cell 118:591–605
Ellis RB, Hawthorne JN (1961) The structures of beef-brain phosphoinositides. Biochim Biophys Acta 51:385–387
Fallon L, Belanger CM, Corera AT, Kontogiannea M, Regan-Klapisz E, Moreau F, Voortman J, Haber M, Rouleau G, Thorarinsdottir T et al (2006) A regulated interaction with the UIM protein Eps15 implicates parkin in EGF receptor trafficking and PI(3)K-Akt signalling. Nat Cell Biol 8:834–842
Faundez V, Horng JT, Kelly RB (1998) A function for the AP3 coat complex in synaptic vesicle formation from endosomes. Cell 93:423–432
Fergestad T, Davis WS, Broadie K (1999) The stoned proteins regulate synaptic vesicle recycling in the presynaptic terminal. J Neurosci 19:5847–5860
Ferguson SM, De Camilli P (2012) Dynamin, a membrane-remodelling GTPase. Nat Rev Mol Cell Biol 13:75–88
Ferguson SM, Brasnjo G, Hayashi M, Wolfel M, Collesi C, Giovedi S, Raimondi A, Gong LW, Ariel P, Paradise S et al (2007) A selective activity-dependent requirement for dynamin 1 in synaptic vesicle endocytosis. Science 316:570–574

Ferguson SM, Raimondi A, Paradise S, Shen H, Mesaki K, Ferguson A, Destaing O, Ko G, Takasaki J, Cremona O et al (2009) Coordinated actions of actin and BAR proteins upstream of dynamin at endocytic clathrin-coated pits. Dev Cell 17:811–822

Fernandez-Alfonso T, Kwan R, Ryan TA (2006) Synaptic vesicles interchange their membrane proteins with a large surface reservoir during recycling. Neuron 51:179–186

Folch J (1946) Isolation of brain diphosphoinositide, a new phosphatide containing inositol meta diphosphate as a constituent. Fed Proc 5:134

Ford MG, Pearse BM, Higgins MK, Vallis Y, Owen DJ, Gibson A, Hopkins CR, Evans PR, McMahon HT (2001) Simultaneous binding of PtdIns(4,5)P2 and clathrin by AP180 in the nucleation of clathrin lattices on membranes. Science 291:1051–1055

Fotin A, Cheng Y, Grigorieff N, Walz T, Harrison SC, Kirchhausen T (2004) Structure of an auxilin-bound clathrin coat and its implications for the mechanism of uncoating. Nature 432:649–653

Frost A, Unger VM, De Camilli P (2009) The BAR domain superfamily: membrane-molding macromolecules. Cell 137:191–196

Gallop JL, Jao CC, Kent HM, Butler PJ, Evans PR, Langen R, McMahon HT (2006) Mechanism of endophilin N-BAR domain-mediated membrane curvature. Embo J 25:2898–2910

Gandhi SP, Stevens CF (2003) Three modes of synaptic vesicular recycling revealed by single-vesicle imaging. Nature 423:607–613

Glyvuk N, Tsytsyura Y, Geumann C, D'Hooge R, Huve J, Kratzke M, Baltes J, Boening D, Klingauf J, Schu P (2010) AP-1/sigma1B-adaptin mediates endosomal synaptic vesicle recycling, learning and memory. Embo J 29:1318–1330

Gong LW, Di Paolo G, Diaz E, Cestra G, Diaz ME, Lindau M, De Camilli P, Toomre D (2005) Phosphatidylinositol phosphate kinase type I gamma regulates dynamics of large dense-core vesicle fusion. Proc Natl Acad Sci U S A 102:5204–5209

Graham TR (2004) Flippases and vesicle-mediated protein transport. Trends Cell Biol 14:670–677

Gu M, Schuske K, Watanabe S, Liu Q, Baum P, Garriga G, Jorgensen EM (2008) Mu2 adaptin facilitates but is not essential for synaptic vesicle recycling in Caenorhabditis elegans. J Cell Biol 183:881–892

Gundelfinger ED, Kessels MM, Qualmann B (2003) Temporal and spatial coordination of exocytosis and endocytosis. Nat Rev Mol Cell Biol 4:127–139

Haffner C, Takei K, Chen H, Ringstad N, Hudson A, Butler MH, Salcini AE, Di Fiore PP, De Camilli P (1997) Synaptojanin 1: localization on coated endocytic intermediates in nerve terminals and interaction of its 170 kDa isoform with Eps15. FEBS Lett 419:175–180

Harata NC, Choi S, Pyle JL, Aravanis AM, Tsien RW (2006) Frequency-dependent kinetics and prevalence of kiss-and-run and reuse at hippocampal synapses studied with novel quenching methods. Neuron 49:243–256

Hayashi M, Raimondi A, O'Toole E, Paradise S, Collesi C, Cremona O, Ferguson SM, De Camilli P (2008) Cell- and stimulus-dependent heterogeneity of synaptic vesicle endocytic recycling mechanisms revealed by studies of dynamin 1-null neurons. Proc Natl Acad Sci U S A 105:2175–2180

He L, Wu LG (2007) The debate on the kiss-and-run fusion at synapses. Trends Neurosci 30:447–455

He L, Wu XS, Mohan R, Wu LG (2006) Two modes of fusion pore opening revealed by cell-attached recordings at a synapse. Nature 444:102–105

Heldwein EE, Macia E, Wang J, Yin HL, Kirchhausen T, Harrison SC (2004) Crystal structure of the clathrin adaptor protein 1 core. Proc Natl Acad Sci U S A 101:14108–14113

Henne WM, Boucrot E, Meinecke M, Evergren E, Vallis Y, Mittal R, McMahon HT (2010) FCHo proteins are nucleators of clathrin-mediated endocytosis. Science 328:1281–1284

Heuser JE, Reese TS (1973) Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. J Cell Biol 57:315–344

Ho HY, Rohatgi R, Lebensohn AM, Le M, Li J, Gygi SP, Kirschner MW (2004) Toca-1 mediates Cdc42-dependent actin nucleation by activating the N-WASP-WIP complex. Cell 118:203–216

Hokin MR, Hokin LE (1953) Enzyme secretion and the incorporation of P32 into phospholipides of pancreas slices. J Biol Chem 203:967–977

Holt M, Cooke A, Wu MM, Lagnado L (2003) Bulk membrane retrieval in the synaptic terminal of retinal bipolar cells. J Neurosci 23:1329–1339

Hosoi N, Holt M, Sakaba T (2009) Calcium dependence of exo- and endocytotic coupling at a glutamatergic synapse. Neuron 63:216–229

Irvine RF (2005) Inositide evolution – towards turtle domination? J Physiol 566:295–300

Itoh T, De Camilli P (2006) BAR, F-BAR (EFC) and ENTH/ANTH domains in the regulation of membrane-cytosol interfaces and membrane curvature. Biochim Biophys Acta 1761:897–912

Jackson AP, Flett A, Smythe C, Hufton L, Wettey FR, Smythe E (2003) Clathrin promotes incorporation of cargo into coated pits by activation of the AP2 adaptor micro2 kinase. J Cell Biol 163:231–236

Janssens PM (1988) The evolutionary origin of eukaryotic transmembrane signal transduction. Comp Biochem Physiol A Comp Physiol 90:209–223

Jiang J, Maes EG, Taylor AB, Wang L, Hinck AP, Lafer EM, Sousa R (2007) Structural basis of J cochaperone binding and regulation of Hsp70. Mol Cell 28:422–433

Jost M, Simpson F, Kavran JM, Lemmon MA, Schmid SL (1998) Phosphatidylinositol-4,5-bisphosphate is required for endocytic coated vesicle formation. Curr Biol 8:1399–1402

Jung N, Wienisch M, Gu M, Rand JB, Muller SL, Krause G, Jorgensen EM, Klingauf J, Haucke V (2007) Molecular basis of synaptic vesicle cargo recognition by the endocytic sorting adaptor stonin 2. J Cell Biol 179:1497–1510

Keen JH, Willingham MC, Pastan IH (1979) Clathrin-coated vesicles: isolation, dissociation and factor-dependent reassociation of clathrin baskets. Cell 16:303–312

Kessels MM, Qualmann B (2004) The syndapin protein family: linking membrane trafficking with the cytoskeleton. J Cell Sci 117:3077–3086

Kim SH, Ryan TA (2009) A distributed set of interactions controls mu2 functionality in the role of AP-2 as a sorting adaptor in synaptic vesicle endocytosis. J Biol Chem 284:32803–32812

Kirchhausen T (1999) Adaptors for clathrin-mediated traffic. Annu Rev Cell Dev Biol 15:705–732

Kirchhausen T (2000) Clathrin. Annu Rev Biochem 69:699–727

Kirchhausen T (2009) Imaging endocytic clathrin structures in living cells. Trends Cell Biol 19:596–605

Kirchhausen T, Harrison SC (1981) Protein organization in clathrin trimers. Cell 23:755–761

Kittelmann M, Liewald JF, Hegermann J, Schultheis C, Brauner M, Steuer Costa W, Wabnig S, Eimer S, Gottschalk A (2013) In vivo synaptic recovery following optogenetic hyperstimulation. Proc Natl Acad Sci U S A 110:E3007–E3016

Klingauf J, Kavalali ET, Tsien RW (1998) Kinetics and regulation of fast endocytosis at hippocampal synapses. Nature 394:581–585

Klyachko VA, Jackson MB (2002) Capacitance steps and fusion pores of small and large-dense-core vesicles in nerve terminals. Nature 418:89–92

Koenig JH, Yamaoka K, Ikeda K (1998) Omega images at the active zone may be endocytotic rather than exocytotic: implications for the vesicle hypothesis of transmitter release. Proc Natl Acad Sci U S A 95:12677–12682

Kononenko NL, Diril MK, Puchkov D, Kintscher M, Koo SJ, Pfuhl G, Winter Y, Wienisch M, Klingauf J, Breustedt J et al (2013) Compromised fidelity of endocytic synaptic vesicle protein sorting in the absence of stonin 2. Proc Natl Acad Sci U S A 110:E526–E535

Koroglu C, Baysal L, Cetinkaya M, Karasoy H, Tolun A (2013) DNAJC6 is responsible for juvenile parkinsonism with phenotypic variability. Parkinsonism Relat Disord 19:320–324

Krauss M, Kinuta M, Wenk MR, De Camilli P, Takei K, Haucke V (2003) ARF6 stimulates clathrin/AP-2 recruitment to synaptic membranes by activating phosphatidylinositol phosphate kinase type I gamma. J Cell Biol 162:113–124

Larrabee MG, Klingman JD, Leicht WS (1963) Effects of temperature, calcium and activity on phospholipid metabolism in a synaptic ganglion. J Neurochem 10:549–570
Lee DW, Wu X, Eisenberg E, Greene LE (2006) Recruitment dynamics of GAK and auxilin to clathrin-coated pits during endocytosis. J Cell Sci 119:3502–3512
Lemmon MA (2003) Phosphoinositide recognition domains. Traffic 4:201–213
Lippincott J, Li R (2000) Involvement of PCH family proteins in cytokinesis and actin distribution. Microsc Res Tech 49:168–172
Liu JP, Sim AT, Robinson PJ (1994) Calcineurin inhibition of dynamin I GTPase activity coupled to nerve terminal depolarization. Science 265:970–973
Loyet KM, Kowalchyk JA, Chaudhary A, Chen J, Prestwich GD, Martin TF (1998) Specific binding of phosphatidylinositol 4,5-bisphosphate to calcium-dependent activator protein for secretion (CAPS), a potential phosphoinositide effector protein for regulated exocytosis. J Biol Chem 273:8337–8343
Lundmark R, Carlsson SR (2003) Sorting nexin 9 participates in clathrin-mediated endocytosis through interactions with the core components. J Biol Chem 278:46772–46781
Lundmark R, Carlsson SR (2009) SNX9 – a prelude to vesicle release. J Cell Sci 122:5–11
Maritzen T, Podufall J, Haucke V (2010) Stonins–specialized adaptors for synaptic vesicle recycling and beyond? Traffic 11:8–15
Maritzen T, Koo SJ, Haucke V (2012) Turning CALM into excitement: AP180 and CALM in endocytosis and disease. Biol Cell 104:588–602
Martin TF (2001) PI(4,5)P(2) regulation of surface membrane traffic. Curr Opin Cell Biol 13:493–499
Massol RH, Boll W, Griffin AM, Kirchhausen T (2006) A burst of auxilin recruitment determines the onset of clathrin-coated vesicle uncoating. Proc Natl Acad Sci U S A 103:10265–10270
McMahon HT, Boucrot E (2011) Molecular mechanism and physiological functions of clathrin-mediated endocytosis. Nat Rev Mol Cell Biol 12:517–533
McPherson PS, Garcia EP, Slepnev VI, David C, Zhang X, Grabs D, Sossin WS, Bauerfeind R, Nemoto Y, De Camilli P (1996) A presynaptic inositol-5-phosphatase. Nature 379:353–357
Merrifield CJ, Feldman ME, Wan L, Almers W (2002) Imaging actin and dynamin recruitment during invagination of single clathrin-coated pits. Nat Cell Biol 4:691–698
Merrifield CJ, Perrais D, Zenisek D (2005) Coupling between clathrin-coated-pit invagination, cortactin recruitment, and membrane scission observed in live cells. Cell 121:593–606
Mettlen M, Pucadyil T, Ramachandran R, Schmid SL (2009) Dissecting dynamin's role in clathrin-mediated endocytosis. Biochem Soc Trans 37:1022–1026
Miller TM, Heuser JE (1984) Endocytosis of synaptic vesicle membrane at the frog neuromuscular junction. J Cell Biol 98:685–698
Milosevic I, Sorensen JB, Lang T, Krauss M, Nagy G, Haucke V, Jahn R, Neher E (2005) Plasmalemmal phosphatidylinositol-4,5-bisphosphate level regulates the releasable vesicle pool size in chromaffin cells. J Neurosci 25:2557–2565
Milosevic I, Giovedi S, Lou X, Raimondi A, Collesi C, Shen H, Paradise S, O'Toole E, Ferguson S, Cremona O et al (2011) Recruitment of endophilin to clathrin-coated pit necks is required for efficient vesicle uncoating after fission. Neuron 72:587–601
Mooren OL, Galletta BJ, Cooper JA (2012) Roles for actin assembly in endocytosis. Annu Rev Biochem 81:661–686
Morlot S, Roux A (2013) Mechanics of dynamin-mediated membrane fission. Annu Rev Biophys 42:629–649
Mullen GP, Grundahl KM, Gu M, Watanabe S, Hobson RJ, Crowell JA, McManus JR, Mathews EA, Jorgensen EM, Rand JB (2012) UNC-41/stonin functions with AP2 to recycle synaptic vesicles in *Caenorhabditis elegans*. PLoS One 7:e40095
Murthy VN, De Camilli P (2003) Cell biology of the presynaptic terminal. Annu Rev Neurosci 26:701–728
Murthy VN, Stevens CF (1998) Synaptic vesicles retain their identity through the endocytic cycle. Nature 392:497–501

Nakatsu F, Okada M, Mori F, Kumazawa N, Iwasa H, Zhu G, Kasagi Y, Kamiya H, Harada A, Nishimura K et al (2004) Defective function of GABA-containing synaptic vesicles in mice lacking the AP-3B clathrin adaptor. J Cell Biol 167:293–302

Newell-Litwa K, Salazar G, Smith Y, Faundez V (2009) Roles of BLOC-1 and adaptor protein-3 complexes in cargo sorting to synaptic vesicles. Mol Biol Cell 20:1441–1453

Okamoto M, Sudhof TC (1997) Mints, Munc18-interacting proteins in synaptic vesicle exocytosis. J Biol Chem 272:31459–31464

Owen DJ, Vallis Y, Noble ME, Hunter JB, Dafforn TR, Evans PR, McMahon HT (1999) A structural explanation for the binding of multiple ligands by the alpha-adaptin appendage domain. Cell 97:805–815

Owen DJ, Vallis Y, Pearse BM, McMahon HT, Evans PR (2000) The structure and function of the beta 2-adaptin appendage domain. Embo J 19:4216–4227

Owen DJ, Collins BM, Evans PR (2004) Adaptors for clathrin coats: structure and function. Annu Rev Cell Dev Biol 20:153–191

Paillart C, Li J, Matthews G, Sterling P (2003) Endocytosis and vesicle recycling at a ribbon synapse. J Neurosci 23:4092–4099

Pearse BM (1975) Coated vesicles from pig brain: purification and biochemical characterization. J Mol Biol 97:93–98

Pearse BM (1976) Clathrin: a unique protein associated with intracellular transfer of membrane by coated vesicles. Proc Natl Acad Sci U S A 73:1255–1259

Pechstein A, Shupliakov O, Haucke V (2010) Intersectin 1: a versatile actor in the synaptic vesicle cycle. Biochem Soc Trans 38:181–186

Perera RM, Zoncu R, Lucast L, De Camilli P, Toomre D (2006) Two synaptojanin 1 isoforms are recruited to clathrin-coated pits at different stages. Proc Natl Acad Sci U S A 103:19332–19337

Phillips AM, Ramaswami M, Kelly LE (2010) Stoned. Traffic 11:16–24

Polo S, Sigismund S, Faretta M, Guidi M, Capua MR, Bossi G, Chen H, De Camilli P, Di Fiore PP (2002) A single motif responsible for ubiquitin recognition and monoubiquitination in endocytic proteins. Nature 416:451–455

Posor Y, Eichhorn-Gruenig M, Puchkov D, Schoneberg J, Ullrich A, Lampe A, Muller R, Zarbakhsh S, Gulluni F, Hirsch E et al (2013) Spatiotemporal control of endocytosis by phosphatidylinositol-3,4-bisphosphate. Nature 499:233–237

Pyle JL, Kavalali ET, Piedras-Renteria ES, Tsien RW (2000) Rapid reuse of readily releasable pool vesicles at hippocampal synapses. Neuron 28:221–231

Quan A, Robinson PJ (2013) Syndapin–a membrane remodelling and endocytic F-BAR protein. FEBS J 280:5198–5212

Raimondi A, Ferguson SM, Lou X, Armbruster M, Paradise S, Giovedi S, Messa M, Kono N, Takasaki J, Cappello V et al (2011) Overlapping role of dynamin isoforms in synaptic vesicle endocytosis. Neuron 70:1100–1114

Ralser M, Nonhoff U, Albrecht M, Lengauer T, Wanker EE, Lehrach H, Krobitsch S (2005) Ataxin-2 and huntingtin interact with endophilin-A complexes to function in plastin-associated pathways. Hum Mol Genet 14:2893–2909

Rana RS, Hokin LE (1990) Role of phosphoinositides in transmembrane signaling. Physiol Rev 70:115–164

Ringstad N, Nemoto Y, De Camilli P (1997) The SH3p4/Sh3p8/SH3p13 protein family: binding partners for synaptojanin and dynamin via a Grb2-like Src homology 3 domain. Proc Natl Acad Sci U S A 94:8569–8574

Rizzoli SO, Jahn R (2007) Kiss-and-run, collapse and 'readily retrievable' vesicles. Traffic 8:1137–1144

Roth TF, Porter KR (1964) Yolk protein uptake in the Oocyte of the mosquito *Aedes aegypti* L. J Cell Biol 20:313–332

Rothman JE, Schmid SL (1986) Enzymatic recycling of clathrin from coated vesicles. Cell 46(1):5–9

Ryan TA, Reuter H, Smith SJ (1997) Optical detection of a quantal presynaptic membrane turnover. Nature 388:478–482

Saarikangas J, Zhao H, Lappalainen P (2010) Regulation of the actin cytoskeleton-plasma membrane interplay by phosphoinositides. Physiol Rev 90:259–289

Saffarian S, Cocucci E, Kirchhausen T (2009) Distinct dynamics of endocytic clathrin-coated pits and coated plaques. PLoS Biol 7:e1000191

Saheki Y, De Camilli P (2012) Synaptic vesicle endocytosis. Cold Spring Harb Perspect Biol 4: a005645

Sato K, Ernstrom GG, Watanabe S, Weimer RM, Chen CH, Sato M, Siddiqui A, Jorgensen EM, Grant BD (2009) Differential requirements for clathrin in receptor-mediated endocytosis and maintenance of synaptic vesicle pools. Proc Natl Acad Sci U S A 106:1139–1144

Schafer DA (2002) Coupling actin dynamics and membrane dynamics during endocytosis. Curr Opin Cell Biol 14:76–81

Schiavo G, Gu QM, Prestwich GD, Sollner TH, Rothman JE (1996) Calcium-dependent switching of the specificity of phosphoinositide binding to synaptotagmin. Proc Natl Acad Sci U S A 93:13327–13332

Schuske KR, Richmond JE, Matthies DS, Davis WS, Runz S, Rube DA, van der Bliek AM, Jorgensen EM (2003) Endophilin is required for synaptic vesicle endocytosis by localizing synaptojanin. Neuron 40:749–762

Sen A, Madhivanan K, Mukherjee D, Aguilar RC (2012) The epsin protein family: coordinators of endocytosis and signaling. Biomol Conc 3:117–126

Shin N, Lee S, Ahn N, Kim SA, Ahn SG, YongPark Z, Chang S (2007) Sorting nexin 9 interacts with dynamin 1 and N-WASP and coordinates synaptic vesicle endocytosis. J Biol Chem 282:28939–28950

Slepnev VI, De Camilli P (2000) Accessory factors in clathrin-dependent synaptic vesicle endocytosis. Nat Rev Neurosci 1:161–172

Smythe E (2002) Regulating the clathrin-coated vesicle cycle by AP2 subunit phosphorylation. Trends Cell Biol 12:352–354

Soulet F, Yarar D, Leonard M, Schmid SL (2005) SNX9 regulates dynamin assembly and is required for efficient clathrin-mediated endocytosis. Mol Biol Cell 16:2058–2067

Stamnes M (2002) Regulating the actin cytoskeleton during vesicular transport. Curr Opin Cell Biol 14:428–433

Stimson DT, Estes PS, Rao S, Krishnan KS, Kelly LE, Ramaswami M (2001) Drosophila stoned proteins regulate the rate and fidelity of synaptic vesicle internalization. J Neurosci 21:3034–3044

Sudhof TC (2013) Neurotransmitter release: the last millisecond in the life of a synaptic vesicle. Neuron 80:675–690

Suh BC, Hille B (2005) Regulation of ion channels by phosphatidylinositol 4,5-bisphosphate. Curr Opin Neurobiol 15:370–378

Takei K, Mundigl O, Daniell L, De Camilli P (1996) The synaptic vesicle cycle: a single vesicle budding step involving clathrin and dynamin. J Cell Biol 133:1237–1250

Takei K, Haucke V, Slepnev V, Farsad K, Salazar M, Chen H, De Camilli P (1998) Generation of coated intermediates of clathrin-mediated endocytosis on protein-free liposomes. Cell 94:131–141

Takei K, Slepnev VI, Haucke V, De Camilli P (1999) Functional partnership between amphiphysin and dynamin in clathrin-mediated endocytosis. Nat Cell Biol 1:33–39

Tan MS, Yu JT, Tan L (2013) Bridging integrator 1 (BIN1): form, function, and Alzheimer's disease. Trends Mol Med 19:594–603

Tavaria M, Gabriele T, Anderson RL, Mirault ME, Baker E, Sutherland G, Kola I (1995) Localization of the gene encoding the human heat shock cognate protein, HSP73, to chromosome 11. Genomics 29:266–268

Taylor MJ, Perrais D, Merrifield CJ (2011) A high precision survey of the molecular dynamics of mammalian clathrin-mediated endocytosis. PLoS Biol 9:e1000604

Tebar F, Sorkina T, Sorkin A, Ericsson M, Kirchhausen T (1996) Eps15 is a component of clathrin-coated pits and vesicles and is located at the rim of coated pits. J Biol Chem 271:28727–28730

Teng H, Lin MY, Wilkinson RS (2007) Macroendocytosis and endosome processing in snake motor boutons. J Physiol 582:243–262

ter Haar E, Harrison SC, Kirchhausen T (2000) Peptide-in-groove interactions link target proteins to the beta-propeller of clathrin. Proc Natl Acad Sci U S A 97:1096–1100

Tomlinson RV, Ballou CE (1961) Complete characterization of the myo-inositol polyphosphates from beef brain phosphoinositide. J Biol Chem 236:1902–1906

Torri-Tarelli F, Haimann C, Ceccarelli B (1987) Coated vesicles and pits during enhanced quantal release of acetylcholine at the neuromuscular junction. J Neurocytol 16:205–214

Traub LM (2003) Sorting it out: AP-2 and alternate clathrin adaptors in endocytic cargo selection. J Cell Biol 163:203–208

Traub LM, Downs MA, Westrich JL, Fremont DH (1999) Crystal structure of the alpha appendage of AP-2 reveals a recruitment platform for clathrin-coat assembly. Proc Natl Acad Sci U S A 96:8907–8912

Trempe JF, Chen CX, Grenier K, Camacho EM, Kozlov G, McPherson PS, Gehring K, Fon EA (2009) SH3 domains from a subset of BAR proteins define a Ubl-binding domain and implicate parkin in synaptic ubiquitination. Mol Cell 36:1034–1047

Tsyba L, Nikolaienko O, Dergai O, Dergai M, Novokhatska O, Skrypkina I, Rynditch A (2011) Intersectin multidomain adaptor proteins: regulation of functional diversity. Gene 473:67–75

Umasankar PK, Sanker S, Thieman JR, Chakraborty S, Wendland B, Tsang M, Traub LM (2012) Distinct and separable activities of the endocytic clathrin-coat components Fcho1/2 and AP-2 in developmental patterning. Nat Cell Biol 14:488–501

Umeda A, Meyerholz A, Ungewickell E (2000) Identification of the universal cofactor (auxilin 2) in clathrin coat dissociation. Eur J Cell Biol 79:336–342

Ungewickell E, Ungewickell H, Holstein SE, Lindner R, Prasad K, Barouch W, Martin B, Greene LE, Eisenberg E (1995) Role of auxilin in uncoating clathrin-coated vesicles. Nature 378:632–635

Van Jaarsveld PP, Nandi PK, Lippoldt RE, Saroff H, Edelhoch H (1981) Polymerization of clathrin protomers into basket structures. Biochemistry 20:4129–4135

Verstreken P, Koh TW, Schulze KL, Zhai RG, Hiesinger PR, Zhou Y, Mehta SQ, Cao Y, Roos J, Bellen HJ (2003) Synaptojanin is recruited by endophilin to promote synaptic vesicle uncoating. Neuron 40:733–748

Vinatier J, Herzog E, Plamont MA, Wojcik SM, Schmidt A, Brose N, Daviet L, El Mestikawy S, Giros B (2006) Interaction between the vesicular glutamate transporter type 1 and endophilin A1, a protein essential for endocytosis. J Neurochem 97:1111–1125

Voglmaier SM, Kam K, Yang H, Fortin DL, Hua Z, Nicoll RA, Edwards RH (2006) Distinct endocytic pathways control the rate and extent of synaptic vesicle protein recycling. Neuron 51:71–84

von Kleist L, Stahlschmidt W, Bulut H, Gromova K, Puchkov D, Robertson MJ, MacGregor KA, Tomilin N, Pechstein A, Chau N et al (2011) Role of the clathrin terminal domain in regulating coated pit dynamics revealed by small molecule inhibition. Cell 146:471–484

Wang Q, Navarro MV, Peng G, Molinelli E, Goh SL, Judson BL, Rajashankar KR, Sondermann H (2009) Molecular mechanism of membrane constriction and tubulation mediated by the F-BAR protein Pacsin/Syndapin. Proc Natl Acad Sci U S A 106:12700–12705

Watanabe S, Liu Q, Davis MW, Hollopeter G, Thomas N, Jorgensen NB, Jorgensen EM (2013) Ultrafast endocytosis at Caenorhabditis elegans neuromuscular junctions. eLife 2:e00723

Wenk MR, De Camilli P (2004) Protein-lipid interactions and phosphoinositide metabolism in membrane traffic: insights from vesicle recycling in nerve terminals. Proc Natl Acad Sci U S A 101:8262–8269

Wienisch M, Klingauf J (2006) Vesicular proteins exocytosed and subsequently retrieved by compensatory endocytosis are nonidentical. Nat Neurosci 9:1019–1027

Willig KI, Rizzoli SO, Westphal V, Jahn R, Hell SW (2006) STED microscopy reveals that synaptotagmin remains clustered after synaptic vesicle exocytosis. Nature 440:935–939

Woodward MP, Roth TF (1978) Coated vesicles: characterization, selective dissociation, and reassembly. Proc Natl Acad Sci U S A 75:4394–4398

Wu W, Wu LG (2007) Rapid bulk endocytosis and its kinetics of fission pore closure at a central synapse. Proc Natl Acad Sci U S A 104:10234–10239

Wu LG, Hamid E, Shin W, Chiang HC (2014) Exocytosis and endocytosis: modes, functions, and coupling mechanisms. Annu Rev Physiol 76:301–331

Xiao J, Kim LS, Graham TR (2006) Dissection of Swa2p/auxilin domain requirements for cochaperoning Hsp70 clathrin-uncoating activity in vivo. Mol Biol Cell 17:3281–3290

Xing Y, Böcking T, Wolf M, Grigorieff N, Kirchhausen T, Harrison SC (2010) Structure of clathrin coat with bound Hsc70 and auxilin: mechanism of Hsc70-facilitated disassembly. EMBO J 29:655–665

Yim YI, Sun T, Wu LG, Raimondi A, De Camilli P, Eisenberg E, Greene LE (2010) Endocytosis and clathrin-uncoating defects at synapses of auxilin knockout mice. Proc Natl Acad Sci U S A 107:4412–4417

Zhang Q, Cao YQ, Tsien RW (2007) Quantum dots provide an optical signal specific to full collapse fusion of synaptic vesicles. Proc Natl Acad Sci U S A 104:17843–17848

Zhang Q, Li Y, Tsien RW (2009) The dynamic control of kiss-and-run and vesicular reuse probed with single nanoparticles. Science 323:1448–1453

Chapter 11
Dynamin Is a Key Molecule to Decode Action Potential Firing

Shota Tanifuji and Sumiko Mochida

Abstract The maintenance of neurotransmission relies on the replenishment of synaptic vesicles (SVs) adapted to wide variations in the number and frequency of incoming action potentials (APs). A candidate mechanism for SV recycling indexed to AP firing activity could involve a protein-initiating endocytosis. Dynamin is a GTPase, which mediates fission of SVs from the presynaptic terminal membrane. There are three dynamin isoforms, dynamin 1, 2 and 3, in mammalian neurons. Knockout of dynamin 1 and 3 in central neurons suggests a role of each dynamin isoform in neuronal activity. We have carefully accessed the SV replenishment into the release site (readily releasable pool, RRP) in relation to AP firing activity and dynamin 1, 2 and 3 mediation. The three isoforms in sympathetic superior cervical ganglion neurons, an ideal model for direct physiological measurement of synaptic transmission combined with genetic knockdown, mediate the RRP replenishment, having distinct rate and time constants. Individual isoforms regulate distinct SV recycling pathways that cover the full range of physiological AP frequency. Thus, dynamin 1, 2 and 3 decode AP firing for SV recycling in sympathetic neurons. In this chapter, we review dynamin, in mammalian central and peripheral neurons, that is a key molecule for the selection and regulation of distinct SV recycling pathways in sensing of presynaptic AP firing patterns.

Keywords Dynamin • Action potential firing • Membrane recycling • Synaptic vesicle replenishment

11.1 Introduction

Neurons in both central and peripheral nervous systems encounter a wide range of activities in the form of action potential (AP) firing patterns. In accord, membrane transport systems, in particular, synaptic vesicle (SV) recycling pathways in synapses that mediate fast neurotransmission, must maintain the ability to rapidly react to ongoing changes in AP firing and adjust membrane trafficking to provide sufficient membrane replenishment. SV recycling through repetitive cycles of

S. Tanifuji • S. Mochida (✉)
Department of Physiology, Tokyo Medical University, 6-1-1 Shinjuku, Shinjuku-ku, Tokyo 160-8402, Japan
e-mail: tanifuji@tokyo-med.ac.jp; mochida@tokyo-med.ac.jp

S. Mochida (ed.), *Presynaptic Terminals*, DOI 10.1007/978-4-431-55166-9_11

exocytosis and endocytosis is well established to operate under different conditions of AP firing at presynaptic terminals.

AP firing triggers exocytosis of SVs. The SV membrane, fused with the presynaptic nerve terminal membrane, is retrieved by various morphological forms of endocytosis (Ceccarelli et al. 1973; Heuser and Reese 1973; Wu 2004; Wu et al. 2009). For endocytosis, dynamin is an essential protein that mediates fission of SVs from the presynaptic nerve terminal membrane (Shpetner and Vallee 1989; Obar et al. 1990; Takei et al. 1995; Roux et al. 2006). The functional role of dynamin was revealed by *Drosophila* mutant flies carrying a temperature-sensitive allele of dynamin (*Drosophila shibire*) at the nonpermissive temperature (Koenig and Ikeda 1989; Chen et al. 1991; Kuromi and Kidokoro 2002). Since then, numerous evidences have demonstrated for dynamin as a key molecule in the SV endocytosis (Ferguson and De Camilli 2012; Saheki and De Camilli 2012).

In mammals, three isoforms, dynamin 1, 2 and 3, are present (Sontag et al. 1994; Cook et al. 1996; Cao et al. 1998). Each dynamin isoform has distinct expression pattern; dynamin 1 is expressed primarily in brain, whereas dynamin 2 is expressed ubiquitously (Cook et al. 1994, 1996; Cao et al. 1998; Ferguson et al. 2007). Dynamin 3 is also highly expressed in brain and testis (Nakata et al. 1993; Cook et al. 1996; Cao et al. 1998). In the central nervous system (CNS), knockout of dynamin 1 in the mice cortical neurons demonstrated a selective activity-dependent requirement for dynamin 1 in SV endocytosis (Ferguson et al. 2007). Together with following study of dynamin 1 and 3, double knockout suggests that dynamin 1 is required for SV recycling during high-frequency stimulation, and dynamin 3 cooperates with dynamin 1 to support optimal rates of SV recycling (Ferguson et al. 2007; Raimondi et al. 2011). The evidence of dynamin 1 knockout also suggested that dynamin 2 is required for SV recycling after cessation of stimulation (Ferguson et al. 2007).

In the mammalian peripheral nervous system, we have shown roles of dynamin in the fast cholinergic synaptic transmission between rat superior cervical ganglion (SCG) neurons applying the inhibitors (Lu et al. 2009) and genetic knockdown (Tanifuji et al. 2013) combined with direct physiological measurements of the readily releasable pool (RRP) in the active zone. The effects of dynamin inhibitor, dynasore, and peptide perturbing dynamin interaction with amphiphysin suggest that dynamin and synaptic activity regulate SV recycling into the RRP through two distinct pathways (Lu et al. 2009). Moreover, knockdown of each dynamin isoform confirmed the specific selection of SV recycling pathways by activation of individual dynamin in response to various neuronal firing patterns (Tanifuji et al. 2013). With dynamin isoform mediation, a presynaptic terminal covers synaptic transmission in response to full range of physiological AP firing.

This chapter reviews the role of dynamin and its isoforms in SV recycling, especially the molecular mechanisms linking variation in presynaptic neuronal activity to SV recycling. We focus on progressed functional studies of dynamin and its isoforms at presynaptic terminals in the mammalian CNS, at the calyx of Held, hippocampal and cortical neurons and peripheral sympathetic neurons.

11.2 Dynamin Activation in the CNS

Ca^{2+} influx after APs arrived at presynaptic nerve terminal triggers SV exocytosis and also initiates various forms of endocytosis (Gad et al. 1998; Hosoi et al. 2009; Wu et al. 2009). Recent studies at the calyx of Held nerve terminal using Ca^{2+} chelators EGTA and BAPTA suggest the essential role of Ca^{2+} in triggering endocytosis both at the active zone and the peri-active zone (Yamashita et al. 2010). In addition, numerous studies have revealed a modulatory role for Ca^{2+} in endocytosis at nerve terminals in different synaptic preparations (Balaji et al. 2008; Wu et al. 2009; Yao et al. 2009; Yamashita et al. 2010). Interestingly, the rate of SV exocytosis is controlled by Ca^{2+} accumulation following consecutive AP firing (Armbruster et al. 2013). High-sensitivity vGlut 1-pHluorin assays of SV endocytosis in cultured hippocampal and cortical neurons demonstrated that acceleration of endocytosis is followed by the stalling during AP bursts and that the biphasic dependence on electrical activity is blocked by mutation of dynamin at the phosphorylation site (Armbruster et al. 2013). These observations suggest that the rate of endocytosis varies with number and frequency of APs due to dephosphorylation of dynamin following Ca^{2+} influx with repeated AP firing. Thus, dynamin controls optimization of the SVs recycling rate for varying synaptic activity.

Most forms of endocytosis in the physiological range require the protein dynamin, a large GTPase that oligomerizes around the neck of clathrin-coated pits and catalyzes SV fission triggered by GTP hydrolysis (Takei et al. 1995; Marks et al. 2001; Praefcke and McMahon 2004). With the use of the nonhydrolyzable GTP analogue, GTPγS, to inhibit endocytosis (Takei et al. 1995; Song et al. 2004), it reduced synaptic transmission in an activity-dependent manner at the calyx of Held (Yamashita et al. 2005; Xu et al. 2008); however, endocytosis independent on dynamin GTP hydrolysis was also suggested (Xu et al. 2008).

11.2.1 Selective Requirement of Dynamin Isoforms in Activity-Dependent and -Independent SV Recycling

Dynamin 1 is present at high concentrations in presynaptic nerve terminals of the CNS. Ferguson et al. (2007) demonstrated a selective requirement of dynamin 1 in the SV endocytosis only during high-frequency stimulation, but not after cessation of the stimulus train. Genetically engineered mice that lack dynamin 1 are able to form functional synapses, even though their postnatal viability is limited. Dynamin 1 knockout mice appear normal at birth, with near-normal numbers of neurons and SVs. However, they exhibit failure to thrive within several hours after birth and die within 2 weeks of birth. At inhibitory presynaptic nerve terminals of dynamin 1 knockout cortical neurons, electron microscopy analysis revealed massive increase in clathrin-coated pit abundance under spontaneous network activity. Their higher levels of tonic activity lead to a build-up of clathrin-coated

intermediates with perturbation of endocytic protein functions (Hayashi et al. 2008). Under intense stimulation with high-potassium buffer, larger-branched tubular networks connected to the plasma membrane capped by clathrin-coated buds were increased (Ferguson et al. 2007). Visualization of endocytosis by expressing synapto-pHluorin in cultured dynamin 1 knockout cortical neurons shows completely blocked endocytosis during stimulation with 300 APs at 10 Hz, yet resumes at a normal rate after termination of the stimulus. This endocytic blockade during stimulation is fully rescued by dynamin 1 transfection. Synaptic vesicle endocytosis is also rescued by the overexpression of dynamin 3 in dynamin 1 knockout neurons, whereas partially rescued by dynamin 2. Thus, gene knockout studies with the cortical neurons demonstrate that dynamin 1 is required during stimulation, but not indispensable after cessation of stimulation, and that dynamin 3 has a greater functional similarity with dynamin 1. The efficient formation of endosome-like structures in dynamin 1 KO synapses during the high-potassium stimulation, whereas SV recycling in these mutant neurons detected profound endocytic defect during the field stimulation (300 APs at 10 Hz) suggests that different endocytic pathways are activated by dynamin 1/3 and 2 in response to different modes of stimulation (Hayashi et al. 2008).

Furthermore, dynamin 3 plays a role in supporting optimal rates of SV endocytosis by cooperating with dynamin 1 (Raimondi et al. 2011). Dynamin 3 knockout mice do not show any obvious pathological phenotype observed in dynamin 1 knockout mice; however, dynamin 1 and dynamin 3 double-knockout mice have a more severe phenotype than dynamin 1 single-knockout mice. They exhibit lack of milk in the stomach and hunched posture 2 h after birth and die within several hours after birth. Compared to dynamin 1 knockout synapses, the double-knockout nerve terminals in cultured cortical neurons show more severe endocytic defect, confirmed by a massive accumulation of clathrin-coated pits at presynaptic nerve terminals and by a delay in compensatory endocytosis in response to a stimulus monitored with vGlut 1-pHluorin, a pH-sensitive probe that is very efficiently targeted to SVs (Balaji and Ryan 2007). However, consecutive rounds of stimulation at 10 Hz with the interval of 10 min show that, given sufficient time, vGlut 1-pHluorin signals recover in dynamin 1- and dynamin 3 double-knockout neurons albeit at a much reduced rate, indicating a contribution of dynamin 2 to SV recycling. Overall, in the CNS, overlapping roles of dynamin 1 and dynamin 3 in SV endocytosis related to high synaptic activity and a role of dynamin 2 and/or dynamin-independent mechanisms in basic SV endocytosis have been suggested.

11.2.2 Dynamin-Mediated Ultrafast Endocytosis

SVs are thought to be regenerated from plasma membrane approximately 20 s after fusion by the classic clathrin-mediated endocytosis (Heuser and Reese 1973) or within 1 s by kiss-and-run endocytosis that retrieves fusing vesicles by reversing their neck (Ceccarelli et al. 1973; Zhang et al. 2009). However, combination of

optogenetics and rapid high-pressure freezing, 'flash-and-freeze' approach, allows for visualizing synaptic ultrastructure with electron microscopy following a single physiological stimulation. This approach revealed an ultrafast mode of endocytosis, which occurs within 50 to 100 ms after the onset of the stimulus, requiring actin and dynamin (Watanabe et al. 2013a). In cultured mouse hippocampal neurons expressing channelrhodopsin-2, a light-activated channel protein, a 10-ms pulse of blue light elicits an influx of sodium ions, leading to an AP. Within 30 ms of the stimulus, docked vesicles fuse and collapse into the membrane. These fusions are observed within the active zone, and vesicle-depleted active zones are repopulated with a full complement of docked vesicles with a time constant of 3.8 s. This value is consistent with that for the recovery of the RRP previously measured electrophysiologically in mouse hippocampal neurons (Pyott and Rosenmund 2002). Following exocytosis, the shallow invaginated pits occur at locations outside the active zone within 50 ms after stimulation. In addition, within 50–100 ms, large vesicles which have about fourfold greater surface area than normal SVs are generated. This compensatory endocytosis seems not to be mediated by clathrin coats and is inhibited by latrunculin A which disrupts polymerization of actin and dynasore which interferes with the GTPase activity of dynamin. Thus, the ultrafast endocytosis appears to compensate the membrane added to the plasma membrane immediately after exocytosis, in clathrin-independent but actin- and dynamin-dependent manner.

11.2.3 Role for Dynamin in Exocytic-Endocytic Coupling

Several recent studies interfering with the function of endocytic proteins have reported inhibitory effects on SV exocytosis, indicating a role for endocytic proteins in regulation of SV exocytosis (Saheki and De Camilli 2012). Dynamin dysfunction with *Drosophila shibire* mutant (Kawasaki et al. 2000) and that in the calyx of Held (Hosoi et al. 2009) demonstrated that rapid dynamin action is required for synaptic transmission in response to repetitive firing of APs, indicating that clearance of used SVs including the cargo proteins from the active zone is triggered by dynamin activation. This step is the rate limiting for transmitter release, and delayed clearance leads to the short-term synaptic depression (i.e. gradual decrease in transmitter release in response to each AP firing) (Hosoi et al. 2009; Neher 2010). Dynamin dysfunction by dynasore, anti-dynamin antibody or dynamin inhibitory peptide which perturbs dynamin binding to the SH3 domain of amphiphysin delayed SV recruitment to docking sites and enhanced short-term synaptic depression at the calyx of Held. Another study with measurements of capacitance, which is proportional to net changes in neuron surface area and thus constitute measurement of synaptic exocytosis and endocytosis, also showed that dynasore slows down the RRP replenishment 100 ms after the first pulse (Wu et al. 2009). Clearance of the active zone could be achieved by rapid lateral diffusion of SV proteins towards the peri-active zone, an area that surrounds the

active zone and into which SV membranes are recycled following exocytosis (Roos and Kelly 1999; Haucke et al. 2011). In consistent, ultrafast endocytic invaginations are outside the active zone and are often observed flanking the active zone (Watanabe et al. 2013a). Thus, exocytic-endocytic coupling could be achieved by the direct dynamin-mediated retrieval of SVs near the exocytic site.

11.3 Dynamin Activation in SCG Neurons

Evidence in a large presynaptic terminal of the calyx of Held (Yamashita et al. 2005, 2010; Hosoi et al. 2009; Neher 2010) and other central neurons (Newton et al. 2006; Ferguson et al. 2007; Raimondi et al. 2011; Hayashi et al. 2008) provides a general role for dynamin and its isoforms in SV recycling to regulate synaptic transmission in the CNS. We have used a model system for the genetic analysis of fast cholinergic transmission between rat SCG neurons to examine the role of dynamin isoforms in activity sensing (Tanifuji et al. 2013). The SCG neurons are a useful model to study presynaptic terminal proteins because of the large cell body and nucleus for the manipulation of gene expression and function via acute microinjection of siRNA, peptides and antibodies (Mochida et al. 1994, 2008; Ma et al. 2009), an approach not technically feasible for cultured neurons from the central nervous system. Dynamin function was disrupted by inhibitors (Sect. 11.3.1), or each isoform was knocked down by the specific siRNAs (Sects. 11.3.2, 11.3.3, 11.3.4, and 11.3.5) injected into presynaptic neurons. These neurons were challenged with various AP firing patterns, and the resultant changes in the replenishment of the RRP were monitored by recording evoked excitatory postsynaptic potentials (EPSPs).

11.3.1 Sympathetic Neurons Maintain Synaptic Transmission via the SV Recycling Through Dynamin-Mediated Pathways

In SCG neurons, dynamin dysfunction by either P4 peptide (QVPSRPNRAP) which interferes linkage of dynamin to clathrin coats through interaction with amphiphysin or dynasore, a specific cell-permeable dynamin inhibitor, impaired synaptic transmission with various presynaptic AP firing patterns, such as paired AP and consecutive APs at low or high frequency (Lu et al. 2009). These electrophysiological recordings from P4- or dynasore-treated SCG neurons demonstrated that sympathetic neurons have two dynamin-mediated SV replenishment pathways, one activity dependent and the other activity independent (Lu et al. 2009). In addition, the differential mechanisms of P4 and dynasore for dysfunction of dynamin suggest that the RRP refills at two time constants, fast and slow, with

the RRP refilling through distinct dynamin-mediated pathways. The fast refilling rate reflects a rapid SV replenishment into the RRP from the reserve pool (RP) in an amphiphysin-dependent endocytic pathway, whereas the slow refilling rate involves a gradual SV replenishment through an amphiphysin-independent endocytic pathway (Lu et al. 2009). Together, these data provide evidence for physiological and molecular heterogeneity in endocytosis.

11.3.2 Dynamin 1, 2 and 3 Differentially Mediate Rapid SV Recycling in Sympathetic Neurons

All three isoforms of dynamin are expressed in peripheral SCG neurons (Tanifuji et al. 2013), similarly to central neurons (Cao et al. 1998; Ferguson et al. 2007). Dynamin isoforms were specifically knocked down in presynaptic neurons by microinjection of siRNA, and the transfected neurons were challenged with various AP firing patterns. Applying a paired-pulse protocol to assess rapid replenishment of the RRP, changes in EPSP amplitude after an AP-evoked transmitter release were monitored by eliciting two consecutive APs at various interstimulus intervals (ISIs) in the siRNA-injected neuron (Fig. 11.1a, left). Under control conditions, at the synapses injected with control siRNA (control), paired-pulse ratio (2nd/1st EPSP ratio) values were less than 1.0 (paired-pulse depression) at short ISIs ($\leq$100 ms), whereas at longer ISIs paired-pulse ratio values were ~1.0, in agreement with our previous studies (Lu et al. 2009; Ma et al. 2009). Shown in Fig. 11.1a, right, dynamin 1 knockdown (KD) increased paired-pulse depression at short ISIs (50–100 ms), but did not increase it at ISIs of 20 or 30 ms. On the other hand, dynamin 2 or 3 KD increased paired-pulse depression at short ISIs (20–100 ms). At longer ISIs (100 ms $\leq$), however, paired-pulse ratio values at dynamin 1, 2 or 3 KD synapses were 60–70 % of that for control. It is unlikely that basal release probability is affected by dynamin loss of function because the mean EPSP amplitude of the first recording was unchanged. Therefore, in contrast to findings in central neurons of dynamin knockout mouse, each dynamin isoform differently contributes to rapid SV recycling.

The rate of endocytosis is generally thought to range from hundreds of milliseconds to hundreds of seconds (Royle and Lagnado 2003; Wu 2004), estimated by capacity measurements (Sun et al. 2002) and synapto-pHluorin imaging (Fernandez-Alfonso and Ryan 2004). However, recent studies in cultured hippocampal neurons (Watanabe et al. 2013a) and at *Caenorhabditis elegans* neuromuscular junctions (Watanabe et al. 2013b) have provided evidence that, by optogenetics coupled with high-pressure freezing, dynamin-mediated SV endocytosis occurs on a millisecond timescale following a single physiological stimulus (see Sect. 11.2.2). The 'flash-and-freeze' electron microscopy revealed that the ultrafast mode of endocytosis occurs within 50–100 ms after a single pulse of blue light, consistent with rapid temporal windows of dynamin-mediated SV recycling in SCG neurons.

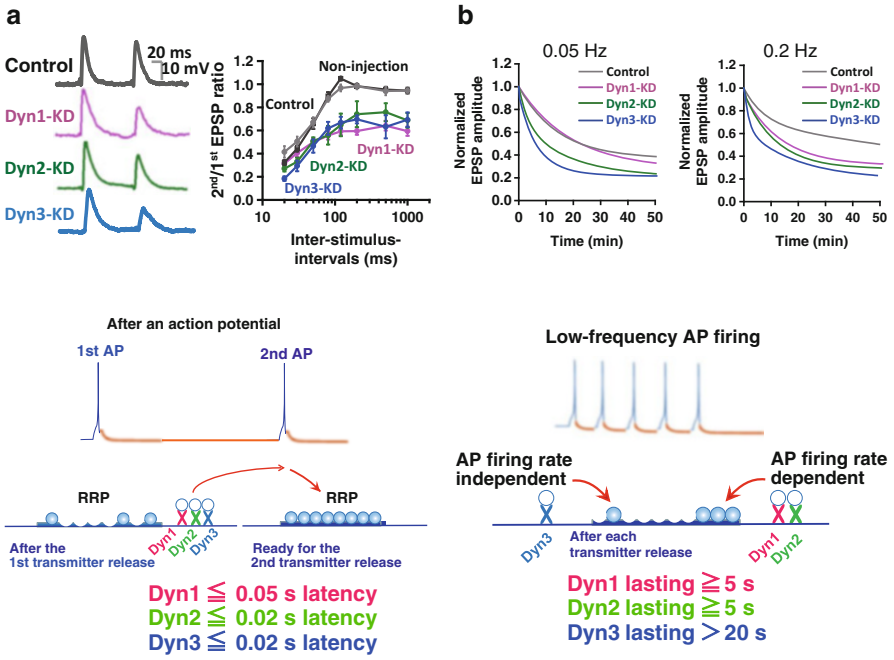

Fig. 11.1 The RRP replenishment mediated by dynamin 1, 2 or 3 under low-frequency AP firing. Presynaptic neurons were injected with control or dynamin siRNA (control, Dyn1-KD, Dyn2-KD or Dyn3-KD). (**a**) Changes in EPSP amplitude after an AP-evoked transmitter release were monitored by eliciting two consecutive APs at various ISIs (20–1,000 ms), every 1 min, in the siRNA-injected neuron. (*left*) EPSPs from a representative recording with an interstimulus interval of 120 ms. (*right*) The averaged paired-pulse ratio (2nd/1st EPSP ratio) is plotted against the ISIs. Dyn1-KD increased paired-pulse depression (PPD) at ISIs of 50–1,000 ms, whereas Dyn2- or 3-KD increased PPD at ISIs of 20–1,000 ms. (*Scheme*) Results of paired-pulse ratio demonstrate an asymmetry in the involvement of dynamin in SV recycling with a distinct latency after AP generation. After the first neurotransmitter release, dynamin 1, 2 or 3 is activated with a latency of $\leq$0.05, $\leq$0.02 and $\leq$0.02 s, respectively, to replenish the RRP ready for the second transmitter release. (**b**) Changes in the EPSP amplitude elicited by consecutive presynaptic APs at 0.05 or 0.2 Hz were monitored over 50 min. (*left*) Single exponential decay curves fitted with the averaged EPSP amplitudes at 0.05 Hz were reduced more rapidly with Dyn3-KD than Dyn1- or 2-KD. (*right*) Single (control, Dyn1-KD or Dyn2-KD) or double (Dyn3-KD) exponential decay curves fitted with averaged EPSP amplitudes at 0.2 Hz were reduced more rapidly with each dynamin-KD. (*Scheme*) During low-frequency AP firing, dynamin 1 and 2 are activated dependent on AP firing rate $\geq$0.2 Hz, while dynamin 3 is activated independent on the AP firing rate. The RRP replenishment process mediated by dynamin 1 and 2 lasts 5 s, while that mediated by dynamin 3 lasts more than 20 s

11.3.3 Distinct Roles of Dynamin 1, 2 and 3 During High- or Low-Frequency AP Firing in Sympathetic Neurons

Endocytosis is essential for maintaining transmitter release during repetitive AP firing (Wu 2004). At cultured hippocampal synapses expressing synapto-pHluorin

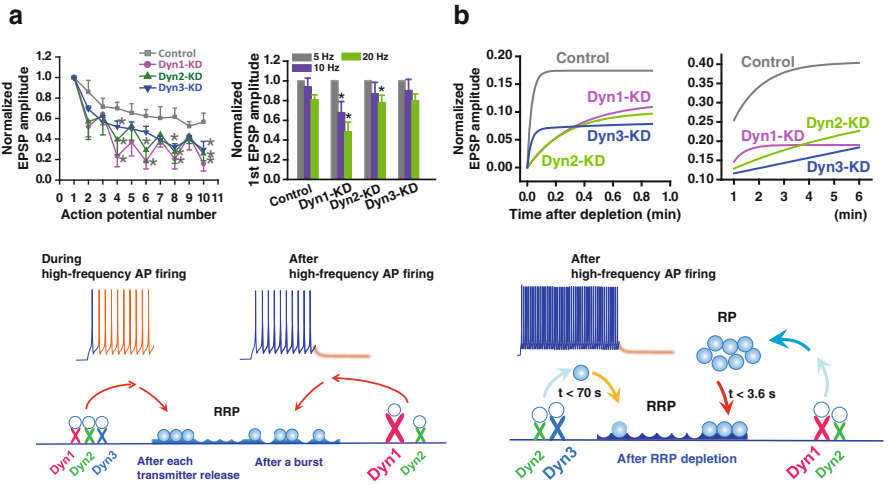

Fig. 11.2 The RRP replenishment mediated by dynamin 1, 2 or 3 under high-frequency AP firing. (**a**) EPSPs elicited by 2-s AP trains at 5, 10 or 20 Hz were monitored every 2 min, 3 times for each frequency. (*left*) Normalized and averaged peak amplitudes from the end of the previous EPSP at 10 Hz were plotted against AP number, indicating that each dynamin isoform contributes to SV recycling during high-frequency AP firing. (*right*) Normalized 1st EPSP amplitudes of each train to that of the 5-Hz train decreased 2 min after a 5- or 10-Hz AP train in Dyn1-KD neurons and after 10-Hz AP train in Dyn2-KD neurons. (*scheme*) During high-frequency AP firing, each dynamin isoform contributes to SV recycling in the RRP, while dynamin 1 and 2 also mediate it after high-frequency AP firing. (**b**) The recovery of SVs in the RRP after full depletion due to a 4-min AP train at 5 Hz was monitored by measuring the EPSP amplitude every 1 s. EPSP amplitudes normalized to the mean value before the depletion were averaged and fitted with single exponential growth curves to estimate the recovery rate and the time constant. (*left*) Dyn1- or 2-KD delayed the fast recovery phase. (*right*) Dyn2- or 3-KD delayed the slow recovery phase. (*Scheme*) After high-frequency AP firing dynamin 1 drives the fast RRP replenishment pathway via RP, whereas dynamin 3 drives the slow replenishment pathway. Dynamin 2 contributes to driving both fast and slow replenishment pathways. Time constant for the fast replenishment of empty RRP with SVs from the RP is <3.6 s, while that of the slow RRP replenishment is <70 s in presynaptic SCG neurons

(Fernandez-Alfonso and Ryan 2004), fast endocytosis occurs during AP firing, although it is slowed down after repetitive stimulation (Sun et al. 2002), suggesting that AP firing frequency at nerve terminal determines the time course of endocytosis. In SCG neurons, dynamin 1, 2 or 3 KD induced severe synaptic depression at 5 or 10 Hz (Fig. 11.2a, left) AP train, in contrast to control synapses that only induced synaptic depression at higher frequency of 20 Hz (Tanifuji et al. 2013). In addition, dynamin 1 KD increased the number of EPSP failures during 2-s AP trains at high-frequency AP firing. Therefore, our results suggest that each dynamin isoform contributes to maintain SV replenishment during high-frequency AP firing. At 2 min after 5 or 10-Hz AP train, the recovery of first EPSP amplitude of each train from depression was impaired with dynamin 1 KD and weakly with dynamin

2 KD (Fig. 11.2a, right), suggesting that dynamin 1 and 2 mediate longer-lasting, at least 2 min, SV recycling after high-frequency AP firing.

In addition to the effect of dynamin isoform KD on SV replenishment during high-frequency AP firing, we also found that at control synapses, synaptic transmission gradually depressed over the 50-min-recording session under low-frequency presynaptic AP firing at 0.05 or 0.2 Hz (Fig. 11.1b) but was reduced more rapidly with dynamin 1, 2 or 3 KD than control level of depression following repetitive APs at 0.2 Hz (Fig. 11.1b, right). Dynamin 3 KD also showed a rapid reduction of the EPSP amplitudes at a much lower AP firing frequency, 0.05 Hz (Fig. 11.1b, left). Together, these data suggest that dynamin 1- or 2-mediated SV replenishment is activated by AP firing at 0.2 Hz and completes within 5 s after AP generation, in an AP firing rate-dependent manner, whereas dynamin 3-mediated SV replenishment is activated by AP at lower than 0.05 Hz and takes more than 20 s, in an AP firing rate-independent manner.

11.3.4 Dynamin 1, 2 and 3 Have Distinct Roles in SV Recycling Pathways with Distinct Kinetics

Multiple recycling pathways are used by SVs with the selection of pathway depending on AP firing frequency (Granseth et al. 2006; Lu et al. 2009; Zhang et al. 2009; Zhu et al. 2009; Cheung et al. 2010). The classic clathrin-mediated endocytic pathway is responsible for the RRP refilling via an RP, whereas the fast mode of endocytosis involves rapid reuse pathway of SVs that bypass the RP (Lu et al. 2009). As discussed above, the fast SV replenishment of the RRP involves SV transport from the RP via dynamin-dependent and clathrin-mediated pathway or de novo sorting via an endosomal pool, whereas the slow SV replenishment of the RRP involves another mode of SV recycling, which bypasses the RP via dynamin-mediated pathway (Lu et al. 2009). The kinetics of refilling of the RRP with SVs following full RRP depletion due to a train of 4-min APs at 5 Hz was monitored by measuring the EPSP amplitude every 1 s. The refilling rate of the fast phase was delayed with dynamin 1 or 2 KD, whereas that of the slow phase was delayed with dynamin 2 or 3 KD, respectively (Fig. 11.2b). Thus, in sympathetic neurons, dynamin isoforms have distinct specificity for fast and slow recycling pathways. Taken together, dynamin 1 mainly regulates the RRP replenishment with a rapid rate for the selective recovery of SV depletion during and after high-frequency AP firing, whereas dynamin 3 mainly regulates the RRP replenishment at a slow rate, mediating a separate endocytic pathway in an AP firing frequency-independent manner. Dynamin 2 exhibits a contribution equally to both rapid and slow replenishment.

11.3.5 Dynamin 1, 2 and 3 Decode AP Firing for SV Replenishment into the RRP

How dynamin 1, 2 and 3 contribute to the RRP replenishment via distinct SV recycling pathways linking variation in neuronal activity is speculated in the schemes (Figs. 11.1 and 11.2). In SCG neurons, individual dynamin isoforms regulate distinct SV reuse pathway that covers the full range of physiological AP firing frequency patterns for the selection of specific vesicle reuse modes that replenish a shared RRP. Dynamin 1-mediated SV recycling pathway is primarily activated during and after high-frequency AP firing at >5 Hz with a latency of 0.05 s and lasting 5 s after AP generation. In contrast, dynamin 3-mediated SV recycling pathway supports a separate mode of the RRP replenishment that was independent of AP firing frequency and rapidly activated within 0.02 s after AP generation. Dynamin 2 mediates both SV recycling pathways, with hybrid properties between the other isoforms. Overall, fine-tuning mechanisms of SV recycling dependent on dynamin isoforms allow synapses to maintain stable neurotransmission during dynamic changes in AP firing properties.

11.4 Concluding Remarks

In mammals, dynamin is encoded by three different genes, whose products undergo further alternative splicing to generate a multiplicity of variants (Cao et al. 1998). Given the differences in the expression pattern between dynamin isoforms and the unique protein-protein interactions of each isoform, additional studies are required to fully address the issue of allocation between dynamin isoforms in the nervous systems, even though some studies in non-neuronal cells demonstrate that each dynamin isoform regulates distinct endocytic pathways (Shpetner and Vallee 1989; Artalejo et al. 2002; Lu et al. 2008). At central synapses, knockout studies of dynamin isoforms revealed that clathrin-mediated endocytosis is tailored to work well under different conditions of synaptic activity which activates specific dynamin (Ferguson et al. 2007; Raimondi et al. 2011). However, while these studies collectively establish a general role for dynamin and its isoforms in membrane recycling, several important questions remain unanswered. First, how do the three dynamin isoforms collaborate in single synapses to maintain stable SV recycling pools in response to AP firing properties? To address this question would require synaptic electrophysiological methods to measure ongoing synaptic transmission and quantitate the recovery of the RRP after stimulation. Second, how do individual dynamin isoforms allocate specific time windows and rate constants to allow a cell to respond to variable patterns of electrical stimulation? To address this question would necessitate an acute dynamin knockdown rather than a mouse knockout approach, for which genetic compensation may occur. Moreover, to address the role of dynamin isoforms in RRP maintenance, the study should be done in a system in

which dynamin loss-of-function specifically occurs in the presynaptic but not postsynaptic cell to avoid complications from dynamin-dependent postsynaptic regulation. In SCG neuron, dynamin isoforms were specifically knocked down in presynaptic neurons by microinjection of siRNA. The transfected neurons were challenged with various AP firing patterns, and the resultant changes in the recovery of readily releasable SVs were monitored by recording evoked EPSPs. The studies revealed distinct dynamin isoforms controlling unique vesicle pools may allow synapses to dynamically respond to rapid or complex APs burst into EPSPs that reflect a cell's history of synaptic firing, while simultaneously maintaining the general capability for fast and stable SV recycling and maintenance after sparse single APs. Whether there is a spatial synaptic organization to dynamin isoform-dependent recycling pathways remains a challenging future issue.

Ultrastructually, the recent study using 'flash-and-freeze' microscopy (Watanabe et al. 2013a) supports the proposal that dynamin mediates exocytic-endocytic coupling to clear the vesicle release site for the next set of exocytosis (Hosoi et al. 2009). Dynasore used for the study on exocytic-endocytic coupling is reported to inhibit the GTPase activity of dynamin 1, dynamin 2 and Drp 1, the mitochondrial dynamin (Macia et al. 2006), but not dynamin 3. Thus, which dynamin isoform mediates the clearance pathway remains to be determined. The new 'flash-and-freeze' microscopy technique (Watanabe et al. 2013a) also supports our findings that dynamin isoforms differently drive SV recycling with a more rapid time window than that estimated by membrane capacity measurements (Sun et al. 2002) and vesicle trafficking with fluorescence imaging (Fernandez-Alfonso and Ryan 2004). The rapid activation of dynamin isoforms covers the range of AP frequency patterns for the selection of specific vesicle reuse modes; however, the molecular mechanism underlying the relationship of dynamin isoforms and sensing cell activity will require further investigation.

Increase in intracellular Ca^{2+} accompanying AP firing at presynaptic nerve terminals plays a critical role in SV endocytosis and efficient replenishment of the RRP (Wu et al. 2009; Denker and Rizzoli 2010; Yamashita 2012). Thus, identification of Ca^{2+} sensor proteins is essential to understand the molecular mechanism for the selection of dynamin isoforms, sensing cell activity, to drive the specific SV recycling pathway. In this regard, synaptotagmin is one of the candidate molecules to play a role in fast endocytosis (Poskanzer et al. 2003; Nicholson-Tomishima and Ryan 2004; Watanabe et al. 2010), which forms complexes with presynaptic proteins such as AP2 (Takei et al. 1999), a clathrin coat protein that interacts with dynamin. On the other hand, endocytic proteins including dynamin are phosphoproteins. Upon AP firing at presynaptic nerve terminals in the brain, dynamin is dephosphorylated by the Ca^{2+}/calmodulin (CaM)-dependent phosphatase calcineurin (Cousin and Robinson 2001; Sun et al. 2010), suggesting that CaM, calcineurin and endocytic phosphoproteins represent Ca^{2+} sensors, mediators, and effectors, respectively, for slow endocytosis. The low- and high-affinity Ca^{2+} sensors seem to be used to drive endocytosis at different synapses or different developmental stages. At the calyx of Held synapses, Ca^{2+} elevation in the Ca^{2+} nano-domain around Ca^{2+} channels triggers dynamin-mediated endocytosis

via low-affinity Ca^{2+} binding molecules, whereas bulk Ca^{2+} elevation outside of the Ca^{2+} nano-domain, peri-active zone triggers clathrin-mediated endocytosis via Ca^{2+}/CaM/calcineurin mediation at immature synapses, but not in mature synapses (Yamashita et al. 2010). In summary, given that specific interactions between dynamin isoforms and Ca^{2+} sensor proteins must initiate SV endocytosis dependent on presynaptic activity, it remains a challenging future issue to elucidate the molecular mechanisms.

References

Armbruster M, Messa M, Ferguson SM, De Camilli P, Ryan TA (2013) Dynamin phosphorylation controls optimization of endocytosis for brief action potential bursts. Elife 2:e00845

Artalejo CR, Elhamdani A, Palfrey HC (2002) Sustained stimulation shifts the mechanism of endocytosis from dynamin-1-dependent rapid endocytosis to clathrin- and dynamin-2-mediated slow endocytosis in chromaffin cells. Proc Natl Acad Sci U S A 99:6358–6363

Balaji J, Ryan TA (2007) Single-vesicle imaging reveals that synaptic vesicle exocytosis and endocytosis are coupled by a single stochastic mode. Proc Natl Acad Sci U S A 104:20576–20581

Balaji J, Armbruster M, Ryan TA (2008) Calcium control of endocytic capacity at a CNS synapse. J Neurosci 28:6742–6749

Cao H, Garcia F, McNiven MA (1998) Differential distribution of dynamin isoforms in mammalian cells. Mol Biol Cell 9:2595–2609

Ceccarelli B, Hurlbut WP, Mauro A (1973) Turnover of transmitter and synaptic vesicles at the frog neuromuscular junction. J Cell Biol 57:499–524

Chen MS, Obar RA, Schroeder CC, Austin TW, Poodry CA, Wadsworth SC, Vallee RB (1991) Multiple forms of dynamin are encoded by *shibire*, a *Drosophila* gene involved in endocytosis. Nature 351:583–586

Cheung G, Jupp OJ, Cousin MA (2010) Activity-dependent bulk endocytosis and clathrin-dependent endocytosis replenish specific synaptic vesicle pools in central nerve terminals. J Neurosci 30:8151–8161

Cook TA, Urrutia R, McNiven MA (1994) Identification of dynamin 2, an isoform ubiquitously expressed in rat tissues. Proc Natl Acad Sci U S A 91:644–648

Cook T, Mesa K, Urrutia R (1996) Three dynamin-encoding genes are differentially expressed in developing rat brain. J Neurochem 67:927–931

Cousin MA, Robinson PJ (2001) The dephosphins: dephosphorylation by calcineurin triggers synaptic vesicle endocytosis. Trends Neurosci 24:659–665

Denker A, Rizzoli SO (2010) Synaptic vesicle pools: an update. Front Synaptic Neurosci 2:135

Ferguson SM, De Camilli P (2012) Dynamin, a membrane-remodelling GTPase. Nat Rev Mol Cell Biol 13:75–88

Ferguson SM, Brasnjo G, Hayashi M, Wolfel M, Collesi C, Giovedi S, Raimondi A, Gong LW, Ariel P, Paradise S, O'Toole E, Flavell R, Cremona O, Miesenbock G, Ryan TA, De Camilli P (2007) A selective activity-dependent requirement for dynamin 1 in synaptic vesicle endocytosis. Science 316:570–574

Fernandez-Alfonso T, Ryan TA (2004) The kinetics of synaptic vesicle pool depletion at CNS synaptic terminals. Neuron 41:943–953

Gad H, Low P, Zotova E, Brodin L, Shupliakov O (1998) Dissociation between Ca^{2+}-triggered synaptic vesicle exocytosis and clathrin-mediated endocytosis at a central synapse. Neuron 21:607–616

Granseth B, Odermatt B, Royle SJ, Lagnado L (2006) Clathrin-mediated endocytosis is the dominant mechanism of vesicle retrieval at hippocampal synapses. Neuron 51:773–786

Haucke V, Neher E, Sigrist SJ (2011) Protein scaffolds in the coupling of synaptic exocytosis and endocytosis. Nat Rev Neurosci 12:127–138

Hayashi M, Raimondi A, O'Toole E, Paradise S, Collesi C, Cremona O, Ferguson SM, De Camilli P (2008) Cell- and stimulus-dependent heterogeneity of synaptic vesicle endocytic recycling mechanisms revealed by studies of dynamin 1-null neurons. Proc Natl Acad Sci U S A 105:2175–2180

Heuser JE, Reese TS (1973) Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. J Cell Biol 57:315–344

Hosoi N, Holt M, Sakaba T (2009) Calcium dependence of exo- and endocytotic coupling at a glutamatergic synapse. Neuron 63:216–229

Kawasaki F, Hazen M, Ordway RW (2000) Fast synaptic fatigue in *shibire* mutants reveals a rapid requirement for dynamin in synaptic vesicle membrane trafficking. Nat Neurosci 3:859–860

Koenig JH, Ikeda K (1989) Disappearance and reformation of synaptic vesicle membrane upon transmitter release observed under reversible blockage of membrane retrieval. J Neurosci 9:3844–3860

Kuromi H, Kidokoro Y (2002) Selective replenishment of two vesicle pools depends on the source of Ca^{2+} at the *Drosophila* synapse. Neuron 35:333–343

Lu J, He Z, Fan J, Xu P, Chen L (2008) Overlapping functions of different dynamin isoforms in clathrin-dependent and -independent endocytosis in pancreatic β cells. Biochem Biophys Res Commun 371:315–319

Lu W, Ma H, Sheng ZH, Mochida S (2009) Dynamin and activity regulate synaptic vesicle recycling in sympathetic neurons. J Biol Chem 284:1930–1937

Ma H, Cai Q, Lu W, Sheng ZH, Mochida S (2009) KIF5B motor adaptor syntabulin maintains synaptic transmission in sympathetic neurons. J Neurosci 29:13019–13029

Macia E, Ehrlich M, Massol R, Boucrot E, Brunner C, Kirchhausen T (2006) Dynasore, a cell-permeable inhibitor of dynamin. Dev Cell 10:839–850

Marks B, Stowell MH, Vallis Y, Mills IG, Gibson A, Hopkins CR, McMahon HT (2001) GTPase activity of dynamin and resulting conformation change are essential for endocytosis. Nature 410:231–235

Mochida S, Nonomura Y, Kobayashi H (1994) Analysis of the mechanism for acetylcholine release at the synapse formed between rat sympathetic neurons in culture. Microsc Res Tech 29:94–102

Mochida S, Few AP, Scheuer T, Catterall WA (2008) Regulation of presynaptic $Ca_V2.1$ channels by Ca^{2+} sensor proteins mediates short-term synaptic plasticity. Neuron 57:210–216

Nakata T, Takemura R, Hirokawa N (1993) A novel member of the dynamin family of GTP-binding proteins is expressed specifically in the testis. J Cell Sci 105(Pt 1):1–5

Neher E (2010) What is rate-limiting during sustained synaptic activity: vesicle supply or the availability of release sites. Front Synaptic Neurosci 2:144

Newton AJ, Kirchhausen T, Murthy VN (2006) Inhibition of dynamin completely blocks compensatory synaptic vesicle endocytosis. Proc Natl Acad Sci U S A 103:17955–17960

Nicholson-Tomishima K, Ryan TA (2004) Kinetic efficiency of endocytosis at mammalian CNS synapses requires synaptotagmin I. Proc Natl Acad Sci U S A 101:16648–16652

Obar RA, Collins CA, Hammarback JA, Shpetner HS, Vallee RB (1990) Molecular cloning of the microtubule-associated mechanochemical enzyme dynamin reveals homology with a new family of GTP-binding proteins. Nature 347:256–261

Poskanzer KE, Marek KW, Sweeney ST, Davis GW (2003) Synaptotagmin I is necessary for compensatory synaptic vesicle endocytosis in vivo. Nature 426:559–563

Praefcke GJ, McMahon HT (2004) The dynamin superfamily: universal membrane tubulation and fission molecules? Nat Rev Mol Cell Biol 5:133–147

Pyott SJ, Rosenmund C (2002) The effects of temperature on vesicular supply and release in autaptic cultures of rat and mouse hippocampal neurons. J Physiol 539:523–535

Raimondi A, Ferguson SM, Lou X, Armbruster M, Paradise S, Giovedi S, Messa M, Kono N, Takasaki J, Cappello V, O'Toole E, Ryan TA, De Camilli P (2011) Overlapping role of dynamin isoforms in synaptic vesicle endocytosis. Neuron 70:1100–1114

Roos J, Kelly RB (1999) The endocytic machinery in nerve terminals surrounds sites of exocytosis. Curr Biol 9:1411–1414

Roux A, Uyhazi K, Frost A, De Camilli P (2006) GTP-dependent twisting of dynamin implicates constriction and tension in membrane fission. Nature 441:528–531

Royle SJ, Lagnado L (2003) Endocytosis at the synaptic terminal. J Physiol 553:345–355

Saheki Y, De Camilli P (2012) Synaptic vesicle endocytosis. Cold Spring Harb Perspect Biol 4: a005645

Shpetner HS, Vallee RB (1989) Identification of dynamin, a novel mechanochemical enzyme that mediates interactions between microtubules. Cell 59:421–432

Song BD, Leonard M, Schmid SL (2004) Dynamin GTPase domain mutants that differentially affect GTP binding, GTP hydrolysis, and clathrin-mediated endocytosis. J Biol Chem 279:40431–40436

Sontag JM, Fykse EM, Ushkaryov Y, Liu JP, Robinson PJ, Sudhof TC (1994) Differential expression and regulation of multiple dynamins. J Biol Chem 269:4547–4554

Sun JY, Wu XS, Wu LG (2002) Single and multiple vesicle fusion induce different rates of endocytosis at a central synapse. Nature 417:555–559

Sun T, Wu XS, Xu J, McNeil BD, Pang ZP, Yang W, Bai L, Qadri S, Molkentin JD, Yue DT, Wu LG (2010) The role of calcium/calmodulin-activated calcineurin in rapid and slow endocytosis at central synapses. J Neurosci 30:11838–11847

Takei K, McPherson PS, Schmid SL, De Camilli P (1995) Tubular membrane invaginations coated by dynamin rings are induced by GTP-γS in nerve terminals. Nature 374:186–190

Takei K, Slepnev VI, Haucke V, De Camilli P (1999) Functional partnership between amphiphysin and dynamin in clathrin-mediated endocytosis. Nat Cell Biol 1:33–39

Tanifuji S, Funakoshi-Tago M, Ueda F, Kasahara T, Mochida S (2013) Dynamin isoforms decode action potential firing for synaptic vesicle recycling. J Biol Chem 288:19050–19059

Watanabe H, Yamashita T, Saitoh N, Kiyonaka S, Iwamatsu A, Campbell KP, Mori Y, Takahashi T (2010) Involvement of Ca^{2+} channel synprint site in synaptic vesicle endocytosis. J Neurosci 30:655–660

Watanabe S, Rost BR, Camacho-Perez M, Davis MW, Sohl-Kielczynski B, Rosenmund C, Jorgensen EM (2013a) Ultrafast endocytosis at mouse hippocampal synapses. Nature 504:242–247

Watanabe S, Liu Q, Davis MW, Hollopeter G, Thomas N, Jorgensen NB, Jorgensen EM (2013b) Ultrafast endocytosis at *Caenorhabditis elegans* neuromuscular junctions. Elife 2:e00723

Wu LG (2004) Kinetic regulation of vesicle endocytosis at synapses. Trends Neurosci 27:548–554

Wu XS, McNeil BD, Xu J, Fan J, Xue L, Melicoff E, Adachi R, Bai L, Wu LG (2009) Ca^{2+} and calmodulin initiate all forms of endocytosis during depolarization at a nerve terminal. Nat Neurosci 12:1003–1010

Xu J, McNeil B, Wu W, Nees D, Bai L, Wu LG (2008) GTP-independent rapid and slow endocytosis at a central synapse. Nat Neurosci 11:45–53

Yamashita T (2012) Ca^{2+}-dependent regulation of synaptic vesicle endocytosis. Neurosci Res 73:1–7

Yamashita T, Hige T, Takahashi T (2005) Vesicle endocytosis requires dynamin-dependent GTP hydrolysis at a fast CNS synapse. Science 307:124–127

Yamashita T, Eguchi K, Saitoh N, von Gersdorff H, Takahashi T (2010) Developmental shift to a mechanism of synaptic vesicle endocytosis requiring nanodomain Ca^{2+}. Nat Neurosci 13:838–844

Yao CK, Lin YQ, Ly CV, Ohyama T, Haueter CM, Moiseenkova-Bell VY, Wensel TG, Bellen HJ (2009) A synaptic vesicle-associated Ca^{2+} channel promotes endocytosis and couples exocytosis to endocytosis. Cell 138:947–960

Zhang Q, Li Y, Tsien RW (2009) The dynamic control of kiss-and-run and vesicular reuse probed with single nanoparticles. Science 323:1448–1453

Zhu Y, Xu J, Heinemann SF (2009) Two pathways of synaptic vesicle retrieval revealed by single-vesicle imaging. Neuron 61:397–411

Part V
Synaptic Vesicle Refilling and Storage

Chapter 12
Transport of Amino Acid Neurotransmitters into Synaptic Vesicles

Shigeo Takamori

Abstract Fast chemical neurotransmission at the synapse is mediated by the release of neurotransmitters from synaptic vesicles (SVs) by exocytosis. In the mammalian central nervous system, the majority of neurons utilize amino acids such as glutamate, γ-aminobutyric acid (GABA), and glycine. Glutamate is the major excitatory neurotransmitter, whereas GABA and glycine are inhibitory. These amino acids are present at relatively high levels in the cytoplasm of presynaptic terminals and are accumulated into SVs for their exocytotic release. Over the past several decades, this essential process has been biochemically characterized and proteins responsible for neurotransmitter loading have been molecularly identified. Analysis of knockout animals has elucidated physiological significance of this process and moreover has deepened our understanding of glutamatergic and GABAergic neural circuits. However, the precise mechanism of the transport system remains largely unknown. In this chapter, I overview advances in the vesicular loading process and discuss some controversial concepts that may have important consequences for synaptic transmission.

Keywords Synaptic vesicles • Vesicular glutamate transporter • Vesicular GABA transporter • Vacuolar-type H^+ ATPase • Cl^- channels

12.1 V-ATPase Drives Neurotransmitter Uptake

Unlike plasma membrane neurotransmitter transporters that utilize the Na^+ gradient across the membrane as a driving force, transporters responsible for neurotransmitter refilling into SVs rely on a proton electrochemical gradient across the vesicle membrane, generated by the vacuolar-type H^+ ATPase (V-ATPase) (Moriyama et al. 1992). The V-ATPase consists of at least 13 subunits, comprising the largest protein complex (~800 kDa complex) present on SVs (Takamori et al. 2006) (see Chap. 7). It is divided into two functional domains: V1 is a peripheral sector and V0 is a membrane sector. The overall structure resembles the mitochondrial F_0F_1-ATP

S. Takamori (✉)
Laboratory of Neural Membrane Biology, Graduate School of Brain Science, Doshisha University, Kyoto, Japan
e-mail: stakamor@mail.doshisha.ac.jp

S. Mochida (ed.), *Presynaptic Terminals*, DOI 10.1007/978-4-431-55166-9_12

synthase which catalyzes ATP synthesis from ADP by using the H^+ gradient (Nelson et al. 2002). V-ATPase performs the exact opposite task. It uses the hydrolyzing energy of ATP and translocates H^+ from the cytoplasm into the vesicle lumen. As a result, the lumen becomes more acidic and the voltage more positive, both of which act to drive neurotransmitter transport into SVs. While the potential across the SV membrane has not been experimentally determined, the pH gradient across SV membrane has been estimated from vesicular pH measurements (Maycox et al. 1988; Moriyama and Nelson 1987). By using pH-sensitive GFP variants, which are targeted to the luminal region of SVs in neuronal culture preparations, vesicular pH was determined as 5.6–5.8 (Miesenbock et al. 1998). Assuming that the cytoplasmic pH is close to neutral (7.0–7.4), ΔpH was estimated to be approximately ~1.5 pH unit (~30-fold gradient). It should be noted that because of the small size of SVs (diameter of 40 nm), a pH of 5.5 ($[H^+] = 10^{-5.5}$) corresponds to less than one free H^+ in the lumen (Takamori et al. 2006). Although little is known about the intrinsic buffering of the vesicle lumen, this simple calculation indicates that few H^+ ions are required to establish the proton electrochemical gradient. This markedly contrasts to Na^+, or other ion gradients such as K^+ or Cl^-, across the plasma membrane. Moreover, in contrast to the relatively stable ion gradient of Na^+ and K^+ across the plasma membrane, the proton electrochemical gradient across the SV membrane is made and dissipated during repeated exocytosis and endocytosis. Given the continuous activity at synapses, it is probably more economical for SVs to utilize minor ion species such as H^+, which involves less ion movement than major ions such as Na^+.

12.2 The Regulation of $\Delta\mu H^+$

The proton electrochemical gradient ($\Delta\mu H^+$) generated by V-ATPase consists of an electrical gradient ($\Delta\Psi$, inside positive) and a chemical gradient (ΔpH, inside acidic) (Fig. 12.1). Their relative influence on the $\Delta\mu H^+$ can be modulated by other ion channels or transporters present on SVs. The extent of both components has been assessed in isolated SVs by fluorometric assays using either voltage-sensitive dyes (i.e., oxanol) for $\Delta\Psi$ or membrane-permeable weak-base dyes such as acridine orange for ΔpH (Maycox et al. 1988; Tabb et al. 1992). V-ATPase is an electrogenic pump; thus, in the absence of counterion movements, H^+ influx is restricted by the voltage developed as a result of H^+ entry, and thereby a large membrane potential ($\Delta\Psi$) is established. On the other hand, when counterions are present, their movement across the SV membrane compensates for charges associated with H^+ influx. While this facilitates greater H^+ influx into the lumen to establish a greater pH gradient (ΔpH), $\Delta\Psi$ is attenuated (Fig. 12.1a, b). The physiological counterion that affects $\Delta\mu H^+$ is chloride. It has been shown that $\Delta\Psi$ is maximal in the absence of Cl^- (and other haloids), while ΔpH is very small. As extravesicular Cl^- increases, $\Delta\Psi$ is attenuated, whereas a larger pH

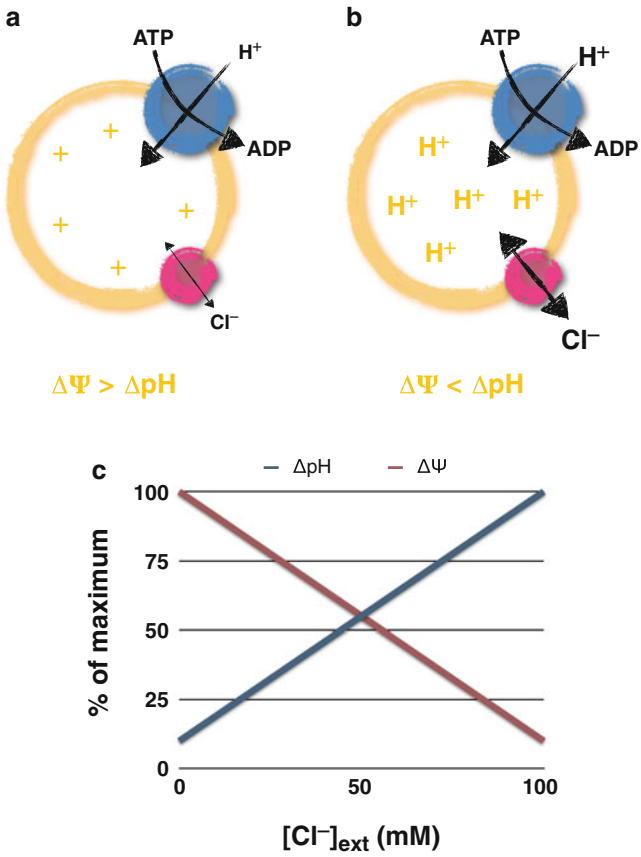

Fig. 12.1 Electrogenic property of the vacuolar-type H^+ ATPase. Vacuolar-type H^+ ATPase (V-ATPase) (*blue*) utilizes an energy produced by ATP hydrolysis and generates a proton electrochemical gradient that consists of two components, ΔΨ and ΔpH. Since the V-ATPase is electrogenic, H^+ influx is critically restricted by ΔΨ, and either accompanying anon influx or efflux of cation is necessary to facilitate proton transport into the lumen. SVs contain a putative Cl^- channel (*pink*) that affects the balance of ΔΨ and ΔpH. (**a**) In the presence of low Cl^-, H^+ influx is small and more ΔΨ is generated. (**b**) In the presence of high Cl^-, Cl^- influx would dissipate ΔΨ; therefore, more H^+ can flow into SVs. This facilitates ΔpH buildup and attenuates ΔΨ. (**c**) The relationship between ΔΨ and ΔpH as a function of external Cl^- concentrations. Both components have been monitored by using fluorescent indicators for the voltage and ΔpH

gradient is generated (Fig. 12.1c). The molecular identity of this Cl^- pathway has been elusive (discussed below).

Experimentally, it is possible to manipulate the two components of $\Delta\mu H^+$ in isolation. For instance, ammonia (NH_3) is a proton acceptor and can penetrate into the SV lumen where it attenuates ΔpH in a concentration-dependent manner (Hell et al. 1988). Likewise, the K^+/H^+ exchanger, nigericin, produces proton efflux in concert with K^+ influx, to selectively dissipate ΔpH yet facilitate ΔΨ (Tabb

et al. 1992). The Mg^{2+}/H^+ exchanger, A23187, has also been used as a substitute for nigericin (Wolosker et al. 1996). In contrast, the K^+ ionophore, valinomycin, eliminates the formation of $\Delta\Psi$ and therefore strongly augments ΔpH (Moriyama and Nelson 1987). Given that changes in the composition of $\Delta\mu H^+$ have been indirectly monitored, the absolute values of intravesicular pH and transmembrane voltage in any conditions are unknown. Yet, it is generally believed that for cationic neurotransmitters such as acetylcholine and monoamines, uptake depends largely on ΔpH. By contrast, the uptake of anionic neurotransmitter such as glutamate depends predominantly on $\Delta\Psi$. The zwitterionic transmitters GABA and glycine both depend on ΔpH and $\Delta\Psi$ for efficient uptake (Edwards 2007). As will be discussed in the following sections, the precise mechanism of how H^+ drives neurotransmitter transport is not completely understood.

12.3 Mechanisms of Neurotransmitter Uptake into Synaptic Vesicles

The biochemical mechanism underlying the uptake of amino acid neurotransmitters into SVs has been characterized using isolated SVs from mammalian brains. Glutamate uptake into SV preparations was shown to be dependent on ATP hydrolysis, because proton ionophores such as CCCP or FCCP abolished ATP-dependent glutamate accumulation, demonstrating that the process is dependent on a proton gradient (Naito and Ueda 1983, 1985). A prominent feature of glutamate uptake into SV preparations is an unusual biphasic dependence on chloride concentrations in the assay medium (Naito and Ueda 1985). Glutamate uptake activity was found to be maximal in the presence of ~mM of Cl^-, yet relatively depressed at lower or higher Cl^- concentrations. The extent of depression depended on glutamate concentrations used for the assays. In terms of the composition of $\Delta\mu H^+$, at low mM Cl^-, $\Delta\Psi$ predominates over ΔpH. Manipulations to selectively dissipate ΔpH, such as nigericin and NH_4^+ applications, had no overwhelming effect on glutamate uptake, whereas those that abolished $\Delta\Psi$ such as valinomycin strongly reduced it (Hell et al. 1988; Moriyama et al. 1990; Naito and Ueda 1985). Given that the majority of glutamate at neutral pH exists in the negatively charged form, these results led to a long-standing belief that glutamate uptake is predominantly dependent on $\Delta\Psi$ across the SV membrane. In fact, when $\Delta\Psi$ was artificially generated by VAL and Rb^+, significant glutamate transport was detected, albeit to a lesser extent (~50-fold or more) than that achieved by the activation of V-ATPase (Shioi and Ueda 1990). This led to the extreme hypothesis that $\Delta\Psi$ alone is sufficient to drive glutamate transport. Furthermore, the nonselective anion channel blocker, DIDS, was found to attenuate V-ATPase activity and glutamate transport with different IC_{50} values, leading to another hypothesis that Cl^- directly binds to the glutamate transporter and allosterically activates it (Hartinger and Jahn 1993). This theory was also able to explain why low

transport activity was observed in the absence of Cl^-. In this context, the attenuation of glutamate transport in high Cl^- conditions can be explained by the attenuation of $\Delta\Psi$ as Cl^- increases. On the other hand, there is substantial evidence to suggest the pH gradient is required for glutamate transport. First, 20 mM NH_4^+ dramatically reduced uptake, a concentration that is thought to completely abolish ΔpH yet facilitate $\Delta\Psi$ (Carlson et al. 1989). Second, application of either NH_4^+ or A23187 completely dissipated ΔpH, but effects on glutamate uptake were dependent on the Cl^- and glutamate concentrations used to measure glutamate transport activity. Both treatments facilitated glutamate transport at low Cl^- concentrations but attenuated it at higher Cl^- concentrations (Wolosker et al. 1996), indicating a complex regulation of glutamate transport by both Cl^- and $\Delta\mu H^+$. Third, when intravesicular pH was clamped with external solution by using nigericin, glutamate transport was sensitive to pH, reaching a maximum at pH ~6.8 and thus indicating that not only ΔpH but also optimal pH is required to drive glutamate transport (Tabb et al. 1992). These results suggest that pH is an essential factor for glutamate transport and optimal protonation of amino acid side chains of the transporter is crucial for its function. Investigations to clarify the mechanisms involved have largely used reconstitution systems (see below).

There has been some evidence that outward leak of neurotransmitter from the synaptic vesicle may affect transport kinetics (Wolosker et al. 1996). In vitro radiotracer flux assays have demonstrated that this dissipation of ΔpH promotes glutamate efflux. Furthermore, glutamate efflux was promoted by the application of Cl^- at relatively high concentrations similar to that attained at steady state following glutamate transport. Collectively, such evidence indicates that changes in intravesicular pH affect glutamate storage. Although the mechanism and pathway for this glutamate leak has not been established, these results suggest that the combination of glutamate efflux and influx determine the rate of glutamate transport. Despite these biochemical observations, whether $\Delta\mu H^+$ is essential for neurotransmitter maintenance in the SVs remains controversial. Application of the V-ATPase inhibitor, Bafilomycin A_1 ($BafA_1$), in hippocampal autaptic cultures resulted in activity-dependent attenuation of EPSCs supporting the idea that there is almost no glutamate leak from already filled SVs (Ikeda and Bekkers 2009). In contrast, glutamate transported into isolated SVs in vitro was found to be somewhat more leaky. Acidification of SVs by glutamate can be monitored by acridine orange, thus providing a proxy measure for glutamate transport into SVs (Maycox et al. 1988). Upon application of $BafA_1$ or FCCP, such acidification was rapidly reversed, indicating that glutamate as well as H^+ leaks out by disrupting $\Delta\mu H^+$ (Hnasko et al. 2010; Maycox et al. 1988). Consistent with these observations, glutamate leak was also observed in an assay of radio-labeled glutamate flux, although the rate and extent of the efflux therein seemed to be dependent on $BafA_1$ in a concentration-dependent manner (our unpublished observations). In addition to glutamate, proton leakage exhibited different properties both in vitro when monitored by acridine orange and also in vivo measured with a pH-sensitive luminal probe (i.e., pHluorin). As mentioned, $BafA_1$ rapidly reversed acridine orange quenching, while $BafA_1$ application did not dramatically alter the acidity

of the SV lumen, measured as fluorescent changes from pHluorin expressing SVs (Sankaranarayanan and Ryan 2001). Therefore, it is unclear if glutamate efflux, as well as H^+ leak, is relevant in physiological conditions.

In contrast to glutamate transport, transport of inhibitory neurotransmitters such as GABA and glycine into SVs is less well characterized. Due to the low affinity of the carrier (>5 mM) and the meager amount of GABA-containing SVs isolated from whole brain tissues, the signal-to-noise ratio of GABA/glycine uptake assays is much lower than that for glutamate (Burger et al. 1991; Takamori et al. 2000b). Nevertheless, GABA/glycine uptake also depends on a proton electrochemical gradient generated by the V-ATPase (Fykse et al. 1989; Hell et al. 1991; Kish et al. 1989). Selective dissipation of $\Delta\Psi$ and ΔpH revealed that GABA and glycine transport into SVs was moderately inhibited by both modifications. As with glutamate transport, the uptake of GABA and glycine was dependent on Cl^-, albeit to a lesser degree (Hell et al. 1990; Kish et al. 1989). Collectively, GABA and glycine transport has been proposed to be dependent equally on $\Delta\Psi$ and ΔpH. How these gradients drive GABA and glycine transport is uncertain, though a recent study that reconstituted vesicular GABA transporter protein has proposed a novel model in which GABA transport is driven solely by $\Delta\Psi$ and is stoichiometrically coupled to Cl^- transport (Juge et al. 2009) (see more details below in Sect. 12.6).

12.4 Molecular Identities of Vesicular Glutamate and GABA Transporters

12.4.1 VGLUTs

The vesicular glutamate transporters were originally cloned as Na^+-dependent inorganic phosphate transporters and categorized into the group of type I phosphate transporters (Takamori 2006). The gene encoding one of the phosphate transporter family members has been identified to be upregulated in cortical neuron preparations following exposure to sub-toxic levels of a glutamate receptor agonist. The gene was highly homologous to the founding member of the phosphate transporter family, NaPi-1, and highly expressed in the central nervous system. When mRNA of the gene was heterologously expressed in *Xenopus oocytes*, substantial Pi uptake was observed in a Na^+-dependent manner. This evidence led the gene responsible to be named brain-specific Na^+-dependent inorganic phosphate transporter 1 (BNP1). However, subsequent studies on BNP1 protein suggested it was localized to SVs rather than the plasma membrane (Bellocchio et al. 1998; Takamori et al. 2000a). Moreover, BNP1 was not ubiquitously expressed in all neurons in the CNS, but restricted to those containing glutamate (Bellocchio et al. 1998). When BNP1 was heterologously expressed in neuroendocrine cell lines, vesicles isolated from these cell lines were capable of transporting glutamate. Intriguingly, this process was akin to that observed in a biochemical transport assay of isolated SVs, suggesting

that BNP1 indeed functions as a vesicular glutamate transporter (Bellocchio et al. 2000; Takamori et al. 2000a). Notably, by expressing BNP1 in non-glutamatergic neurons in culture, the cells were subsequently capable of quantal glutamate release. Such data clearly supports a primary role of BNP1 for glutamate transport into SVs, and consequentially, BNP1 was renamed as vesicular glutamate transporter 1 (VGLUT1).

VGLUT1 expression was not detected in all brain regions where glutamatergic synaptic transmission is thought to occur. In areas such as the hypothalamus and striatum, the absence of VGLUT1 expression promoted the hypothesis that other VGLUT isoforms may be responsible for glutamate uptake. Indeed, two more VGLUT isoforms were identified and named: VGLUT2 and VGLUT3 (Takamori 2006 and references therein). The genes for VGLUT1, VGLUT2, and VGLUT3 are now categorized into the solute carrier protein (SLC17) family and coded as SLC17A7, SLC17A6, and SLC17A8, respectively. The amino acid sequences of VGLUT1~3 share nearly 80 % identities. Specifically, their transmembrane regions are almost identical to one another (>95 % identities), yet their N- and C-terminal cytoplasmic tails are highly variable, indicating that all three share a common mechanism in glutamate transport and diversities among VGLUTs may exist in their trafficking that requires distinct protein-protein interactions with cytosolic components. Indeed, the glutamate transport activity of VGLUT isoforms did not significantly vary in measurements from heterologous systems, whereas the subcellular distributions of individual VGLUTs in neuroendocrine cell lines were somewhat different. When VGLUT1 and VGLUT2 were expressed in PC12 cells, a greater quantity of VGLUT2 was localized at the cell surface compared to VGLUT1 (Fremeau et al. 2001). In addition, throughout the CNS, VGLUT1 and VGLUT2 are almost exclusively expressed at the presynaptic terminals of glutamatergic neurons, whereas VGLUT3 is expressed not only at presynaptic terminals but, in some interneurons, is also present at somatodendritic locations (Fremeau et al. 2002). Some of the key factors that determine cellular localization of VGLUTs will be discussed separately in Sect. 12.7.

In addition to the distinct subcellular localization of VGLUT isoforms within neurons, the three isoforms show complementary expression patterns in the mammalian CNS with very little overlap. In the adult brain, excitatory neurons in the cortex, hippocampus, and cerebellar cortex (granule cells) mainly express VGLUT1, whereas excitatory neurons in the thalamus and brainstem predominantly express VGLUT2 (Fremeau et al. 2001; Fujiyama et al. 2001). Consistent with their expression patterns, glutamatergic transmission in the hippocampus was largely shut down in VGLUT1 knockout mice (Fremeau et al. 2004; Wojcik et al. 2004), whereas in the thalamus, transmission was lost in VGLUT2 knockout mice (Moechars et al. 2006). Some residual glutamatergic transmission was observed in hippocampal preparations (both cultured hippocampal neurons and hippocampal slices) to suggest that VGLUT2 is transiently expressed during early postnatal development. Indeed, the developmental regulation of VGLUT1 and VGLUT2 expression occurs in many brain regions (Miyazaki et al. 2003; Nakamura et al. 2005). Overall, the expression of VGLUT1 is very low at birth

and increases during postnatal development up to 3 weeks, whereas the expression of VGLUT2 is detectable already at birth and stays relatively constant throughout postnatal development. Consistent with the low expression of VGLUT1 in early postnatal development, VGLUT1 knockout mice survive up to 3 weeks after birth without any apparent deficiency, suggesting its limited importance for glutamatergic transmission during development. VGLUT1 knockout mice begin to suffer from feeding and die, in most cases, during postnatal 4th week (Fremeau et al. 2004; Wojcik et al. 2004). In contrast, VGLUT2 knockout mice die immediately after birth presumably due to the lack of glutamatergic transmission in the brainstem involved in the generation of respiratory rhythm (Moechars et al. 2006; Wallen-Mackenzie et al. 2006). In contrast to the expression of VGLUT1 and VGLUT2, VGLUT3 is expressed in a small population of neurons not traditionally considered to be glutamatergic neurons, including cholinergic neurons in the striatum, a small set of GABAergic interneurons in the cortex and the hippocampus, and dopaminergic neurons in the raphe (Fremeau et al. 2002). VGLUT3 knockout mice exhibit non-convulsant seizures and deafness (Ruel et al. 2008; Seal et al. 2008). The latter is likely caused by a loss of glutamate release from inner hair cells in the cochlea where VGLUT3 is highly expressed (Akil et al. 2012). Furthermore, VGLUT3 knockout mice exhibited an unexpected hypercholinergic phenotype, suggesting that, together with the fact that VGLUT3 is coexpressed with vesicular acetylcholine transporter, glutamate transport by VGLUT3 affected the amount of acetylcholine in the same vesicles (Gras et al. 2008).

12.4.2 VGAT

Vesicular GABA transporter was identified as a rat orthologue of unc-47, one of the *C. elegans* genes responsible for the GABAergic transmission (McIntire et al. 1997; Sagne et al. 1997). In the worm, the 26 GABA-expressing neurons are required to inhibit contractions of the head muscles during foraging, to inhibit contractions of the body muscles during locomotion, and to stimulate contraction of the enteric muscles that mediate the defecation cycles. The unc-47 mutant showed impairments of all behaviors and, importantly, responded normally to GABA receptor agonists, indicating the role of unc-47 gene product at the presynaptic site. Furthermore, the unc-47 gene product was selectively localized to SVs. When a rat orthologue of unc-47 was transfected into PC12 cells, the isolated intracellular membrane acquired the ability to transport GABA, in a similar manner to that observed in isolated synaptic vesicles, adding weight to the idea that the unc-47 gene product functions as a vesicular GABA transporter (VGAT). Similar to synaptic vesicles, GABA uptake by VGAT was competitively inhibited by glycine, albeit with lower efficiency (Burger et al. 1991), supporting the notion that these inhibitory neurotransmitters in the CNS share a common transporter (thereby, VGAT is also referred to as vesicular inhibitory amino acid transporter, VIAAT) (Sagne et al. 1997). In fact, VGAT is not only localized at glutamic acid

dehydrogenase (GAD)-positive GABAergic terminals but also at putative glycinergic terminals (Chaudhry et al. 1998; Wang et al. 2009). Furthermore, loss of VGAT caused a drastic reduction of neurotransmitter release in both GABAergic and glycinergic neurons, further suggesting that GABA and glycine are transported by the same transporter (Wojcik et al. 2006). Recent studies revealed that the plasma membrane glycine transporters (GlyT2) ensured high levels of cytoplasmic glycine at glycinergic terminals (Rousseau et al. 2008), whereas the presence of GAD was associated with high levels of cytoplasmic GABA at GABAergic terminals. In this respect, although they share the same vesicular transporter to accumulate the respective neurotransmitters, the type of neurotransmitter is defined by the relative concentration of transmitter in the cytoplasm. Little is known about how VGAT is regulated and the subsequent effects on inhibitory neurotransmission. Although VGAT is constitutively phosphorylated in vivo, dephosphorylation of VGAT did not alter its transport activity (Bedet et al. 2000).

Inactivation of VGAT led to embryonic lethality as a result of an abdominal defect known as omphalocele (Saito et al. 2010; Wojcik et al. 2006), which was also observed in GAD67 knockout mice. Both effects indicate a crucial role for GABAergic transmission during prenatal development. As expected, the loss of VGAT caused a drastic reduction in both GABAergic and glycinergic neurotransmission. However, as a reduced number of IPSCs remained, it is possible that GABA release could have occurred independently of VGAT (Wojcik et al. 2006). Whether another VGAT isoform was responsible or the residual IPSCs were caused by non-vesicular GABA release remains uncertain.

12.5 Channels and Transporters: $\Delta\mu H^+$ Modulators?

Since vesicle refilling is a secondary active transport process, modulating the driving force would have profound consequences. Little is known on the regulation of V-ATPase function; however, it is thought that the proportion of ΔpH and $\Delta\Psi$ is affected by the permeability of counterions that accompany H^+ flux (Grabe and Oster 2001). As described above, one of the factors that potentially affect $\Delta\mu H^+$ is a chloride channel that supports vesicle acidification. From earlier evidence it was proposed that the ClC-3 chloride channel was partly responsible for the acidification of SVs (Stobrawa et al. 2001). This was supported by observations from crude SV fractions isolated from ClC-3 knockout mouse brains, which exhibited significantly lower Cl^--induced acidification. However, this conclusion was complicated by the fact that ClC-3 knockout mice underwent severe neuronal degeneration after 2 weeks, with complete loss of hippocampal structures in adulthood. Moreover, expression of VGLUT1, glutamate-induced acidification, and glutamate uptake were all reduced in the SV fraction. Thus, the alternative hypothesis is that the impairment of Cl^--induced SV acidification resulted from a reduction in VGLUT1-laden SVs, rather than the loss of ClC-3. Indeed, the amplitude of miniature EPSCs recorded from CA1 hippocampal pyramidal cells did not differ between wild-type

and ClC-3 knockout brains, further casting doubt that ClC-3 was responsible for SV acidification and, by association, Cl^--dependent glutamate uptake. This hypothesis was supported by subsequent evidence from ClC-3-deficient SVs isolated prior to the onset of neural degeneration, which showed normal Cl^--dependent acidification, glutamate uptake, and VGLUT1 expression (Schenck et al. 2009). In contrast, vesicles isolated from VGLUT1-overexpressing PC12 cells exhibited an enhanced Cl^--induced acidification, raising the possibility that VGLUT1 itself has a Cl^- conductance (Bellocchio et al. 2000). Indeed, SVs isolated from VGLUT1 knockout mice showed a marked reduction in Cl^--induced SV acidification, suggesting that VGLUT1 has a chloride conductance responsible for SV acidification (Schenck et al. 2009). However, this model has been recently challenged (Juge et al. 2010) (see below). In any case, the Cl^- influx into SVs has only been monitored indirectly using acridine dye measurements of Cl^--induced acidification. A direct demonstration of Cl^- transport via VGLUT1 will be necessary to clarify the model.

As with glutamate transport, a role for ClC-3 in GABA loading into SVs has been proposed (Riazanski et al. 2011). When miniature IPSCs in the hippocampal CA1 region of ClC-3 KO mice were analyzed, there was a clear reduction in both their amplitude and frequency. Furthermore, the SV fraction devoid of ClC-3 showed deficient Cl^--induced acidification as revealed by an acridine orange assay. Collectively, it was suggested that the additional acidification of GABA vesicles resulting from ClC-3 activity would facilitate proton-dependent GABA loading into SVs. As with glutamate, the role of CIC-3 in GABA uptake and its significance to synaptic transmission remains controversial (Stauber and Jentsch 2013).

The cation/H^+ exchange has been proposed to decrease ΔpH and thus increase $\Delta\Psi$. Such a process clearly diverges from anion conductance that would facilitate ΔpH. In an in vitro acidification assay, addition of Ca^{2+} reduced ΔpH once a stable baseline was established (Goncalves et al. 1999a, b). More recently, a cation/H^+ exchange mechanism was demonstrated in isolated SVs, and a Na^+/H^+ exchanger (NHE) detected in the SV proteome (Goh et al. 2011; Gronborg et al. 2010). NHE activity pushes out protons at the expense of Na^+ (or K^+) influx. As a result, ΔpH was decreased and $\Delta\Psi$ was facilitated, providing favorable conditions for glutamate uptake. Moreover, replacing cytoplasmic K^+ with a non-permeable cation ($NMDG^+$) attenuated quantal release of glutamate at the calyx of Held synapse, indicating the importance of cation/H^+ exchange for normal glutamate loading into SVs (Goh et al. 2011).

In addition to biochemical demonstrations of vesicular channels/transporters, it is possible to take electrophysiological recordings on SV membranes once they have been incorporated into liposomes or planner bilayers. From these preparations three cation conductances and one anion conductance were detected (Sato et al. 1992). Intriguingly, although not demonstrated in mammalian SVs, the ion conductances in the Torpedo electric organ SVs were sensitive to pH (Ahdut-Hacohen et al. 2004). Specifically, these channels were active at neutral pH and inactivated in acidic conditions. To date, the relationship between the ion conductances and indications from biochemical experiments remains enigmatic; furthermore, how these ion permeations translate into neurotransmitter loading is unknown.

12.6 Reconstitution: Simpler Is Better?

The cloning of VGAT and the three VGLUT isoforms has allowed for detailed investigation into their mechanism of action. By expressing the proteins in isolation, this system avoids complexities resulting from other SV proteins. For instance, their effect on $\Delta\mu H^+$ could be studied in the absence of potentially confounding influences from putative Cl^- channels and cation/H^+ exchanger proteins. The reconstitution of recombinant VGLUT1 protein with a F_0F_1-ATP synthase (which can be used as a proton pump) facilitated Cl^--induced acridine orange quenching in a dose-dependent manner (Schenck et al. 2009). This is consistent with the fact that overexpression and gene knockout of VGLUT1 resulted in facilitated and reduced Cl^--induced acidification, respectively (Bellocchio et al. 2000; Schenck et al. 2009), supporting that VGLUT1 has a Cl^- conductance. Having demonstrated that glutamate transport and the Cl^- conductance coexist in VGLUT1 proteins, it was subsequently shown that $\Delta\Psi$-driven (nigericin-resistant) glutamate uptake was markedly facilitated when liposomes were filled with high concentration of Cl^-. Thus, the Cl^- efflux pathway appears to be required for efficient glutamate influx by maintaining charge neutrality during transport cycle. In addition, VGLUT1 liposomes also exhibited a significant nigericin-sensitive uptake that depended on external Cl^- concentrations, indicating that VGLUT can also be driven by ΔpH. Remarkably, Cl^--filled VGLUT1 liposomes took up glutamate very efficiently even in the absence of external Cl^- and thus strongly opposed the previous hypothesis that Cl^- directly binds to the carrier and allosterically activates it (Hartinger and Jahn 1993). One intrinsic caveat of the experimental strategies of the study was that the composition of $\Delta\mu H^+$ could be affected when Cl^- was replaced with other anions such as gluconate. More recent reconstitution studies, however, have argued against several important conclusions summarized above. Juge and colleagues used a reconstitution system in which the proton pump was omitted (Juge et al. 2010). Instead, the authors created $\Delta\Psi$ by manipulating internal and external K^+ concentrations in the presence of the K^+ ionophore valinomycin. They demonstrated glutamate uptake occurred transiently and peaked within 2 min. Interestingly, glutamate transport elicited by the Val-induced K^+-diffusion potential was greatly reduced in the absence of external Cl^- and drastically increased by several mM of Cl^-, with a Hill coefficient of 3.3. Furthermore, when ΔpH was imposed by simply loading liposomes with a solution of pH 5.5 in the assay buffer (pH 7.0), there was no significant glutamate uptake. This led the authors to propose that VGLUT functions as a Cl^--activated glutamate uniporter whose driving force was solely provided by $\Delta\Psi$ (Fig. 12.2).

Juge and colleagues also investigated GABA transport by reconstituting VGAT into liposomes (Juge et al. 2009). Similar to glutamate transport by VGLUTs, GABA transport by VGAT also showed a biphasic dependency on external Cl^- when VGAT was co-reconstituted with a proton pump. However, they demonstrated that GABA uptake could be driven by $\Delta\Psi$ generated by the combination of K^+ gradient and valinomycin and, unexpectedly, stimulated by the presence of

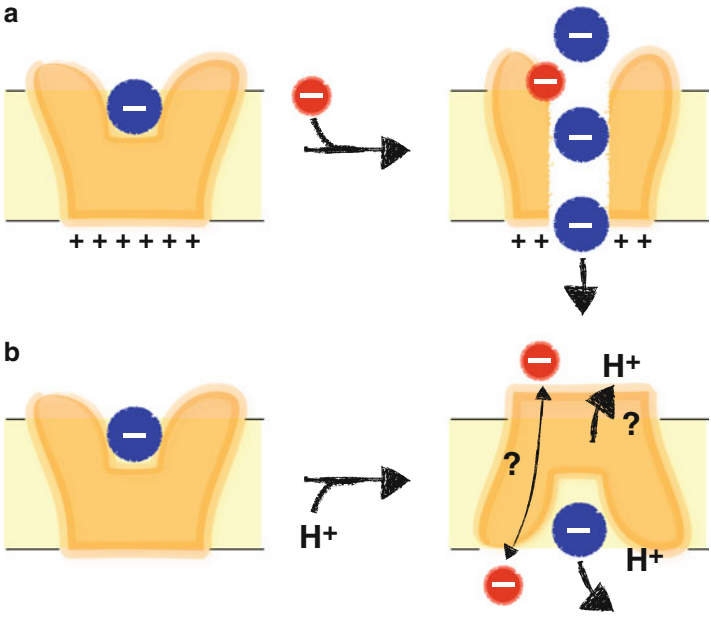

Fig. 12.2 Models for glutamate transport by VGLUT. (**a**) ΔΨ-driven transport. In this model, ΔΨ solely drives glutamate transport. Negatively charged glutamate binds to VGLUT, but is not transported efficiently even when there is ΔΨ. Cl^- directly binds to VGLUT and promotes conformational change of VGLUT protein. Glutamate is transported electrophoretically as if VGLUT is a Cl^--activated glutamate uniporter. (**b**) ΔpH-driven transport. In outward-facing conformation, glutamate binds to the substrate-binding site that can shift VGLUT to inward-facing conformation. Protonation of the luminal side of VGLUT causes a release of bound glutamate released from VGLUT. Protons that are once bound to VGLUT are then released either the same side (glutamate symport) or the other side (glutamate/H^+ antiport) of VGLUT protein. VGLUT protein has a putative Cl^- permeation pathway which may help to facilitate ΔpH or to compensate charge movement associated with glutamate influx. *Blue balls* and *red balls* indicate glutamate and Cl^-, respectively

external Cl^-. Whereas Cl^- was proposed to directly activate VGLUTs, Cl^- was shown to be cotransported with GABA or glycine via VGAT with a GABA-Cl^- stoichiometry of 1:2. This led the authors to a compelling hypothesis that VGAT is a Cl^-/GABA cotransporter. Although the liposomes contained only VGAT protein and therefore represented the simplest assay system to monitor VGAT activity, it is not easy to reconcile all the biochemical data that clearly indicated ΔpH-driven GABA uptake into isolated SVs (Fykse et al. 1989; Hell et al. 1991; Kish et al. 1989) (Fig. 12.3). One possible drawback of the aforementioned reconstitution experiments is their use of high acetate concentrations as a substitute anion for Cl^-. The protonated form of acetate (CH_3COOH) is to some extent membrane permeable and can release H^+ when deprotonated. Careful consideration of the effects of acetate on pH is required to exclude the involvement of ΔpH on GABA transport. Furthermore, although the authors attempted to establish ΔpH by preloading a low acidic buffer into liposomes, number of free protons in a limited space, especially if

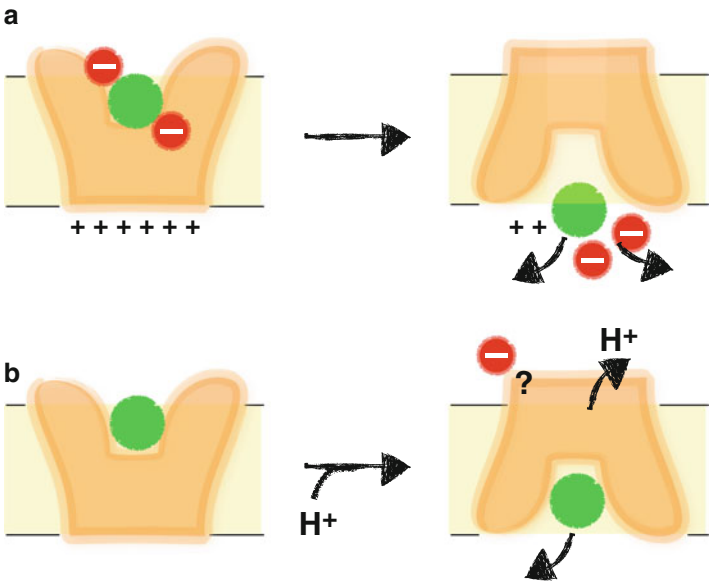

Fig. 12.3 Models for GABA transport by VGAT. (**a**) ΔΨ-driven transport. In this model, ΔΨ solely drives GABA transport. Unlike the ΔΨ-driven glutamate transport, GABA transport is coupled to Cl^- transport with a stoichiometry of GABA-Cl^- = 1:2. As such, GABA transport involves two negative charge movements, which would consume ΔΨ and facilitate ΔpH formation. (**b**) ΔpH-driven transport. A line of evidence suggested that ΔpH can drive GABA transport via GABA/H^+ antiport mechanism. Although Cl^- affects the efficacy of GABA transport, the role of Cl^- is largely unknown. *Green balls* and *red balls* indicate GABA and Cl^-, respectively

protons were coupled to substrate transport by an antiport mechanism, the reaction would have stopped immediately as the efflux of a single proton would evoke massive alkalization of the liposome lumen.

In summary, despite recent advances provided by the reconstitution of vesicular transporters, the exact mechanisms and bioenergetics of the transport processes are largely unknown. To improve understanding, experimental conditions require further refinement, including methods to produce proteoliposomes, buffer compositions, and a quantitative assay for the proton electrochemical gradient. Additionally, if pH plays a role in transport process, structural information of the transporters at various pH will open a new avenue for experimental design.

12.7 Regulations of Synaptic Vesicle Refilling and Quantal Size

Since postsynaptic receptors at the CNS synapses are not always saturated by neurotransmitters released from a single SV, alterations in the amount of neurotransmitter in a SV may potentially influence the efficacy of synaptic transmission.

Heterologous expression of VGLUT1 in hippocampal neurons increased both the amplitude and frequency of miniature EPSCs, suggesting that SVs with higher VGLUT1 expression can accumulate more glutamate (Wilson et al. 2005; Wojcik et al. 2004). However, increased expression of the transporter would in theory affect the kinetics of vesicle refilling rather than steady-state level of neurotransmitters (Edwards 2007). One explanation is that increased expression of VGLUT1 resulted in an enlargement of vesicles, leading to greater glutamate accumulation without changing its concentration. In fact, overexpression of *Drosophila* VGLUT (DVGLUT) in the neuromuscular junction of larvae increased the postsynaptic response in parallel with vesicle volume (Daniels et al. 2004). Yet, gradual reductions in DVGLUT expression did not alter mEPSP amplitude, but did produce a decrease in their frequency, indicating that only a single copy of DVGLUT was sufficient to fill SVs with glutamate to the normal level (Daniels et al. 2006). In this context, it should be noted that there were no detectable differences in mEPSCs in VGLUT1 heterozygous mice (Fremeau et al. 2004; Wojcik et al. 2004), supporting the idea that the expression of VGLUTs is not related to steady-state glutamate content. However, as VGLUT heterozygous mice exhibited a number of behavioral phenotypes, including clonic seizures, increased fear, and depression (Leo et al. 2009; Schallier et al. 2009; Tordera et al. 2007), the possibility remains that a reduction in VGLUT expression influences synaptic transmission.

Another parameter that might affect quantal size is concentration of neurotransmitter in the cytoplasm. Evidence from a radiotracer assay of isolated SVs revealed that increasing substrate concentration resulted in greater substrate accumulation such that transport kinetics could be adequately described by a Michaelis-Menten equation (Wilson et al. 2005; Wolosker et al. 1996). Furthermore, when the concentration of cytosolic glutamate was increased at the calyx of Held, glutamate content was also increased, demonstrated by an enhancement in EPSC amplitude (Ishikawa et al. 2002). Also at the calyx of Held, vesicle refilling rates were measured by flash photolysis of caged glutamate following washout of cytoplasmic glutamate (Hori and Takahashi 2012). The time constant of glutamate refilling was approximately 15 s, much faster than previous estimated from biochemical assays. As observed for glutamate uptake in isolated SVs, neurotransmitter-refilling rate at the synapse was also affected by cytoplasmic Cl^- concentrations, albeit to a lesser extent.

12.8 Trafficking of VGLUTs and VGAT to and Within Presynaptic Terminals

The expression of vesicular transporters on SVs can be regulated either at the point of biosynthesis in the soma or by factors that modulate their sorting and/or targeting to SVs. Given their crucial role in neurotransmitter release, the efficacy of their trafficking to required destinations may have profound physiological implications.

Although little is known concerning how VGLUTs and VGAT are conveyed from the soma to locations throughout the endoplasmic reticulum (ER)-Golgi network, VGLUT3 appears to utilize a unique sorting mechanism. Unlike VGLUT1, VGLUT2, and VGAT, which all preferentially localize on SVs at presynaptic sites, VGLUT3 also localizes at dendrites in multiple brain regions including the striatum, where it may promote retrograde glutamate signaling from the postsynaptic cells (Fremeau et al. 2002). Molecular mechanisms underlying this difference in subcellular localization are totally unexplored to date. At presynaptic sites, the ratio of the transporters on SVs and at the plasma membrane can be differentially regulated among VGLUT isoforms. When heterologously expressed in PC12 cells, VGLUT1 is targeted to secretory vesicles more efficiently than VGLUT2 (Fremeau et al. 2001). These vesicular transporters transiently remain within the plasma membrane upon exocytosis. Thus, the mechanism by which the endocytic machinery recognizes and initiates the incorporation of the transporters into newly endocytosed vesicles would determine their distributions at the presynaptic terminals. Interestingly, the kinetics of VGLUT1 recycling during and after stimulation is faster than that of VGLUT2 (Foss et al. 2013). How the recycling kinetics are controlled molecularly has been investigated through mutagenesis experiments. Essentially, both typical and atypical dileucine-like endocytic motifs in VGLUTs and VGAT moieties control transporter trafficking, albeit to different degrees (Foss et al. 2013; Santos et al. 2013). All VGLUT isoforms contain a dileucine motif at their C-terminus and disruption of this motif differentially affects distribution of VGLUT1 and VGLUT2 (Foss et al. 2013; Voglmaier et al. 2006). In case of VGLUT1, disruption of the motif slows down its activity-dependent recycling, although it does not entirely eliminate its endocytosis. In contrast, the disruption of the motif in VGLUT2 resulted in a dramatic redistribution of VGLUT2 proteins on the cell surface, suggesting that the reliance on the motif for effective recycling differs between VGLUT isoforms. Indeed, VGLUT1 contains two additional dileucine-like motifs at its N-terminus, which may also contribute to its endocytosis. Disruption of all three motifs resulted in limited accumulation of VGLUT1 at synaptic sites and impairment of its activity-dependent recycling, indicating that N-terminus dileucine motifs cooperatively function to sort VGLUT1 to presynaptic sites and, along with the C-terminal motif, promote its recycling. In contrast, when the N-terminus of VGLUT1 was mutated, no abnormalities in SV recycling were reported, suggesting these three motifs are functionally redundant. Yet, differences in surface expression of N-term and C-term mutants indicate that the motifs do have distinct and probably complex mechanisms that underlie transporter targeting and recycling. It has just emerged that VGAT also contains an atypical dileucine-like motif in its N-terminus, which is similarly required for its distribution at presynaptic sites, localization on SVs, and activity-dependent recycling (Santos et al. 2013). The question remains as to how these dileucine motifs dictate trafficking and recycling of vesicular transporter proteins, while other SV proteins can be correctly targeted in the absence of these structures.

While the general principle of vesicle targeting remains enigmatic, features of the specific trafficking mechanism for VGLUT1 have been determined. VGLUT1

has unique proline-rich domains (PRDs) at the C-terminus that interact with endophilin A family proteins at their SH3-domain. Disruption of this interaction had a subtle effect on the steady-state distribution of VGLUT1, but slowed its retrieval from the plasma membrane during high-frequency stimulations (Voglmaier et al. 2006). Furthermore, this interaction may confer lower release probability of VGLUT1-laden SVs compared to that of VGLUT2-laden SVs (Weston et al. 2011).

12.9 Concluding Remarks

Over the past several decades, we have learned much about the molecular basis of neurotransmitter refilling into SVs. Identification of vesicular transporters for glutamate and GABA has provided molecular probes suitable for studying the anatomy of neurotransmitter-specific neural circuits in the mammalian CNS. In addition, the ability to inactivate individual transporters in vivo has allowed researchers to impair synaptic transmission from specific neural populations, thereby greatly enhancing knowledge of how certain glutamatergic and GABAergic circuits contribute to brain function. However, much remains to be solved regarding precise mechanisms of how the transporters work and whether this process is limiting for synaptic physiology and pathology.

References

Ahdut-Hacohen R, Duridanova D, Meiri H, Rahamimoff R (2004) Hydrogen ions control synaptic vesicle ion channel activity in Torpedo electromotor neurones. J Physiol 556:347–352

Akil O, Seal RP, Burke K, Wang C, Alemi A, During M, Edwards RH, Lustig LR (2012) Restoration of hearing in the VGLUT3 knockout mouse using virally mediated gene therapy. Neuron 75:283–293

Bedet C, Isambert MF, Henry JP, Gasnier B (2000) Constitutive phosphorylation of the vesicular inhibitory amino acid transporter in rat central nervous system. J Neurochem 75:1654–1663

Bellocchio EE, Hu H, Pohorille A, Chan J, Pickel VM, Edwards RH (1998) The localization of the brain-specific inorganic phosphate transporter suggests a specific presynaptic role in glutamatergic transmission. J Neurosci 18:8648–8659

Bellocchio EE, Reimer RJ, Fremeau RT Jr, Edwards RH (2000) Uptake of glutamate into synaptic vesicles by an inorganic phosphate transporter. Science 289:957–960

Burger PM, Hell J, Mehl E, Krasel C, Lottspeich F, Jahn R (1991) GABA and glycine in synaptic vesicles: storage and transport characteristics. Neuron 7:287–293

Carlson MD, Kish PE, Ueda T (1989) Characterization of the solubilized and reconstituted ATP-dependent vesicular glutamate uptake system. J Biol Chem 264:7369–7376

Chaudhry FA, Reimer RJ, Bellocchio EE, Danbolt NC, Osen KK, Edwards RH, Storm-Mathisen J (1998) The vesicular GABA transporter, VGAT, localizes to synaptic vesicles in sets of glycinergic as well as GABAergic neurons. J Neurosci 18:9733–9750

Daniels RW, Collins CA, Gelfand MV, Dant J, Brooks ES, Krantz DE, DiAntonio A (2004) Increased expression of the Drosophila vesicular glutamate transporter leads to excess glutamate release and a compensatory decrease in quantal content. J Neurosci 24:10466–10474

Daniels RW, Collins CA, Chen K, Gelfand MV, Featherstone DE, DiAntonio A (2006) A single vesicular glutamate transporter is sufficient to fill a synaptic vesicle. Neuron 49:11–16

Edwards RH (2007) The neurotransmitter cycle and quantal size. Neuron 55:835–858

Foss SM, Li H, Santos MS, Edwards RH, Voglmaier SM (2013) Multiple dileucine-like motifs direct VGLUT1 trafficking. J Neurosci 33:10647–10660

Fremeau RT Jr, Troyer MD, Pahner I, Nygaard GO, Tran CH, Reimer RJ, Bellocchio EE, Fortin D, Storm-Mathisen J, Edwards RH (2001) The expression of vesicular glutamate transporters defines two classes of excitatory synapse. Neuron 31:247–260

Fremeau RT Jr, Burman J, Qureshi T, Tran CH, Proctor J, Johnson J, Zhang H, Sulzer D, Copenhagen DR, Storm-Mathisen J et al (2002) The identification of vesicular glutamate transporter 3 suggests novel modes of signaling by glutamate. Proc Natl Acad Sci U S A 99:14488–14493

Fremeau RT Jr, Kam K, Qureshi T, Johnson J, Copenhagen DR, Storm-Mathisen J, Chaudhry FA, Nicoll RA, Edwards RH (2004) Vesicular glutamate transporters 1 and 2 target to functionally distinct synaptic release sites. Science 304:1815–1819

Fujiyama F, Furuta T, Kaneko T (2001) Immunocytochemical localization of candidates for vesicular glutamate transporters in the rat cerebral cortex. J Comp Neurol 435:379–387

Fykse EM, Christensen H, Fonnum F (1989) Comparison of the properties of gamma-aminobutyric acid and L-glutamate uptake into synaptic vesicles isolated from rat brain. J Neurochem 52:946–951

Goh GY, Huang H, Ullman J, Borre L, Hnasko TS, Trussell LO, Edwards RH (2011) Presynaptic regulation of quantal size: K+/H + exchange stimulates vesicular glutamate transport. Nat Neurosci 14:1285–1292

Goncalves PP, Meireles SM, Neves P, Vale MG (1999a) Ionic selectivity of the Ca2+/H+ antiport in synaptic vesicles of sheep brain cortex. Brain Res Mol Brain Res 67:283–291

Goncalves PP, Meireles SM, Neves P, Vale MG (1999b) Synaptic vesicle Ca2+/H+ antiport: dependence on the proton electrochemical gradient. Brain Res Mol Brain Res 71:178–184

Grabe M, Oster G (2001) Regulation of organelle acidity. J Gen Physiol 117:329–344

Gras C, Amilhon B, Lepicard EM, Poirel O, Vinatier J, Herbin M, Dumas S, Tzavara ET, Wade MR, Nomikos GG et al (2008) The vesicular glutamate transporter VGLUT3 synergizes striatal acetylcholine tone. Nat Neurosci 11:292–300

Gronborg M, Pavlos NJ, Brunk I, Chua JJ, Munster-Wandowski A, Riedel D, Ahnert-Hilger G, Urlaub H, Jahn R (2010) Quantitative comparison of glutamatergic and GABAergic synaptic vesicles unveils selectivity for few proteins including MAL2, a novel synaptic vesicle protein. J Neurosci 30:2–12

Hartinger J, Jahn R (1993) An anion binding site that regulates the glutamate transporter of synaptic vesicles. J Biol Chem 268:23122–23127

Hell JW, Maycox PR, Stadler H, Jahn R (1988) Uptake of GABA by rat brain synaptic vesicles isolated by a new procedure. EMBO J 7:3023–3029

Hell JW, Maycox PR, Jahn R (1990) Energy dependence and functional reconstitution of the gamma-aminobutyric acid carrier from synaptic vesicles. J Biol Chem 265:2111–2117

Hell JW, Edelmann L, Hartinger J, Jahn R (1991) Functional reconstitution of the gamma-aminobutyric acid transporter from synaptic vesicles using artificial ion gradients. Biochemistry 30:11795–11800

Hnasko TS, Chuhma N, Zhang H, Goh GY, Sulzer D, Palmiter RD, Rayport S, Edwards RH (2010) Vesicular glutamate transport promotes dopamine storage and glutamate corelease in vivo. Neuron 65:643–656

Hori T, Takahashi T (2012) Kinetics of synaptic vesicle refilling with neurotransmitter glutamate. Neuron 76:511–517

Ikeda K, Bekkers JM (2009) Counting the number of releasable synaptic vesicles in a presynaptic terminal. Proc Natl Acad Sci U S A 106:2945–2950

Ishikawa T, Sahara Y, Takahashi T (2002) A single packet of transmitter does not saturate postsynaptic glutamate receptors. Neuron 34:613–621

Juge N, Muroyama A, Hiasa M, Omote H, Moriyama Y (2009) Vesicular inhibitory amino acid transporter is a Cl-/gamma-aminobutyrate Co-transporter. J Biol Chem 284:35073–35078

Juge N, Gray JA, Omote H, Miyaji T, Inoue T, Hara C, Uneyama H, Edwards RH, Nicoll RA, Moriyama Y (2010) Metabolic control of vesicular glutamate transport and release. Neuron 68:99–112

Kish PE, Fischer-Bovenkerk C, Ueda T (1989) Active transport of gamma-aminobutyric acid and glycine into synaptic vesicles. Proc Natl Acad Sci U S A 86:3877–3881

Leo S, Moechars D, Callaerts-Vegh Z, D'Hooge R, Meert T (2009) Impairment of VGLUT2 but not VGLUT1 signaling reduces neuropathy-induced hypersensitivity. Eur J Pain 13:1008–1017

Maycox PR, Deckwerth T, Hell JW, Jahn R (1988) Glutamate uptake by brain synaptic vesicles. Energy dependence of transport and functional reconstitution in proteoliposomes. J Biol Chem 263:15423–15428

McIntire SL, Reimer RJ, Schuske K, Edwards RH, Jorgensen EM (1997) Identification and characterization of the vesicular GABA transporter. Nature 389:870–876

Miesenbock G, De Angelis DA, Rothman JE (1998) Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. Nature 394:192–195

Miyazaki T, Fukaya M, Shimizu H, Watanabe M (2003) Subtype switching of vesicular glutamate transporters at parallel fibre-Purkinje cell synapses in developing mouse cerebellum. Eur J Neurosci 17:2563–2572

Moechars D, Weston MC, Leo S, Callaerts-Vegh Z, Goris I, Daneels G, Buist A, Cik M, van der Spek P, Kass S et al (2006) Vesicular glutamate transporter VGLUT2 expression levels control quantal size and neuropathic pain. J Neurosci 26:12055–12066

Moriyama Y, Nelson N (1987) The purified ATPase from chromaffin granule membranes is an anion-dependent proton pump. J Biol Chem 262:9175–9180

Moriyama Y, Maeda M, Futai M (1990) Energy coupling of L-glutamate transport and vacuolar H (+)-ATPase in brain synaptic vesicles. J Biochem 108:689–693

Moriyama Y, Maeda M, Futai M (1992) The role of V-ATPase in neuronal and endocrine systems. J Exp Biol 172:171–178

Naito S, Ueda T (1983) Adenosine triphosphate-dependent uptake of glutamate into protein I-associated synaptic vesicles. J Biol Chem 258:696–699

Naito S, Ueda T (1985) Characterization of glutamate uptake into synaptic vesicles. J Neurochem 44:99–109

Nakamura K, Hioki H, Fujiyama F, Kaneko T (2005) Postnatal changes of vesicular glutamate transporter (VGluT)1 and VGluT2 immunoreactivities and their colocalization in the mouse forebrain. J Comp Neurol 492:263–288

Nelson N, Sacher A, Nelson H (2002) The significance of molecular slips in transport systems. Nat Rev Mol Cell Biol 3:876–881

Riazanski V, Deriy LV, Shevchenko PD, Le B, Gomez EA, Nelson DJ (2011) Presynaptic CLC-3 determines quantal size of inhibitory transmission in the hippocampus. Nat Neurosci 14:487–494

Rousseau F, Aubrey KR, Supplisson S (2008) The glycine transporter GlyT2 controls the dynamics of synaptic vesicle refilling in inhibitory spinal cord neurons. J Neurosci 28:9755–9768

Ruel J, Emery S, Nouvian R, Bersot T, Amilhon B, Van Rybroek JM, Rebillard G, Lenoir M, Eybalin M, Delprat B et al (2008) Impairment of SLC17A8 encoding vesicular glutamate transporter-3, VGLUT3, underlies nonsyndromic deafness DFNA25 and inner hair cell dysfunction in null mice. Am J Hum Genet 83:278–292

Sagne C, El Mestikawy S, Isambert MF, Hamon M, Henry JP, Giros B, Gasnier B (1997) Cloning of a functional vesicular GABA and glycine transporter by screening of genome databases. FEBS Lett 417:177–183

Saito K, Kakizaki T, Hayashi R, Nishimaru H, Furukawa T, Nakazato Y, Takamori S, Ebihara S, Uematsu M, Mishina M et al (2010) The physiological roles of vesicular GABA transporter during embryonic development: a study using knockout mice. Mol Brain 3:40

Sankaranarayanan S, Ryan TA (2001) Calcium accelerates endocytosis of vSNAREs at hippocampal synapses. Nat Neurosci 4:129–136

Santos MS, Park CK, Foss SM, Li H, Voglmaier SM (2013) Sorting of the vesicular GABA transporter to functional vesicle pools by an atypical dileucine-like motif. J Neurosci 33:10634–10646

Sato M, Inoue K, Kasai M (1992) Ion channels on synaptic vesicle membranes studied by planar lipid bilayer method. Biophys J 63:1500–1505

Schallier A, Massie A, Loyens E, Moechars D, Drinkenburg W, Michotte Y, Smolders I (2009) vGLUT2 heterozygous mice show more susceptibility to clonic seizures induced by pentylenetetrazol. Neurochem Int 55:41–44

Schenck S, Wojcik SM, Brose N, Takamori S (2009) A chloride conductance in VGLUT1 underlies maximal glutamate loading into synaptic vesicles. Nat Neurosci 12:156–162

Seal RP, Akil O, Yi E, Weber CM, Grant L, Yoo J, Clause A, Kandler K, Noebels JL, Glowatzki E et al (2008) Sensorineural deafness and seizures in mice lacking vesicular glutamate transporter 3. Neuron 57:263–275

Shioi J, Ueda T (1990) Artificially imposed electrical potentials drive L-glutamate uptake into synaptic vesicles of bovine cerebral cortex. Biochem J 267:63–68

Stauber T, Jentsch TJ (2013) Chloride in vesicular trafficking and function. Annu Rev Physiol 75:453–477

Stobrawa SM, Breiderhoff T, Takamori S, Engel D, Schweizer M, Zdebik AA, Bosl MR, Ruether K, Jahn H, Draguhn A et al (2001) Disruption of ClC-3, a chloride channel expressed on synaptic vesicles, leads to a loss of the hippocampus. Neuron 29:185–196

Tabb JS, Kish PE, Van Dyke R, Ueda T (1992) Glutamate transport into synaptic vesicles. Roles of membrane potential, pH gradient, and intravesicular pH. J Biol Chem 267:15412–15418

Takamori S (2006) VGLUTs: 'exciting' times for glutamatergic research? Neurosci Res 55:343–351

Takamori S, Rhee JS, Rosenmund C, Jahn R (2000a) Identification of a vesicular glutamate transporter that defines a glutamatergic phenotype in neurons. Nature 407:189–194

Takamori S, Riedel D, Jahn R (2000b) Immunoisolation of GABA-specific synaptic vesicles defines a functionally distinct subset of synaptic vesicles. J Neurosci 20:4904–4911

Takamori S, Holt M, Stenius K, Lemke EA, Gronborg M, Riedel D, Urlaub H, Schenck S, Brugger B, Ringler P et al (2006) Molecular anatomy of a trafficking organelle. Cell 127:831–846

Tordera RM, Totterdell S, Wojcik SM, Brose N, Elizalde N, Lasheras B, Del Rio J (2007) Enhanced anxiety, depressive-like behaviour and impaired recognition memory in mice with reduced expression of the vesicular glutamate transporter 1 (VGLUT1). Eur J Neurosci 25:281–290

Voglmaier SM, Kam K, Yang H, Fortin DL, Hua Z, Nicoll RA, Edwards RH (2006) Distinct endocytic pathways control the rate and extent of synaptic vesicle protein recycling. Neuron 51:71–84

Wallen-Mackenzie A, Gezelius H, Thoby-Brisson M, Nygard A, Enjin A, Fujiyama F, Fortin G, Kullander K (2006) Vesicular glutamate transporter 2 is required for central respiratory rhythm generation but not for locomotor central pattern generation. J Neurosci 26:12294–12307

Wang Y, Kakizaki T, Sakagami H, Saito K, Ebihara S, Kato M, Hirabayashi M, Saito Y, Furuya N, Yanagawa Y (2009) Fluorescent labeling of both GABAergic and glycinergic neurons in vesicular GABA transporter (VGAT)-venus transgenic mouse. Neuroscience 164:1031–1043

Weston MC, Nehring RB, Wojcik SM, Rosenmund C (2011) Interplay between VGLUT isoforms and endophilin A1 regulates neurotransmitter release and short-term plasticity. Neuron 69:1147–1159

Wilson NR, Kang J, Hueske EV, Leung T, Varoqui H, Murnick JG, Erickson JD, Liu G (2005) Presynaptic regulation of quantal size by the vesicular glutamate transporter VGLUT1. J Neurosci 25:6221–6234

Wojcik SM, Rhee JS, Herzog E, Sigler A, Jahn R, Takamori S, Brose N, Rosenmund C (2004) An essential role for vesicular glutamate transporter 1 (VGLUT1) in postnatal development and control of quantal size. Proc Natl Acad Sci U S A 101:7158–7163

Wojcik SM, Katsurabayashi S, Guillemin I, Friauf E, Rosenmund C, Brose N, Rhee JS (2006) A shared vesicular carrier allows synaptic corelease of GABA and glycine. Neuron 50:575–587

Wolosker H, de Souza DO, de Meis L (1996) Regulation of glutamate transport into synaptic vesicles by chloride and proton gradient. J Biol Chem 271:11726–11731

Chapter 13
Synapsins and Synaptic Vesicle Storage

Fabrizia C. Guarnieri, Fabio Benfenati, and Flavia Valtorta

Abstract The synapsins constitute a family of evolutionarily conserved neuronal phosphoproteins associated with the cytosolic surface of synaptic vesicles. In mammals, the family comprises three members encoded by distinct genes that give rise to various splicing isoforms. In the central nervous system, the vast majority of neurons expresses at least one synapsin isoform. However, the functions of these proteins are not fully understood to date. Given their ability to bind both the vesicular membrane and actin filaments in a phosphorylation-dependent manner, the classical role attributed to synapsins is the reversible anchorage of synaptic vesicles to the cytoskeletal matrix present in the presynaptic terminal. However, recent evidences suggest the implication of synapsins in other aspects of the synaptic vesicle life cycle, such as docking, fusion and recycling. Genetic manipulation of synapsins in various in vitro and in vivo models has proved that they are dispensable for the proper development of functional neuronal networks but are essential modulators of synaptic neurotransmission and play differential roles at excitatory and inhibitory synapses. Indeed, mice lacking synapsins are viable and do not display gross brain abnormalities but exhibit generalised epileptic seizures as well as autism-related behavioural abnormalities. Consistently, several mutations have been identified in *SYN1* and *SYN2* genes in patients affected by epilepsy and/or autism spectrum disorders.

This chapter overviews the current knowledge about synapsin structure and function in the modulation of synaptic vesicle release, as well as the mechanisms leading to synaptic pathology when their properties are altered.

Keywords Synapsins • Synaptic vesicles • Synaptic transmission • Epilepsy • Autism

F.C. Guarnieri • F. Valtorta (✉)
Vita-Salute San Raffaele University and Division of Neuroscience, San Raffaele Scientific Institute, Via Olgettina 58, 20132 Milan, Italy
e-mail: valtorta.flavia@hsr.it

F. Benfenati
Department of Neuroscience and Brain Technologies, The Italian Institute of Technology and Department of Experimental Medicine, University of Genoa, Genoa, Italy

S. Mochida (ed.), *Presynaptic Terminals*, DOI 10.1007/978-4-431-55166-9_13

13.1 Introduction

Four decades of research have addressed the study of the synapsins in neuronal physiology, since the first description of Protein I (later renamed synapsin I) as a major target of phosphorylation by cyclic AMP-dependent kinases in synaptic membrane fractions by Paul Greengard in 1972 (Johnson et al. 1972). Though, we are still far from the complete understanding of the multiple functions that these proteins exert in neuronal development and synaptogenesis, synapse organisation, modulation of synaptic vesicle trafficking and plasticity.

Soon after their discovery, it turned out that synapsins I and II, the latter identified in 1978 and initially named Protein III (Forn and Greengard 1978), are presynaptic proteins able to reversibly associate with the cytosolic side of synaptic vesicle membrane (De Camilli et al. 1983a, b; Huttner et al. 1983) and with cytoskeletal components (Bahler and Greengard 1987; Fesce et al. 1992; Valtorta et al. 1992b) in a phosphorylation-dependent manner. Indeed, synapsins are physiological substrates of cyclic AMP-dependent and Ca^{2+}/calmodulin-dependent protein kinases that phosphorylate them upon neuronal activation (Greengard et al. 1993). Given these properties, synapsin I was postulated to be involved in the regulation of neurotransmitter release through the reversible tethering of synaptic vesicles to the actin cytoskeleton (Benfenati et al. 1991; Greengard et al. 1987, 1993; Valtorta et al. 1992a). However, recent evidences also imply synapsins in post-docking steps and vesicle recycling (Bloom et al. 2003; Fassio et al. 2006; Hilfiker et al. 1998, 2005), as well as in neurite extension and synapse formation (Fornasiero et al. 2010). More than 20 years after the identification of synapsin I, a third member of the family was described (Kao et al. 1998). In contrast to synapsins I and II, which are increasingly expressed during neuronal maturation, synapsin III is highly expressed in growth cones at early stages of development (Ferreira et al. 2000) and its specific function is still not completely elucidated. It is now clear that the three members of the family share many features but are not completely redundant. However, the precise roles and pattern of expression of each synapsin isoform remain elusive and need further clarification.

13.2 The Synapsin Family

13.2.1 Genes and Promoter Regions

In mammals the synapsin family is composed of three members encoded by distinct genes: *SYN1*, *SYN2* and *SYN3*. In humans, the *SYN* genes are located on chromosomes X, 3 and 22, respectively. Synapsin genes were identified in vertebrate and invertebrate organisms; however in invertebrates only a single synapsin gene is present. The intron/exon structure is conserved from *C. elegans* to humans, as well as in the three mammalian genes, suggesting that duplication events

occurred from a single ancestor, probably when vertebrates diverged from invertebrates. This interpretation is also supported by the observation that two other gene families, raf and TIMP (tissue inhibitor of metalloproteinases), are in close physical linkage to Syn genes. In fact, each synapsin gene lies in very close proximity to a TIMP and a raf gene family member (Kao et al. 1999).

Synapsins are widely and selectively expressed in neurons of the central and peripheral nervous systems. The proximal promoter of the *SYN1* gene is characterised by the absence of TATA and CAAT consensus motifs and is enriched in CpGs that are subjected to epigenetic regulation (Ekici et al. 2008; Paonessa et al. 2013; Sauerwald et al. 1990). Neuron-specific expression of synapsin I is driven by the presence of a binding site for the transcriptional repressor NRSF/REST (neuron-restrictive silencer factor/RE-1 silencing transcription factor), which negatively regulates *SYN1* expression in non-neuronal cells both in rodent and human promoter regions (Li et al. 1993; Schoch et al. 1996). A positive regulation is instead mediated by the binding of Sp1, a ubiquitous transcriptional activator able to bind to GC-rich regions and to recruit the transcription initiation complex to TATA-less promoters. Interestingly, it has recently been shown that Sp1 and REST binding sites lie in close proximity in the *SYN1* promoter and that the two transcription factors act in a strict functional interplay. During neuronal differentiation and development, Sp1 binding to the *SYN1* promoter is fine-tuned by REST, and the progressive decline in REST levels favours Sp1 binding and synapsin I expression (Paonessa et al. 2013). In addition, a cAMP-responsive element (CRE) is present and confers basal activation by the CRE-binding (CREB) transcription factor but does not modulate *SYN1* expression in a cAMP-sensitive manner (Hoesche et al. 1995). On the contrary, the factor zif268/Egr-1, which recognises specific binding sites (EBS), was shown to mediate transcriptional activation upon forskolin treatment (James et al. 2004).

The *SYN1* and *SYN2* promoters display very little sequence homology, still they share many features (absence of TATA and CAAT boxes, GC enrichment, neuronal specificity) (Chin et al. 1994). In the *SYN2* promoter, binding sites for Sp1 and zif268/Egr-1, but not for REST, have been identified (Petersohn et al. 1995), raising the question as to how synapsin II confinement in neuronal cells could be achieved. Two binding sites for the activating protein 2α (AP-2α) are also present and mediate *SYN2* activation in a cAMP/protein kinase A (PKA)-modulated manner (Skoblenick et al. 2010).

The *SYN3* promoter is completely uncharacterised. Synapsin III differs from the other two members in terms of its expression pattern: two out of the six described splicing isoforms, namely, synapsins IIIe and f, are expressed only in non-neuronal tissues (placenta and spleen), probably thanks to a second promoter that resides upstream exon 9 (Porton et al. 1999). In addition, while synapsins I and II progressively increase in their levels during development, synapsin III is predominantly expressed in the fetal brain, or during the first days in vitro when neurons are cultured, and then declines (Ferreira et al. 2000; Porton et al. 1999). In the adult brain, synapsin III is highly expressed by immature neurons in the neurogenic niches (Pieribone et al. 2002).

13.2.2 Splicing Isoforms and Protein Domain Structure

Alternative splicing events of the three synapsin genes give rise to various isoforms. Two distinct isoforms are produced for synapsins I and II (named a and b), and at least six transcripts are described for synapsin III (a–f). The resulting proteins are highly homologous at their N-termini but greatly differ in the C-terminal portion (see Fig. 13.1) (Porton et al. 1999; Südhof 1990; Südhof et al. 1989). The N-terminal region can be subdivided into three domains, namely, domains A, B and C, which, except for domain B, are highly conserved across different isoforms and species. Conversely, the C-terminal portion is composed of differentially spliced domains (domains D–J).

Domain A is a short N-terminal region that contains a conserved phosphorylation site (site 1) target of PKA and Ca^{2+}/calmodulin-dependent kinases (CaMK) I and IV (Huttner et al. 1981). Domain B is the least conserved of the domains of the

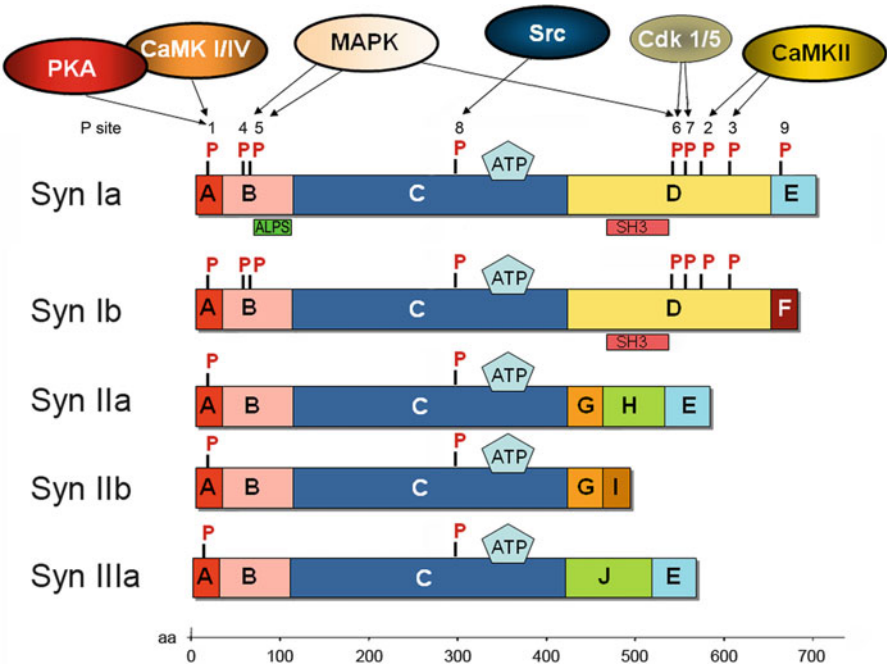

Fig. 13.1 Schematic representation of the domain structure of mammalian synapsin I, II and III proteins. Main splicing isoforms are reported. Known phosphorylation (P) sites (numbered according to the literature), as well as the ALPS motif, the ATP binding site and the proline-rich region mediating the interaction with SH3 domains, are indicated. All isoforms share a conserved N-terminal portion (A, B and C domains), while they differ in alternatively spliced C-terminal domains (D–J). *PKA* protein kinase A, *CaMK* Ca^{2+}/calmodulin-dependent protein kinase, *MAPK* mitogen-activated protein kinase, *Cdk* cyclin-dependent kinase, *ALPS* amphipathic lipid packing sensor, *ATP* adenosine triphosphate, *SH3* Src homology 3 domain (Adapted with authors' permission from Fornasiero et al. 2010)

N-terminal region. It is considered to be a linker region, connecting domain A to domain C. However, mutations in this domain appear to have a strong impact on the functions of this protein (Guarnieri and Valtorta, unpublished results). It is characterised by the presence of small amino acids and probably has an elongated conformation in solution (Brautigam et al. 2004). Recently, an amphipathic lipid packing sensor (ALPS) motif has been recognised in this domain. This motif is evolutionarily conserved and is deputed to the specific binding of highly curved membranes (Krabben et al. 2011). In synapsin I, domain B contains two phosphorylation sites (sites 4 and 5) for mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) (Jovanovic et al. 1996; Matsubara et al. 1996). These two sites are probably present also in synapsin II and III, although their phosphorylation has never been experimentally demonstrated (Jovanovic et al. 2001). Domain C is the core region, highly conserved among the various members of the family. It mediates the principal functions of synapsins, i.e. the interaction with actin filaments and synaptic vesicle phospholipids (Bahler and Greengard 1987; Benfenati et al. 1989b; Benfenati et al. 1989c; Cheetham et al. 2001), as well as synapsin homo- and hetero-dimerisation (Hosaka and Südhof 1999; Monaldi et al. 2010) that probably contributes to synaptic vesicle clustering. A highly conserved phosphorylation site (site 8) is present in this domain and is targeted by the tyrosine kinase Src. The phosphorylation at this site has been experimentally demonstrated for synapsins I and II (Messa et al. 2010; Onofri et al. 2007). Domain C is the only portion for which a crystal structure is available, and it appears folded as a compact structure composed of amphipathic α-helices and β-sheets. Interestingly, it has been shown that the three-dimensional structure of synapsin I strongly resembles that of an ATP-dependent glutathione synthetase and that all synapsins can bind ATP (Esser et al. 1998), even though an enzymatic activity has never been proved. After domain C, the amino acid sequence starts to diverge in the various synapsins. However, all isoforms bear an elongated proline-rich tail, namely, domain D in synapsin I, domain G in synapsin II and domain J in synapsin IIIa. In synapsin I, domain D contains phosphorylation sites 2 and 3 for CaMKII (Czernik et al. 1987). Similar consensus sites are present also in synapsin III, but their accessibility to phosphorylation remains to be determined (Kao et al. 1998). In the same region, site 6 is targeted by MAPK/ERK and cyclin-dependent kinase 5 (Cdk5), and site 7 is specifically targeted by Cdk5 (Jovanovic et al. 1996; Matsubara et al. 1996). The proline-rich domain D mediates the binding to important molecular partners, such as CaMKII (Benfenati et al 1992) and Rab3A (Giovedi et al 2004b), as well as the SH3 (Src homology 3 domain)-containing protein Src (Onofri et al 1997). Downstream of the proline-rich region, domain E, common to all the 'a' isoforms, is highly conserved and it was recently shown to play an important role in modulating synapsin function. In fact, this region is required for the proper targeting of synapsins to the presynaptic terminals (Gitler et al. 2004b), as well as for synapsin I oligomerisation and synaptic vesicle clustering, and is implicated in both the pre- and post-docking steps of synaptic vesicle exocytosis (Fassio et al. 2006; Hilfiker et al. 1998; Monaldi et al. 2010). In addition, a novel phosphorylation site (site 9) targeted by the

ataxia-telangiectasia mutated (ATM) kinase has been recently identified in this domain (Li et al. 2009). The short domains F and I present in synapsin I and II 'b' isoforms, respectively, are poorly conserved and remain as yet uncharacterised.

13.3 Synapsin Expression and Biochemical Properties

13.3.1 Spatial and Temporal Regulation of Synapsin Expression

Virtually all neurons in the central and peripheral nervous system express at least one synapsin isoform, with the notable exception of specialised systems characterised by the presence of ribbon synapses (bipolar cells of the retina, hair cells of the inner ear, taste cells) that use distinct mechanisms of vesicle clustering and exo-endocytosis (Mandell et al. 1990). Many presynaptic terminals appear to contain all I/II isoforms, even if at differential abundance. As an example, cerebellar Purkinje cells express high levels of synapsin Ib, lower levels of synapsin Ia and IIb, while lack synapsin IIa (Südhof et al. 1989). Overall, in the cerebral cortex, synapsin I is more abundant than synapsin II, in a ratio of 2:1, and the ratios of Ia to Ib and IIa to IIb are both consistently 1:2 (De Camilli et al. 1990). In the olfactory cortex, however, synapsin II is higher than synapsin I, and the IIa to IIb ratio is 3:2 (Stone et al. 1994). Another interesting example of differential expression of synapsin isoforms is given by the distinct types of synaptic inputs coming from the retina, the visual cortex and GABAergic interneurons on relay neurons of the lateral geniculate nucleus in the thalamus. Indeed, retinogeniculate synapses do not display synapsin I or II expression, while both isoforms are present in feedback corticogeniculate synapses and only synapsin I is expressed in interneurons (Kielland et al. 2006). This characteristic pattern may reflect specific needs of each type of synaptic connection to fine-tune vesicle release in modulatory inputs or to reach powerful transmission in driver synapses. This interpretation has been strengthened by the observation that the thalamocortical input on visual cortex, which represents another driver synapse in the visual pathway, is devoid of synapsins (Owe et al. 2013). Recently, array tomography experiments confirmed that synapsin I is present in all glutamatergic and GABAergic presynaptic terminals in the mouse cortex (Micheva et al. 2010).

Synapsin III is expressed at very low levels throughout the brain, with high regional variation in the adult mouse. Its presence in presynaptic terminals suggests, at least in part, a similar function to synapsins I and II. However, this third member of the family bears the peculiarity of being expressed at high levels in the dentate gyrus and the olfactory bulb, known to be neurogenic regions of the adult brain. In addition, in these regions, synapsin III localises to the cell soma rather than to nerve terminals, indicating a completely different – but still unclear – function (Pieribone et al. 2002).

In the rat brain, synapsin I expression slightly precedes peaks of synaptogenesis, according to developmental specificity of different regions (Zurmohle et al. 1994). Thus, in the neocortex and cerebellum, synapsin I mRNA starts to increase at postnatal day 7 (P7) and peaks at P17, when synaptogenesis rate is high in these regions. Synapsin I mRNA expression is already detectable in the first postnatal week in the thalamus, piriform cortex and hippocampus (particularly in the CA3 subregion and the dentate gyrus), which mature earlier. High levels of synapsin I mRNA are maintained constant – or slightly decreased – during adulthood, especially in the CA3 region of the hippocampus, suggesting that this protein is important for synapse formation during development as well as for synapse maintenance and remodelling/plasticity during adult life (Zurmohle et al. 1994). At the protein level, synapsins I and II were shown to steadily increase during postnatal development of the mouse brain, with synapsin I being already detectable at P5. Synapsins IIa and IIb are barely detectable during the first week after birth and then increase with different kinetics (slower for IIa and much faster for IIb) (Bogen et al. 2009). These observations also indicate that the progressive increase of synapsin protein is probably due to post-transcriptional/translational mechanisms of regulation, as the rate of mRNA synthesis is about constant. Another possibility, which has been verified in vitro, is that synapsin proteins are progressively stabilised by their incorporation into mature synaptic vesicles produced during neuronal development, despite a constant efficiency in transcription and translation, as their half-life gradually increases (Daly and Ziff 1997).

While the levels of synapsins I and II correlate with the extent of synapse formation in vivo and in vitro, synapsin III is highly expressed in the first week of development and subsequently declines (Ferreira et al. 2000; Pieribone et al. 2002). In vitro, this period corresponds to active neurite elongation, and, consistently with a role in this process rather than in synapse formation, synapsin III is enriched in growth cones and at extrasynaptic sites. Indeed, depletion of synapsin III from hippocampal neurons in culture leads to a defect in axonal elongation, but not in the time course of synapse formation (Ferreira et al. 2000).

Low levels of synapsin I expression have been detected in non-neuronal cell types, such as epithelial cells, pancreatic β-cells, cell lines of neuronal and endocrine origin and, interestingly, astrocytes (see, e.g. Haycock et al. 1988; Maienschein et al. 1999; Romano et al. 1987). In astrocytes, synapsin I appears to be secreted via exosomes and to contribute to neurite outgrowth of neuronal cells (Wang et al. 2011).

13.3.2 Binding to Synaptic Vesicles

Synapsins, and in particular synapsin I, have been extensively characterised in terms of their synaptic vesicle-binding properties. The first evidences for a direct interaction of synapsin I with the cytosolic surface of synaptic vesicles came from electron microscopy immunoferritin/peroxidase staining and from biochemical

purification of the organelles (De Camilli et al. 1983b; Huttner et al. 1983; Valtorta et al. 1988). These studies showed that the interaction is specific, as other membranous compartments in the terminal (such as endoplasmic reticulum cisternae, mitochondria or large dense-core vesicles) were unlabelled, and relatively stable, as it resisted to isotonic, but not to hypotonic, lysis of the nerve ending. Dephosphorylated synapsin I was proved to bind synaptic vesicles as well as artificial phospholipid membrane with high affinity ($K_d \approx 10$ nM) (Benfenati et al. 1989a, 1989b). Phosphorylation of the protein on sites 2 and 3, targets of CaMKII, causes a fivefold decrease in the binding affinity to synaptic vesicles (Schiebler et al. 1986). In intact terminals, also site 1 phosphorylation by PKA and CaMKI induces the dissociation of synapsins from the vesicle membrane (Hosaka and Südhof 1999). Recently, it was estimated that eight molecules of synapsin I are present on the surface of each vesicle (Takamori et al. 2006), confirming that synapsins represent approximately 6 % of the total vesicle proteins (Huttner et al. 1983).

The lipid-binding activity of synapsin I is predominantly mediated by the N-terminal portion of the protein, which is able to partially penetrate into the lipid bilayer after an initial electrostatic interaction with the acidic phospholipids (especially phosphatidylserine and phosphatidylinositol) of the vesicular membrane (Benfenati et al. 1989b). The specific regions involved in membrane insertion were mapped on synapsin I. Three regions, two of which contain an amphipathic α-helix, were found to be able to penetrate the vesicular membrane. They all reside in domain C and are exposed on the surface of one side of the protein that does not overlap with the dimerisation domain (Cheetham et al. 2001). These regions are highly conserved across isoforms and species and probably mediate association with synaptic vesicles also in synapsins II and III.

The C-terminal tail of synapsin I is able to bind the synaptic vesicle surface through the interaction with protein components of the vesicles rather than with phospholipids (Benfenati et al. 1989a). One of these protein binding partners is the regulatory domain of vesicle-associated CaMKII (Benfenati et al. 1992). Synapsin I is also the major binding protein of the tyrosine kinase c-Src on the synaptic vesicle membrane. The interaction between the proline-rich domain D of synapsin I and the SH3 domain of Src triggers a basal level of activation of the kinase, which in turn phosphorylates synapsin I itself on site 8 (Onofri et al. 1997). This phosphorylation confers to the surrounding sequence of synapsin I SH2 domain-binding properties and recruits the SH2 domain of Src. Thus, Src binding is reinforced by combined SH3/SH2 interactions, leading to massive activation of the kinase (Onofri et al. 2007). Through its SH3-binding regions, synapsin I is able to interact with many additional partners, such as the MAPK adaptor Grb2, the p85 subunit of PI3K, phospholipase C-γ, amphiphysins I and II, Crk, p47, α-spectrin (Onofri et al. 2000) and syndapin (Qualmann et al. 1999).

Synapsin I interacts in an SH3-independent manner with Rab3A (Giovedi et al. 2004b). Rab proteins are monomeric G proteins ubiquitously involved in the modulation of membrane trafficking. The Rab3A–C isoforms are specific for synaptic vesicles, and their GTP/GDP cycle is strictly connected to the dynamics of

synaptic vesicle exo-endocytosis (Geppert and Südhof 1998). Similarly to what was described for Src, the interaction of synapsin I and Rab3A influences the functional properties of both proteins: synapsin I stimulates Rab3A GTP binding and GTPase activity, while Rab3A inhibits synapsin I ability to bind and bundle actin and to cluster synaptic vesicles (Giovedi et al. 2004a).

Synapsin II is more tightly bound to synaptic vesicles than synapsin I, possibly because it lacks part of the C-terminal tail that contains highly charged residues, thus making hydrophobic interactions prevalent. An additional binding site for synaptic vesicles has been recognised in domain B of synapsin II, likely involving interaction with protein components (Thiel et al. 1990).

Many approaches have demonstrated that synapsins also have a stabilising activity on the vesicle membrane. Indeed, it was shown that high concentrations of synapsin I – similar to those reached at presynaptic terminals – are able to counteract the effects of destabilising stimuli on artificial liposomes (Benfenati et al. 1993; Cheetham et al. 2003) and to confer break resistance to artificial lipid bilayers (Pera et al. 2004). Given that synaptic vesicles have a very small diameter (around 40–60 nm), they are supposed to be unstable due to high curvature stress. The binding of synapsins may be important to balance the different surface pressures on the external and internal leaflet, to stabilise the small vesicles and to impede unwanted fusion events.

Interestingly, the curvature of the vesicular membrane per se may be a key determinant of the specific binding of synapsins to synaptic vesicles. Synapsin I contains an amphipathic lipid packing sensor (ALPS) motif that specifically senses highly curved membranes and facilitates synapsin binding to (and clustering of) synaptic vesicles (Krabben et al. 2011). This motif is perfectly conserved in all synapsin isoforms and likely exerts similar functions in synapsins II and III.

Thus, the association of synapsins with synaptic vesicles is mediated by multiple domains of the molecule, which interact with either phospholipids or protein components. It is conceivable that binding specificity is achieved by membrane curvature sensing, as well as by interaction with resident synaptic vesicle proteins.

13.3.3 Binding to Actin Cytoskeleton

Synapsin I was shown to interact with several cytoskeletal proteins, but the binding to actin is of particular interest given that actin is the major component of the cytoskeleton in the presynaptic terminal. The ability of synapsins to bind both the membrane of synaptic vesicles and the actin cytoskeleton has led to the hypothesis that they are involved in the cross-linking and anchoring of synaptic vesicles to the cytomatrix of the nerve terminal.

Purified dephosphorylated synapsin I is able to bind ($K_d = 1–2$ μM) and bundle actin filaments in vitro. The bundling activity is reduced when synapsin I is phosphorylated by PKA, almost completely abolished upon CaMKII phosphorylation (Bahler and Greengard 1987), significantly reduced by MAPK (Jovanovic

et al. 1996) and not affected by Cdk5 phosphorylation (Matsubara et al. 1996). The ability to bundle actin implies the presence of multiple binding sites in each synapsin molecule and/or the ability of synapsin molecules bearing a single binding site to dimerise. At least two regions responsible for actin binding reside in the N-terminal (domain B) and middle portion of the protein (end of domain C), but the presence of the C-terminal tail is necessary for maintaining the bundling activity (Bahler et al. 1989). In addition, domain C and E, but not A, peptides interfere with the ability of synapsin I to bind and bundle actin filaments (Hilfiker et al. 2005).

Interestingly, dephospho-synapsin I also promotes monomeric actin nucleation and polymerisation, and phosphorylation by CaMKII strongly inhibits this function (Fesce et al. 1992; Valtorta et al. 1992b).

Synapsin II has a much stronger actin binding and bundling activity, which is completely abolished by PKA phosphorylation (Nielander et al. 1997).

13.3.4 Homo- and Hetero-dimerisation of Synapsins

Analysis of the crystal structure of synapsin I domain C showed that the protein forms tightly associated dimers and tetramers, which are stabilised by ATP binding and physiological Ca^{2+} concentrations (Brautigam et al. 2004; Esser et al. 1998). The contact area between the subunits of the oligomers is very large, indicating a stable interaction. Given that the C-domain is the most conserved region in all isoforms, synapsin hetero-dimerisation was also investigated. Yeast two-hybrid screenings and immunoprecipitation assays proved that the major synapsin binding partner is another synapsin molecule. All three synapsins are able to interact with themselves and with the other isoforms. The interaction is strong and stable, with the exception of a less intense binding between synapsins I and III, and the full-length domain C is required for the association to occur (Hosaka and Südhof 1999). Homo- and hetero-dimerisation is an important feature of synapsins, as it implies that synaptic vesicles are coated by multiple heterogeneous dimers that possibly exert very similar functions but possess different regulatory properties. Since synapsin isoforms are differentially expressed in neurons, the exact composition of the dimers in each terminal may have a functional relevance in the specific regulation of vesicle life cycle.

In addition to domain C, the highly conserved domain E has a prominent role in mediating synapsin dimerisation. Indeed, domain E peptides selectively bind to synapsins I and II and inhibit the formation of synapsin dimers (Monaldi et al. 2010). The involvement of domain E in the dimerisation of synapsins is also relevant for the specific targeting of synapsins to nerve terminals. In fact, it was shown that domain E and dimerisation are two important determinants of synaptic targeting, in particular for the correct transport of isoforms with weak targeting potential such as synapsin IIb (Gitler et al. 2004b).

13.4 Modes of Synapsin Action in the Presynaptic Terminal

13.4.1 Role in Synaptic Vesicle Pool Organisation

In the presynaptic terminal, synaptic vesicles are organised in at least three distinct and functionally relevant pools: the readily releasable pool, the recycling pool and the resting pool (Rizzoli and Betz 2005). The readily releasable pool comprises a small number of vesicles (1–10 % of the total) that are docked at the membrane, in an intermediate state of fusion and ready to be rapidly released in response to a Ca^{2+} influx. The recycling pool comprises 5–20 % of all vesicles and can be recruited after a moderate stimulation in order to substitute docked vesicles that had undergone release. The resting pool accounts for the majority of vesicles in the terminal (typically 80–90 %). This pool is quite inactive and is recruited after intense stimulation; thus it is considered the true depot for synaptic vesicles.

Given the properties described so far, i.e. the ability to bind synaptic vesicles and actin in a phosphorylation-dependent manner, synapsins were hypothesised to be primarily involved in the organisation of synaptic vesicle pools in the presynaptic terminal (Greengard et al. 1993). The first evidences supporting this idea came from microinjection studies. Delivery of dephosphorylated synapsin I into the squid giant synapse inhibited neurotransmitter release, due to a decrease in the quanta released by a constant stimulus, and this effect was abolished upon CaMKII phosphorylation. Conversely, the injection of CaMKII enhanced neurotransmitter release (Llinas et al. 1985, 1991). These observations have suggested a model in which the inhibitory effect of dephosphorylated synapsin I on neurotransmitter release is achieved through the cross-linking of synaptic vesicle to each other and to the actin cytomatrix (Benfenati et al. 1991). Direct visual proof of the anchorage of synaptic vesicles to actin filaments mediated by synapsin I came from video-enhanced microscopy of fluorescently labelled components incubated in vitro. The interaction of synapsin I to actin and synaptic vesicles is rapid, as after 3–20 s of incubation, synapsin I is able to bundle actin filaments or drive the polymerisation of the monomers and to tether synaptic vesicles to the formed cytoskeletal network. Again, these effects are completely abolished by CaMKII phosphorylation (Ceccaldi et al. 1995).

One mechanism by which synapsins could regulate neurotransmitter release is the reversible association/dissociation to synaptic vesicles and F-actin, according to their phosphorylation state. This was biochemically demonstrated, as depolarisation of rat synaptosomes resulted in a rapid translocation of synapsin I from the particulate to the soluble cytosolic fraction. The stoichiometry of phosphorylation of soluble synapsin I was higher with respect to that of synapsin I in the particulate fraction, and the time course of translocation paralleled that of phosphorylation, suggesting that phosphorylation promotes the dissociation of synapsin from synaptic vesicles and actin (Sihra et al. 1989). The translocation of synapsin I upon neuronal activation was also demonstrated in living hippocampal neurons in

culture through immunocytochemistry and video-imaging analysis of GFP-labelled synapsin Ia (Chi et al. 2001). It was shown that a substantial portion of synapsin molecules present in the presynaptic bouton detaches from synaptic vesicles and diffuses in the axon during electrical stimulation. This dissociation is faster than vesicle turnover itself (monitored through the uptake of the FM4-64 dye), indicating a physical separation of synapsin I from synaptic vesicles that precedes their exocytosis. Both synapsin diffusion and vesicle turnover were slowed down by transfection with non-phosphorylatable mutants on sites 1, 2 and 3 (Chi et al. 2001). These results are consistent with ultrastructural analyses performed on the frog neuromuscular junction, which indicated a partial dissociation of synapsin I from synaptic vesicles upon electrical stimulation. Indeed, upon neuronal activation, synaptophysin (a transmembrane vesicular protein) was still associated with vesicle membranes, whereas synapsin I was partially diffused in regions of the terminal devoid of synaptic vesicles (Torri-Tarelli et al. 1990, 1992).

Together these studies pointed to a model in which synapsin I (and probably synapsin II in a similar manner) is involved in the regulation of the number of synaptic vesicles available for exocytosis after a depolarisation event, through the dynamic organisation of the pools of vesicles (Fig. 13.2). In its dephosphorylated form, synapsin I may bind the majority of synaptic vesicles and link them to each other, thanks to synapsin dimerisation, and to actin filaments, both preformed or newly polymerised on vesicle membranes. In this manner, synaptic vesicles are captured in a cytoskeletal network and are unavailable for exocytosis. Upon stimulation, Ca^{2+} influx can activate protein kinases, such as CaMKII, that in turn phosphorylate synapsin I. In its phosphorylated form synapsin I loses its affinity for synaptic vesicles and the actin cytoskeleton, therefore detaching and releasing vesicles that can undergo fusion (Greengard et al. 1993). It is also important to note that not all synapsin molecules detach from synaptic vesicles upon Ca^{2+} entry; otherwise vesicle release would probably be massive rather than fine-tuned. The detachment of synapsin molecules from the resting pool appears to be a stochastic process. CaMKII phosphorylation probably proceeds from the periphery to the centre of the cluster, given that CaMKII immune-reactivity is clearly enriched around – not inside – the vesicle cluster. Thus, the number of vesicles that the kinase is able to reach would probably be dependent on the duration of the depolarising stimulus (Tao-Cheng et al. 2006).

The modulation of synapsin function by phosphorylation is rather complex. As much as nine phosphorylation sites have been extensively characterised for synapsin I up to now, but others have already been identified and wait for functional depiction (John et al. 2007; Li et al. 2009; Nuwal et al. 2011).

Phosphorylation at site 1 (Ser-9 in the rat sequence) by PKA/CaMKI induces only subtle changes in the overall conformation of the synapsin molecule (Benfenati et al. 1990) but significantly reduces the binding of synapsins to synaptic vesicles and actin in both mature (Chi et al. 2001; Menegon et al. 2006) and developing neurons (Bonanomi et al. 2005), leading to increased recycling activity.

Phosphorylation at sites 2 (Ser-566) and 3 (Ser-603) by CaMKII leads to major conformational changes (Benfenati et al. 1990) and to a drastic decrease in the

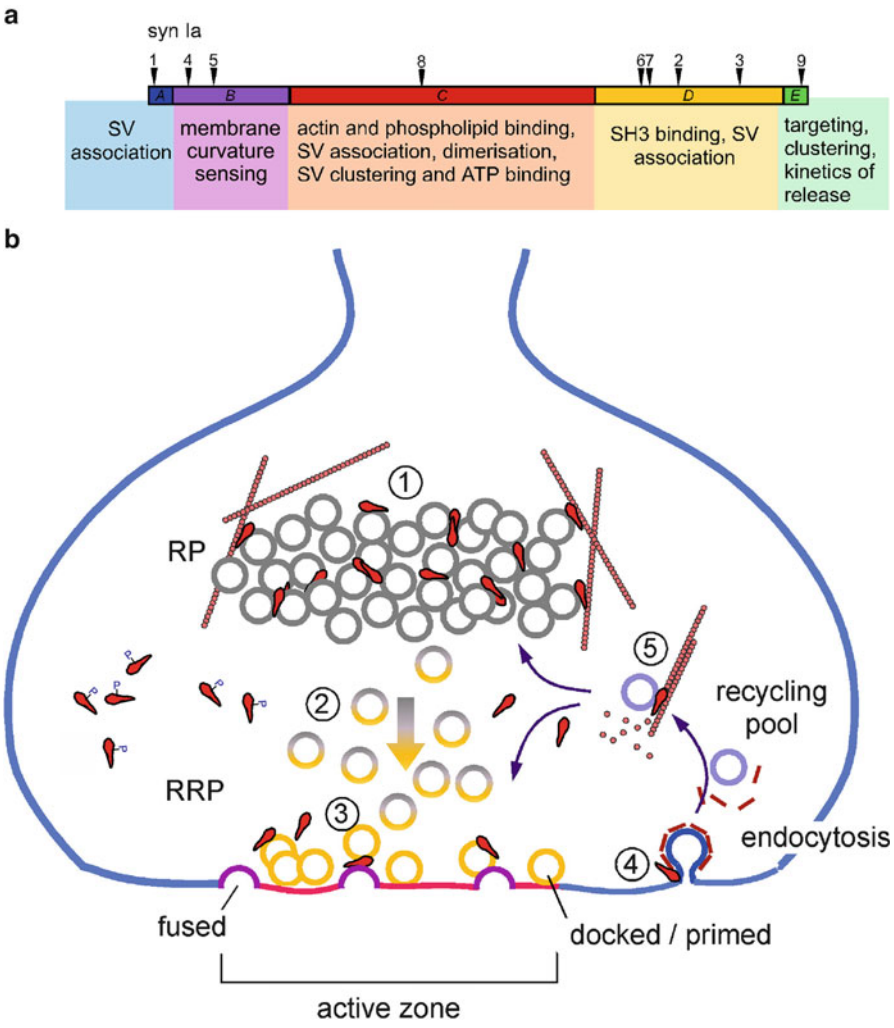

Fig. 13.2 Roles of synapsins in the presynaptic compartment. (**a**) Schematic representation of synapsin Ia, summarising the biological functions attributed to each domain. (**b**) Mechanisms of regulation of the synaptic vesicle cycle. (*1*) Synapsins (in *red*) mediate the clustering of synaptic vesicles of the resting pool (RP, in *grey*), distant from the active zone, by linking adjacent synaptic vesicles to each other and to the actin cytoskeleton. (*2*) Upon stimulation, synapsins get phosphorylated, lose affinity for synaptic vesicles and actin and relieve a subset of vesicles from physical constraint. These vesicles are now free to reach the active zone and undergo fusion. (*3*) Part of the synapsin molecules does not dissociate from synaptic vesicles and is found in close proximity to the active zone membrane, within the readily releasable pool (RRP, in *yellow*). Here, synapsins might contribute to docking, post-docking and fusion events. (*4*) After synaptic vesicle fusion, synapsins re-localise in endocytic regions, where they interact with proteins involved in endocytosis and probably participate in the process. (*5*) After the reuptake, synapsins may stimulate actin polymerisation and contribute to redirecting vesicles back to the RP or RRP. *SV* synaptic vesicle (Reproduced with authors' permission from Cesca et al. 2010)

ability of synapsin I to interact with actin and synaptic vesicles (Bahler and Greengard 1987; Chi et al. 2001; Schiebler et al. 1986). Interestingly, although CaMKII and synapsin I are both expressed starting from early developmental stages, it has been shown that they are functionally coupled only in mature presynaptic terminals (Menegon et al. 2002). It has been hypothesised that, before synapse specification, synapsin I is responsible for the clustering of synaptic vesicles in the growth cone and that its function is primarily modulated by PKA. As synaptogenesis begins, in addition to PKA, synapsin becomes strongly dependent on CaMKII regulation (Bonanomi et al. 2005). In mature neurons, CaMKII phosphorylation was shown to be particularly relevant for controlling synaptic vesicle mobilisation under low-frequency stimulation (Chi et al. 2003).

Similar to site 1 phosphorylation, phosphorylation at sites 4 (Ser-62), 5 (Ser-67) and 6 (Ser-549) by MAPK/ERK (or by Cdk1 at site 6) results in a significant decrease in the ability of synapsin I to bind, bundle and polymerise actin but has no marked effects on its interaction with purified synaptic vesicles in vitro (Jovanovic et al. 1996). Opposite to what was observed with sites 1, 2 and 3, basal levels of phosphorylation on sites 4, 5 and 6 are quite high; thus they possibly mediate a tonic control on synapsin function (Jovanovic et al. 2000). Synapsin phosphorylation by MAPK is promoted by exposure to brain-derived neurotrophic factor (BDNF) and seems to be a key step in the BDNF-driven modulation of neurotransmitter release (Jovanovic et al. 1996, 2000). On the contrary, depolarisation induced by KCl results in a fast Ca^{2+}-dependent decrease in the phosphorylation level of these sites, mediated by calcineurin (Jovanovic et al. 2001). The kinetics of site 4/5/6 phosphorylation is strictly dependent on the frequency of stimulation: at low frequencies (5 Hz), MAPK activation prevails and synapsin I is phosphorylated on these sites; at high frequencies (10–20 Hz), calcineurin is strongly activated and the net effect is synapsin I dephosphorylation. The effects of MAPK/calcineurin on sites 4, 5 and 6 are relevant in modulating synapsin activity over a wide frequency range: synapsin is phosphorylated at low stimulation frequency and dephosphorylated at high frequency, thus mobilising synaptic vesicles for release (Chi et al. 2003). MAPK phosphorylation actively drives synaptic vesicle recruitment, probably through synapsin I detachment from the actin cytoskeleton, both in growth cones and mature synapses (Schenk et al. 2005).

Phosphorylation on sites 6 and 7 by Cdk5 has been less extensively studied (Matsubara et al. 1996). Interestingly, it has been recently shown that Cdk5 has a negative effect on synaptic vesicle mobilisation from the resting pool (Kim and Ryan 2010). Recent data identify synapsin I as the central switch in mediating the synaptic effects of Cdk5. Synapsin I is constitutively phosphorylated on the Cdk5 sites at rest. Phosphorylation of synapsin I by Cdk5 increases its binding to actin, leaving the interaction with synaptic vesicles unaltered (Verstegen, Fassio and Benfenati, personal communication).

Finally, phosphorylation of synapsin I at site 8 (Tyr-301, residing in domain C) by Src results in an increase in its ability to bind actin and synaptic vesicles and to dimerise. The overexpression of a site 8 dephosphomimetic mutant in hippocampal neurons in culture leads to increased vesicle exo-endocytosis upon

electrical stimulation, suggesting that phosphorylation on this site favours the sequestration of synaptic vesicles and negatively influences their recruitment from the resting pool (Messa et al. 2010). Depolarisation induces Src phosphorylation in a Ca^{2+}-dependent manner, with a peak at 2 min upon stimulation and persistence up to 30 min, raising the possibility that tyrosine phosphorylation counteracts the effects of serine phosphorylation late after stimulation in order to reassemble synaptic vesicle pools.

The various phosphorylation sites are subjected to dephosphorylation by distinct protein phosphatases. As already mentioned, sites 4, 5 and 6 are under a dominant control of calcineurin, while sites 1, 2 and 3 are selectively dephosphorylated by protein phosphatase 2A (Jovanovic et al. 2001).

Given the complexity of the network of phosphorylating/dephosphorylating enzymes contributing to synapsin regulation, the final effect of a stimulus might be hard to predict, depending on many concomitant factors such as the intensity and duration of stimulation, the selective signal-transduction pathway activated, the presence of synaptic efficacy modulators such as BDNF and the crosstalk among the various pathways. Recent evidence showed that the blockage of exocytosis as well as of endocytosis, while not hampering phosphorylation of the protein, impairs the diffusion of synapsin I in the axonal shaft that occurs upon stimulation. These observations suggest that, in addition to phosphorylation, other unknown signals coming from the plasma membrane affect synapsin I localisation within the presynaptic terminal (Orenbuch et al. 2012b).

Further complicating the picture, synapsins are subjected to other regulatory post-translational modifications, such as the addition of O-linked N-acetylglucosamine (O-GlcNAc) on serine and threonine residues and fucosylation. O-GlcNAc sites flank phosphorylation sites in domains B and D of synapsin I and modulate the phosphorylation state and functional properties of the protein (Cole and Hart 1999; Luthi et al. 1991; Skorobogatko et al. 2014; Tallent et al. 2009). Fucosylation regulates synapsin I turnover, preventing the protein from being degraded by the Ca^{2+}-activated protease calpain (Murrey et al. 2006).

In mature synapses, many evidences suggest that synapsins are primarily involved in keeping a large proportion of synaptic vesicles unavailable for exocytosis, through reversible anchorage to each other and to the actin cytoskeleton; thereby they are thought to maintain the resting pool of synaptic vesicles (Benfenati et al. 1991). A small subset of vesicles, referred to as the readily releasable pool, is instead docked at the plasma membrane and is relatively depleted of synapsin immunoreactivity (Pieribone et al. 1995; Siksou et al. 2007). Injection of anti-synapsin I antibodies in the lamprey reticulospinal axons resulted in depletion of the distal cluster of synaptic vesicles, while the number of vesicles adjacent to the plasma membrane was unaffected (Pieribone et al. 1995). In addition, disruption of synapsin function by injection of a domain E-derived peptide dramatically decreased the number of vesicles distant from the plasma membrane, leaving docked vesicles unaltered (Hilfiker et al. 1998).

In synapsin knockout (KO) mice, the number of distal synaptic vesicles ($>$100 nm apart from the active zone) was greatly reduced with respect to the

wild type (WT) (Gitler et al. 2004a; Rosahl et al. 1995; Siksou et al. 2007). Analysis of the three-dimensional structure of the presynaptic terminal using advanced electron tomography showed that synaptic vesicles are linked to each other by short 30 nm-long filaments, consistent with synapsin dimension and structure. In mice deleted for all synapsin genes, the connecting filaments among synaptic vesicles were still present (Siksou et al. 2007), indicating that additional players participate in vesicle clustering (Shupliakov et al. 2011). However, in single as well as double or triple KO hippocampal neurons in culture, the reduction in the number of synaptic vesicles at presynaptic terminals was paralleled by vesicle relocalisation in adjacent axonal shafts (Fornasiero et al. 2012; Orenbuch et al. 2012a). Notably, the dispersion of synaptic vesicles could be rescued by chronic inhibition of synaptic activity, indicating that molecules other than synapsins are able to cluster synaptic vesicles at the nerve terminal but that synapsins are essential for retaining vesicles at release sites during neuronal activity (Fornasiero et al. 2012). Overexpression of synapsin IIa appears sufficient to rescue the synaptic vesicle reduction and dispersion observed in triple KO neurons (Gitler et al. 2008; Orenbuch et al. 2012a).

13.4.2 Post-docking Roles

Synapsins have been proposed to have a role also in the late stages of synaptic vesicle life cycle in the presynaptic terminal, after vesicles have docked or fused. As mentioned above, in stimulated nerve terminals, synapsins are to a great extent dissociated from the synaptic vesicle membrane. However, dissociation is not complete (Chi et al. 2001; Torri-Tarelli et al. 1990, 1992). In the lamprey giant synapses, stimulation increases the fraction of synapsin I detected in the pool of vesicles proximal to the active zone (Bloom et al. 2003).

The injection of peptides from synapsin domain E and C inhibited and slowed the kinetics of neurotransmitter release. This effect was accompanied by a reduction in the number of vesicles in the resting pool but not of the docked vesicles, suggesting that synapsins may have a second function much more related to the priming/fusion process itself (Hilfiker et al. 1998, 2005). These observations are consistent with the finding that synapsin I KO mice display glutamate release defects without changes in the number of docked vesicles (Li et al. 1995). Interestingly, in Purkinje cells, the overexpression of domain E peptides still resulted in a decrease of vesicles in the resting pool but not in the readily releasable pool, and, opposite to glutamate, it accelerated GABA release kinetics, suggesting that synapsins may exert differential modulation of vesicle fusion at glutamatergic and GABAergic synapses (Fassio et al. 2006). As previously mentioned, synapsin I, and to a lower extent synapsin II, interacts with the vesicle-associated small G protein Rab3A (Giovedi et al. 2004b). GTP-bound Rab3A is able to associate with the synaptic vesicle membrane, and it negatively influences vesicle priming and fusion. Upon GTP hydrolysis, GDP-Rab3A detaches from synaptic vesicles

and relieves the inhibition. The interaction with synapsin I stimulates the GTPase activity of Rab3A. Thus, synapsin I may stimulate priming/fusion by accelerating GTP hydrolysis and Rab3A detachment from synaptic vesicles (Giovedi et al. 2004a).

Actin filaments predominantly decorate the periphery of the synaptic vesicle cluster, while are rarely detected inside, thus providing a structural scaffold for vesicle accumulation. Upon synaptic activation, actin filaments surrounding the cluster further polymerise, probably contributing to vesicle endocytosis and recycling – even though the actual role of actin in modulating synaptic vesicle recycling is largely debated (Sankaranarayanan et al. 2003; Shupliakov et al. 2002). Interestingly, in stimulated synapses, synapsin was observed within the filamentous actin cytomatrix lateral to the active zone, where endocytosis takes place, in association with synaptic vesicles recycling back to the cluster (Bloom et al. 2003). This observation raised the possibility that synapsins participate in endocytosis of synaptic vesicles.

Synapsin was never detected on clathrin-coated intermediates, indicating that it probably dissociates before endocytosis and re-associates once synaptic vesicles have been released in the cytoplasm (Bloom et al. 2003; Torri Tarelli et al. 1992). Given these observations, the model proposed is the following: synapsin at rest is localised mainly in the resting pool and preserves its clustering; upon stimulation synapsin dissociates and accumulates in the endocytic zone, where it promotes actin polymerisation and contributes to the recycling of synaptic vesicles back to the recycling or resting pool. It has been hypothesised that this function of synapsin I could be modulated by MAPK phosphorylation: MAPK sites are phosphorylated under basal conditions and dephosporylated by calcineurin upon stimulation (Jovanovic et al. 2001). Calcineurin is implicated in the coordinated depolarisation-induced dephosphorylation of other proteins as well, such as amphiphysin I/II, dynamin I and synaptojanin. These proteins are now classified as ‘dephosphins’; they are all involved in synaptic vesicle endocytosis, and their calcineurin-mediated dephosphorylation is necessary for triggering the process (Cousin and Robinson 2001). It is possible that in the endocytic zone synapsin interacts with some of these regulators, thus modulating synaptic vesicle recycling. Indeed, a direct interaction of amphiphysin I/II with the SH3-binding motif present in domain D of synapsin I has been demonstrated (Onofri et al. 2000). Synapsin I is also able to interact with intersectin, which in turn regulates dynamin function in vesicle fission (Evergren et al. 2006).

13.5 Role of Synapsins in Synaptic Transmission and Plasticity

A large number of studies have addressed the contribution of synapsins to synaptic transmission and plasticity, mainly taking advantage of mice deleted for one or more synapsin genes. The data generated are quite complex to interpret, and in

more than one case, they are difficult to reconcile with each other, partly because of differences in the methods used and partly because of possible compensatory mechanisms that may confound the results. Synapsin KO mice are viable and fertile, with normal life expectancy and no gross brain abnormalities (Gitler et al. 2004a; Rosahl et al. 1995), even though this family of proteins has been extensively implicated also in neuronal development and synaptogenesis (Fornasiero et al. 2010). Thus, despite their abundance in neuronal tissue, synapsins are not essential for overall brain function.

Mice deleted for synapsins, with the notable exception of synapsin III KO mice (Feng et al. 2002), experience generalised tonic-clonic seizures starting from the second month of age (Cambiaghi et al. 2013; Etholm et al. 2011; Feng et al. 2002; Ketzef et al. 2011; Li et al. 1995; Rosahl et al. 1993, 1995). Importantly, several nonsense and missense mutations have been identified in the human *SYN1* gene in families and sporadic individuals affected by epilepsy and/or autism spectrum disorders (Fassio et al. 2011; Garcia et al. 2004; Giannandrea et al. 2013; Lignani et al. 2013). The *SYN2* gene has been recognised as a susceptibility locus for epilepsy (Cavalleri et al. 2007; Lakhan et al. 2010), and various mutations have been recently described in patients affected by autism or epilepsy (Corradi et al. 2014). This strong association with epilepsy suggests that synapsins may have a role in the control of the excitability of neuronal networks and that their absence could lead to excitation/inhibition imbalance.

13.5.1 Synapsins and Synaptic Transmission: Differential Role at Excitatory and Inhibitory Synapses

As previously mentioned, injection experiments in which either phospho- or dephosphorylated synapsin I, as well as peptides or anti-peptide antibodies, were delivered into presynaptic terminals support a model whereby dephospho-synapsins act as a physical constraint on the resting pool of synaptic vesicles, while the PKA- or CaM kinase-phosphorylated forms leave synaptic vesicles free to fuse with the plasma membrane. This action, aside from being important for the formation and maintenance of the resting pool of vesicles, implies that synapsins are involved in the fine regulation of the equilibrium between the resting and the recycling pool of vesicles, thus determining the amount of neurotransmitter released (Benfenati et al. 1991; Fassio et al. 2011 [la review di Sem. Cell. Dev. Biol.]). Indeed, the injection of dephosphorylated synapsin I into the squid giant synapse decreased the amplitude and rate of rise of postsynaptic potentials, whereas the injection of either phosphorylated or heat-inactivated protein was ineffective. Conversely, injection of CaMKII increased the amplitude and rate of rise of postsynaptic potentials (Llinas et al. 1985, 1991). The injection of antibodies directed against the domain E of synapsin I in lamprey reticulospinal presynaptic terminals caused the depletion of the resting pool of vesicles and enhanced depression following high-, but not

low-, frequency stimulation (Pieribone et al. 1995). Similar results were obtained after the injection of peptides derived from domain E and C in the squid giant synapse (Hilfiker et al. 1998, 2005).

In agreement with these observations, all synapsin KO mice, with the exception of *Syn3*$^{-/-}$ (Feng et al. 2002), display a reduction of the resting and recycling pools and, consequently, an increased synaptic fatigue under high-frequency stimulation that is particularly pronounced in glutamatergic terminals. The study of synaptic vesicle recycling at single synaptic boutons using FM dyes showed that the number of vesicles exocytosed during brief action potential trains and the total recycling pool were reduced in *Syn1*$^{-/-}$ neurons, while the kinetics of endocytosis was unaltered (Ryan et al. 1996). Similar results were obtained using the pH-sensitive probe synaptopHluorin in triple KO neurons, in which the recycling pool was significantly reduced, while the readily releasable pool was unaffected (Fornasiero et al. 2012). In *Syn1*$^{-/-}$ mice glutamate release from cortical synaptosomes was impaired and the recovery of synaptic transmission after high-frequency stimulation was delayed (Li et al. 1995). A moderate increase of synaptic depression was observed also at inhibitory synapses of cultured *Syn1*$^{-/-}$ neurons at high stimulation frequencies, accompanied by a slowdown of recovery from depression (Baldelli et al. 2007). Synaptic depression after repetitive stimulation was increased at excitatory, but not inhibitory, neurons in *Syn2*$^{-/-}$ (Medrihan et al. 2013; Rosahl et al. 1995) and further enhanced in excitatory terminals of *Syn1/2*$^{-/-}$ (Rosahl et al. 1995), as well as triple KO animals (Farisello et al. 2013; Gitler et al. 2004a; Vasileva et al. 2012), probably because of defective resting pool size and recruitment of vesicles to the readily releasable pool.

Overexpression of synapsin Ia in the rat calyx of Held resulted in the redistribution of vesicles in the terminal and increased depression during repetitive stimulation, possibly because the excess synapsin molecules favoured the formation of dimers not associated with the vesicle membrane, thereby interfering with synaptic vesicle clustering. Recovery from depression was accelerated, possibly through synapsin-actin interactions that speeded up vesicle endocytosis and recycling (Vasileva et al. 2013).

Conversely, mice lacking synapsin III exhibit no change in vesicle density, an increase in the size of the recycling pool and a significant reduction in synaptic depression (Feng et al. 2002), suggesting that synapsin III does not participate to the maintenance of the resting pool but is involved in limiting the number of recycling vesicles.

In addition to the pre-docking roles mainly responsible for the effects described so far, post-docking roles have also been hypothesised for synapsins, contributing to the alteration of neurotransmitter release observed upon isolated action potentials when synapsin function is perturbed. The injection of domain E and C peptides significantly reduced the amplitude and slowed the kinetics of evoked excitatory postsynaptic currents (eEPSCs), without altering the number of docked vesicles (Hilfiker et al. 1998). The lack of synapsin I induced a decrease in the amplitude of the evoked inhibitory postsynaptic currents (eIPSCs) in cultured *Syn1*$^{-/-}$ hippocampal autaptic neurons due to a reduced number of vesicles released per

single action potential and a reduced readily releasable pool, with no change in the probability of release, quantal size or number of synaptic contacts (Baldelli et al. 2007; Chiappalone et al. 2009). Conversely, the lack of synapsin II leads to an increase in the amplitude of eIPSCs in the dentate gyrus granule neurons (Medrihan et al. 2013). A decrease of eIPSC amplitude was observed in autaptic neurons from *Syn3*$^{-/-}$ (Feng et al. 2002).

In triple KO neurons the reduction in eIPSC amplitude was accompanied by a decreased number of docked vesicles in inhibitory terminals, as assessed by electron microscopy (Gitler et al. 2004a). Opposite to inhibitory transmission, *Syn1*$^{-/-}$ cortical autaptic neurons displayed an increase in eEPSC amplitude attributable to an increased readily releasable pool, with no change in release probability (Chiappalone et al. 2009).

It is worth to note that spontaneous synaptic transmission is unaffected in the absence of synapsins, as the amplitude, frequency and kinetics of both miniature EPSCs and IPSCs were essentially unchanged in all synapsin KO mice (Baldelli et al. 2007; Chiappalone et al. 2009; Feliciano et al. 2013; Feng et al. 2002; Gitler et al. 2004a), indicating that the number of synaptic contacts and the postsynaptic apparatus are comparable in WT and KO neurons.

A more specific role for synapsin II in the regulation of GABA release has been recently unravelled. In granule neurons of the dentate gyrus, synapsin II deletion resulted in higher amplitude and faster kinetics of eIPSCs, due to an increase in the synchronously released fraction of the readily releasable pool. High-frequency stimulation causes, in addition to synchronous release, a delayed asynchronous release component that lasts up to seconds. The absence of synapsin II selectively and dramatically hampered asynchronous release at inhibitory, but not excitatory, synapses. Synapsin II controlled the amount of the Ca^{2+}-dependent asynchronous release, through the interaction with the P/Q-type of Ca^{2+} channels. Opposite to synapsin II, synapsin I deletion increased the asynchronous release at inhibitory synapses (Medrihan et al. 2013). In this scenario, synapsins I and II appear to compete at a post-docking step on the same readily releasable pool of inhibitory vesicles, by either facilitating release synchronisation or desynchronising GABA release, respectively.

The results described so far highlight the differential effects of the synapsins in excitatory and inhibitory transmission. This difference may at least partly stem from differential expression levels of synapsin isoforms in the two neuronal subtypes. As an example, excitatory synapses positive for VGLUT1 (vesicular glutamate transporter 1) and inhibitory terminals positive for VGAT (vesicular GABA transporter) were shown to express the highest and the lowest levels of synapsin I, respectively (Micheva et al. 2010). Interestingly, other vesicular/presynaptic proteins, such as SV2B, synaptophysin, syntaxin 1A, SNAP-25 or 23 and synaptotagmins, showed a preferential expression in VGLUT1- compared to VGAT-positive synapses, suggesting that probably the release machineries in the two kinds of synaptic terminals slightly differ in order to match distinct physiological needs (Bragina et al. 2007; Gronborg et al. 2010). Synapsin ablation seems to have a stronger effect on the depression of excitatory synapses, but the milder

effect observed in inhibitory synapses could have a higher impact on the network excitability, because of the high-frequency bursting activity that characterises inhibitory neurons and makes them more sensitive to vesicle depletion. At the post-docking level, synapsin deletion mainly impairs inhibitory transmission, while leaving basal excitatory transmission unaffected or even enhanced.

The final outcome of these effects is an imbalance between excitatory and inhibitory inputs, with a hyperexcitability of the network that is probably at the basis of the epileptic phenotype observed in all synapsin KO mice, but $Syn3^{-/-}$. The absence of the epileptic phenotype in $Syn3^{-/-}$ mice strengthens the hypothesis that Syn III plays a differential role with respect to the other isoforms, as suggested by electrophysiology (Feng et al. 2002) and by the observation that its expression progressively drops in the adult life.

The hyperexcitability of mature Syn-deficient neuronal networks was investigated through the use of the multi-electrode array (MEA) platform. Primary cultures of cortical neurons derived from $Syn1^{-/-}$ mice displayed a diffuse spontaneous hyperactivity characterised by a general increase in firing rate, bursting rate and burst duration, as well as a higher degree of activity synchronisation, that became more evident at later stages of network maturation (Chiappalone et al. 2009). Studies in acute cortico-hippocampal brain slices of 3-week-old triple KO mice revealed that hyperexcitability of synapsin KO circuits is detectable before the incidence of manifest epileptic seizures, which arise in the animals at 2 months of age. Slices treated with the K^+ channel blocker 4-aminopyridine were characterised by a higher incidence of fast glutamatergic inter-ictal events and a wider spread of ictal activity to the surrounding cortical areas with respect to age-matched WT slices (Boido et al. 2010). The membrane potential of CA1 hippocampal triple KO neurons was found to be more depolarised than in WT cells in presymptomatic mice and even more so in adult animals. This observation was attributed to a decrease in tonic GABA currents, due to a defective GABA release and spillover (Farisello et al. 2013).

In addition to the differential effects of synapsin deletion on the intrinsic characteristics of excitatory and inhibitory neurons, the net effect on a formed neuronal network also depends on dynamic adaptations to an altered equilibrium (homeostatic plasticity), synaptic plasticity phenomena and developmental components. The existence of compensatory adaptations was recently proposed, starting from the observation that the GABA-synthesising enzyme GAD67 was upregulated in triple KO animals (Ketzef et al. 2011). An increase in inhibitory transmission in specific developmental windows might partly contribute to suppress the seizures until 2 months of age or, vice versa, it might contribute to epileptogenesis and network hypersynchronisation. Indeed, it was recently shown that the altered inhibitory activity is subjected to developmental modulation in synapsin KO mice: sIPSC amplitude and frequency (recorded from layer 5 pyramidal neurons of the entorhinal cortex) were indeed reduced in 2/3-week-old triple KO slices with respect to WT; the differences between genotypes disappeared between 1.5 and 3.5 months of age; in mice older than 7 months of age, sIPSC amplitude and frequency were larger in KO than in WT (Ketzef and Gitler 2014). This may indicate that the failure of phasic inhibitory transmission at the beginning of KO

mice development may contribute to epileptogenesis, but then compensatory mechanisms emerge that try to counteract the established epilepsy, but ultimately aggravate the network synchronisation.

13.5.2 Involvement of Synapsins in Synaptic Plasticity Phenomena

A vast amount of studies have been performed, aimed at analysing the potential involvement of synapsins in short- and long-term plasticity phenomena in the brain.

Concerning short-term plasticity phenomena, the most studied paradigms are paired-pulse facilitation or depression (PPF or PPD, respectively) and post-tetanic potentiation (PTP) (Zucker and Regehr 2002). PPF was increased in excitatory cortical neurons from *Syn1*$^{-/-}$ mice (Chiappalone et al. 2009), as well as in CA1 pyramidal neurons of *Syn1*$^{-/-}$ hippocampal slices (Rosahl et al. 1993). Synapsin I deletion did not affect the paired-pulse ratio in inhibitory neurons (Baldelli et al. 2007; Chiappalone et al. 2009). *Syn2*$^{-/-}$ CA1 pyramidal neurons (Rosahl et al. 1995) and *Syn3*$^{-/-}$ cultured hippocampal neurons (Feng et al. 2002) showed no changes in PPF. PPD was not affected in *Syn2*$^{-/-}$ at short inter-stimulus intervals but was increased at longer intervals (2–3 s) (Medrihan et al. 2013). Interestingly, in triple KO CA1 pyramidal neurons, PPF was increased in young presymptomatic animals but normalised to the WT condition in adult epileptic mice. PPD was slightly increased in young triple KO mice at short inter-stimulus intervals, while it was significantly lower in adult epileptic mice compared to WT (Farisello et al. 2013). The paired-pulse ratio at short inter-stimulus intervals has an intrinsic kinetics of milliseconds, too fast to be dependent on vesicle mobilisation from the resting pool. Thus, synapsins probably affect PPF and PPD through post-docking mechanisms.

PTP was unchanged in *Syn1*$^{-/-}$ slices with respect to WT, while it was decreased in *Syn2*$^{-/-}$ and even more affected in double KO preparations, indicating that both synapsins I and II participate in modulating this short-term increase in synaptic efficacy (Rosahl et al. 1995). PTP was completely abolished in triple KO slices compared to WT (Farisello et al. 2013). PTP of inhibitory transmission was not affected in *Syn1*$^{-/-}$ neurons (Baldelli et al. 2007). PTP operates in a timescale of seconds and probably involves the Ca^{2+}-dependent mobilisation of synaptic vesicles from the resting pool. Thus, synapsins are likely to affect this short-term plasticity paradigm at a pre-docking step, by facilitating vesicle recruitment upon their phosphorylation.

Recently, a new form of short-term plasticity has been described in the CA3-to-CA1 hippocampal synapses. During prolonged stimulation at 5–20 Hz, after a first rapid increase in synaptic efficacy and a subsequent decay, a second delayed response enhancement (DRE) arises in 3–4 s and lasts for approximately

75 additional stimuli. The DRE is strictly dependent on temperature, as it clearly appears at 37 °C but not at 24 °C, on the presence of an intact actin cytoskeleton and of synapsin I and/or II, as it completely disappeared in synapsin I/II double KO hippocampal slices (Bogen et al. 2009; Jensen et al. 2007). This form of plasticity may be important for maintaining synaptic activity during prolonged stimulation periods and may rely both on the synapsin-mediated recruitment of synaptic vesicles from the resting pool and on post-docking functions of these proteins.

Mice deleted for synapsin genes do not exhibit long-term potentiation (LTP) defects (Li et al. 1995; Rosahl et al. 1995; Spillane et al. 1995) and display mild memory and learning deficits (Corradi et al. 2008; Gitler et al. 2004a; Greco et al. 2013; Silva et al. 1996). However, a primary role of synapsin I in mediating LTP and learning has been recently proposed. Synapsin I and ERK were shown to be phosphorylated during contextual fear conditioning. In addition, transgenic mice overexpressing a constitutively active form of $p21^{Ras}$, a potent upstream synaptic activator of ERK, displayed an increased phosphorylation of both ERK and synapsin I (site 4/5) and enhanced presynaptic short-term plasticity and LTP induced by high-frequency stimulation. These functional effects were dependent on the presence of synapsin I and were probably mediated by a facilitation of neurotransmitter release due to increased recruitment of synaptic vesicles from the resting pool to the readily releasable pool, as the number of docked vesicles and the frequency of miniature EPSCs were higher in transgenic mice with respect to WT mice. The activation of the $p21^{Ras}$/ERK/synapsin I signalling pathway was accompanied by enhancement of hippocampus-dependent learning abilities of transgenic mice mediated by synapsin I, as this effect was abolished by crossing transgenic mice with synapsin I KO animals (Kushner et al. 2005). BDNF may be the trigger of $p21^{Ras}$/ERK/synapsin I signalling activation during learning (Tyler et al. 2002). A role for BDNF has already been proved for PTP, which is enhanced by BDNF-driven MAPK phosphorylation of synapsin I (Valente et al. 2012).

Synapsin I levels were found to be increased during LTP and spatial learning in the rat hippocampus (Gomez-Pinilla et al. 2001; Sato et al. 2000), as well as at the sensorimotor synapse in the invertebrate model *Aplysia* in which potentiation had been induced with serotonin treatment (Hart et al. 2011). In the latter case, the serotonin-induced increase in synapsin levels was required for the establishment of LTP, as acute administration of small interfering RNAs that blocked synapsin induction completely abolished synaptic potentiation (Hart et al. 2011).

Synapsin was also implicated in learning and behavioural paradigms in *Drosophila*. In fact, the protein is necessary for the formation of odour-sugar memory traces in the mushroom bodies and requires functional PKA phosphorylation sites to exert this effect (Michels et al. 2011). Synapsin function is specifically required in GABAergic interneurons for short-term, but not long-term, habituation of olfactory avoidance behaviour, and this effect implies CaMKII phosphorylation (Sadanandappa et al. 2013).

13.6 Concluding Remarks

Synapsins have been classically studied for their contribution to synaptic vesicle storage in the presynaptic terminal. Recent evidences described in this chapter point to the fact that, although synapsins play a crucial role in the maintenance of synaptic vesicle clusters, this phenomenon involves also other mechanisms and that, on the other hand, many other functions can be envisaged for synapsins both at the pre- and the post-docking levels. In addition, differential roles start to be depicted for each synapsin isoform. How the various isoforms distribute in the central nervous system and take part in the definition of the functional nature of each neuronal subpopulation is far from being completely understood.

Mutations in synapsins have been strongly linked to epilepsy and autism spectrum disorders (Corradi et al. 2014; Fassio et al. 2011; Garcia et al. 2004), and several polymorphisms have been positively associated with schizophrenia and other psychosis (Chen et al. 2004a, b; Lee et al. 2005; Saviouk et al. 2007). Thus, the thorough unravelling of the precise roles exerted by synapsins under physiological and pathological conditions is fundamental for envisaging new therapeutic approaches for these unresolved diseases.

References

Bahler M, Greengard P (1987) Synapsin I bundles F-actin in a phosphorylation-dependent manner. Nature 326:704–707

Bahler M, Benfenati F, Valtorta F, Czernik AJ, Greengard P (1989) Characterization of synapsin I fragments produced by cysteine-specific cleavage: a study of their interactions with F-actin. J Cell Biol 108:1841–1849

Baldelli P, Fassio A, Valtorta F, Benfenati F (2007) Lack of synapsin I reduces the readily releasable pool of synaptic vesicles at central inhibitory synapses. J Neurosci 27:13520–13531

Benfenati F, Bahler M, Jahn R, Greengard P (1989a) Interactions of synapsin I with small synaptic vesicles: distinct sites in synapsin I bind to vesicle phospholipids and vesicle proteins. J Cell Biol 108:1863–1872

Benfenati F, Greengard P, Brunner J, Bahler M (1989b) Electrostatic and hydrophobic interactions of synapsin I and synapsin I fragments with phospholipid bilayers. J Cell Biol 108:1851–1862

Benfenati F, Valtorta F, Bahler M, Greengard P (1989c) Synapsin I, a neuron-specific phosphoprotein interacting with small synaptic vesicles and F-actin. Cell Biol Int Rep 13:1007–1021

Benfenati F, Neyroz P, Bahler M, Masotti L, Greengard P (1990) Time-resolved fluorescence study of the neuron-specific phosphoprotein synapsin I. Evidence for phosphorylation-dependent conformational changes. J Biol Chem 265:12584–12595

Benfenati F, Valtorta F, Greengard P (1991) Computer modeling of synapsin I binding to synaptic vesicles and F-actin: implications for regulation of neurotransmitter release. Proc Natl Acad Sci U S A 88:575–579

Benfenati F, Valtorta F, Rubenstein JL, Gorelick FS, Greengard P, Czernik AJ (1992) Synaptic vesicle-associated Ca^{2+}/calmodulin-dependent protein kinase II is a binding protein for synapsin I. Nature 359:417–420

Benfenati F, Valtorta F, Rossi MC, Onofri F, Sihra T, Greengard P (1993) Interactions of synapsin I with phospholipids: possible role in synaptic vesicle clustering and in the maintenance of bilayer structures. J Cell Biol 123:1845–1855

Bloom O, Evergren E, Tomilin N, Kjaerulff O, Low P, Brodin L, Pieribone VA, Greengard P, Shupliakov O (2003) Colocalization of synapsin and actin during synaptic vesicle recycling. J Cell Biol 161:737–747

Bogen IL, Jensen V, Hvalby O, Walaas SI (2009) Synapsin-dependent development of glutamatergic synaptic vesicles and presynaptic plasticity in postnatal mouse brain. Neuroscience 158:231–241

Boido D, Farisello P, Cesca F, Ferrea E, Valtorta F, Benfenati F, Baldelli P (2010) Cortico-hippocampal hyperexcitability in synapsin I/II/III knockout mice: age-dependency and response to the antiepileptic drug levetiracetam. Neuroscience 171:268–283

Bonanomi D, Menegon A, Miccio A, Ferrari G, Corradi A, Kao HT, Benfenati F, Valtorta F (2005) Phosphorylation of synapsin I by cAMP-dependent protein kinase controls synaptic vesicle dynamics in developing neurons. J Neurosci 25:7299–7308

Bragina L, Candiracci C, Barbaresi P, Giovedi S, Benfenati F, Conti F (2007) Heterogeneity of glutamatergic and GABAergic release machinery in cerebral cortex. Neuroscience 146:1829–1840

Brautigam CA, Chelliah Y, Deisenhofer J (2004) Tetramerization and ATP binding by a protein comprising the A, B, and C domains of rat synapsin I. J Biol Chem 279:11948–11956

Cambiaghi M, Cursi M, Monzani E, Benfenati F, Comi G, Minicucci F, Valtorta F, Leocani L (2013) Temporal evolution of neurophysiological and behavioral features of synapsin I/II/III triple knock-out mice. Epilepsy Res 103:153–160

Cavalleri GL, Weale ME, Shianna KV, Singh R, Lynch JM, Grinton B, Szoeke C, Murphy K, Kinirons P, O'Rourke D, Ge D, Depondt C, Claeys KG, Pandolfo M, Gumbs C, Walley N, McNamara J, Mulley JC, Linney KN, Sheffield LJ, Radtke RA, Tate SK, Chissoe SL, Gibson RA, Hosford D, Stanton A, Graves TD, Hanna MG, Eriksson K, Kantanen AM, Kalviainen R, O'Brien TJ, Sander JW, Duncan JS, Scheffer IE, Berkovic SF, Wood NW, Doherty CP, Delanty N, Sisodiya SM, Goldstein DB (2007) Multicentre search for genetic susceptibility loci in sporadic epilepsy syndrome and seizure types: a case-control study. Lancet Neurol 6:970–980

Ceccaldi PE, Grohovaz F, Benfenati F, Chieregatti E, Greengard P, Valtorta F (1995) Dephosphorylated synapsin I anchors synaptic vesicles to actin cytoskeleton: an analysis by videomicroscopy. J Cell Biol 128:905–912

Cesca F, Baldelli P, Valtorta F, Benfenati F (2010) The synapsins: key actors of synapse function and plasticity. Prog Neurobiol 91:313–348

Cheetham JJ, Hilfiker S, Benfenati F, Weber T, Greengard P, Czernik AJ (2001) Identification of synapsin I peptides that insert into lipid membranes. Biochem J 354:57–66

Cheetham JJ, Murray J, Ruhkalova M, Cuccia L, McAloney R, Ingold KU, Johnston LJ (2003) Interaction of synapsin I with membranes. Biochem Biophys Res Commun 309:823–829

Chen Q, He G, Qin W, Chen QY, Zhao XZ, Duan SW, Liu XM, Feng GY, Xu YF, St Clair D, Li M, Wang JH, Xing YL, Shi JG, He L (2004a) Family-based association study of synapsin II and schizophrenia. Am J Hum Genet 75:873–877

Chen Q, He G, Wang XY, Chen QY, Liu XM, Gu ZZ, Liu J, Li KQ, Wang SJ, Zhu SM, Feng GY, He L (2004b) Positive association between synapsin II and schizophrenia. Biol Psychiatry 56:177–181

Chi P, Greengard P, Ryan TA (2001) Synapsin dispersion and reclustering during synaptic activity. Nat Neurosci 4:1187–1193

Chi P, Greengard P, Ryan TA (2003) Synaptic vesicle mobilization is regulated by distinct synapsin I phosphorylation pathways at different frequencies. Neuron 38:69–78

Chiappalone M, Casagrande S, Tedesco M, Valtorta F, Baldelli P, Martinoia S, Benfenati F (2009) Opposite changes in glutamatergic and GABAergic transmission underlie the diffuse hyperexcitability of synapsin I-deficient cortical networks. Cereb Cortex 19:1422–1439

Chin LS, Li L, Greengard P (1994) Neuron-specific expression of the synapsin II gene is directed by a specific core promoter and upstream regulatory elements. J Biol Chem 269:18507–18513

Cole RN, Hart GW (1999) Glycosylation sites flank phosphorylation sites on synapsin I: O-linked N-acetylglucosamine residues are localized within domains mediating synapsin I interactions. J Neurochem 73:418–428

Corradi A, Zanardi A, Giacomini C, Onofri F, Valtorta F, Zoli M, Benfenati F (2008) Synapsin-I- and synapsin-II-null mice display an increased age-dependent cognitive impairment. J Cell Sci 121:3042–3051

Corradi A, Fadda M, Piton A, Patry L, Marte A, Rossi P, Cadieux-Dion M, Gauthier J, Lapointe L, Mottron L, Valtorta F, Rouleau GA, Fassio A, Benfenati F, Cossette P (2014) SYN2 is an autism predisposing gene: loss-of-function mutations alter synaptic vesicle cycling and axon outgrowth. Hum Mol Genet 23:90–103

Cousin MA, Robinson PJ (2001) The dephosphins: dephosphorylation by calcineurin triggers synaptic vesicle endocytosis. Trends Neurosci 24:659–665

Czernik AJ, Pang DT, Greengard P (1987) Amino acid sequences surrounding the cAMP-dependent and calcium/calmodulin-dependent phosphorylation sites in rat and bovine synapsin I. Proc Natl Acad Sci U S A 84:7518–7522

Daly C, Ziff EB (1997) Post-transcriptional regulation of synaptic vesicle protein expression and the developmental control of synaptic vesicle formation. J Neurosci 17:2365–2375

De Camilli P, Cameron R, Greengard P (1983a) Synapsin I (protein I), a nerve terminal-specific phosphoprotein. I. Its general distribution in synapses of the central and peripheral nervous system demonstrated by immunofluorescence in frozen and plastic sections. J Cell Biol 96:1337–1354

De Camilli P, Harris SM Jr, Huttner WB, Greengard P (1983b) Synapsin I (Protein I), a nerve terminal-specific phosphoprotein. II. Its specific association with synaptic vesicles demonstrated by immunocytochemistry in agarose-embedded synaptosomes. J Cell Biol 96:1355–1373

De Camilli P, Benfenati F, Valtorta F, Greengard P (1990) The synapsins. Annu Rev Cell Biol 6:433–460

Ekici M, Schmitz F, Hohl M, Seigel GM, Thiel G (2008) Chromatin structure and expression of synapsin I and synaptophysin in retinal precursor cells. Neurochem Int 53:165–172

Esser L, Wang CR, Hosaka M, Smagula CS, Südhof TC, Deisenhofer J (1998) Synapsin I is structurally similar to ATP-utilizing enzymes. EMBO J 17:977–984

Etholm L, Linden H, Eken T, Heggelund P (2011) Electroencephalographic characterization of seizure activity in the synapsin I/II double knockout mouse. Brain Res 1383:270–288

Evergren E, Zotova E, Brodin L, Shupliakov O (2006) Differential efficiency of the endocytic machinery in tonic and phasic synapses. Neuroscience 141:123–131

Farisello P, Boido D, Nieus T, Medrihan L, Cesca F, Valtorta F, Baldelli P, Benfenati F (2013) Synaptic and extrasynaptic origin of the excitation/inhibition imbalance in the hippocampus of synapsin I/II/III knockout mice. Cereb Cortex 23:581–593

Fassio A, Merlo D, Mapelli J, Menegon A, Corradi A, Mete M, Zappettini S, Bonanno G, Valtorta F, D'Angelo E, Benfenati F (2006) The synapsin domain E accelerates the exoendocytotic cycle of synaptic vesicles in cerebellar Purkinje cells. J Cell Sci 119:4257–4268

Fassio A, Patry L, Congia S, Onofri F, Piton A, Gauthier J, Pozzi D, Messa M, Defranchi E, Fadda M, Corradi A, Baldelli P, Lapointe L, St-Onge J, Meloche C, Mottron L, Valtorta F, Khoa Nguyen D, Rouleau GA, Benfenati F, Cossette P (2011) SYN1 loss-of-function mutations in autism and partial epilepsy cause impaired synaptic function. Hum Mol Genet 20:2297–2307

Feliciano P, Andrade R, Bykhovskaia M (2013) Synapsin II and Rab3a cooperate in the regulation of epileptic and synaptic activity in the CA1 region of the hippocampus. J Neurosci 33:18319–18330

Feng J, Chi P, Blanpied TA, Xu Y, Magarinos AM, Ferreira A, Takahashi RH, Kao HT, McEwen BS, Ryan TA, Augustine GJ, Greengard P (2002) Regulation of neurotransmitter release by synapsin III. J Neurosci 22:4372–4380

Ferreira A, Kao HT, Feng J, Rapoport M, Greengard P (2000) Synapsin III: developmental expression, subcellular localization, and role in axon formation. J Neurosci 20:3736–3744

Fesce R, Benfenati F, Greengard P, Valtorta F (1992) Effects of the neuronal phosphoprotein synapsin I on actin polymerization. II. Analytical interpretation of kinetic curves. J Biol Chem 267:11289–11299

Forn J, Greengard P (1978) Depolarizing agents and cyclic nucleotides regulate the phosphorylation of specific neuronal proteins in rat cerebral cortex slices. Proc Natl Acad Sci U S A 75:5195–5199

Fornasiero EF, Bonanomi D, Benfenati F, Valtorta F (2010) The role of synapsins in neuronal development. Cell Mol Life Sci 67:1383–1396

Fornasiero EF, Raimondi A, Guarnieri FC, Orlando M, Fesce R, Benfenati F, Valtorta F (2012) Synapsins contribute to the dynamic spatial organization of synaptic vesicles in an activity-dependent manner. J Neurosci 32:12214–12227

Garcia CC, Blair HJ, Seager M, Coulthard A, Tennant S, Buddles M, Curtis A, Goodship JA (2004) Identification of a mutation in synapsin I, a synaptic vesicle protein, in a family with epilepsy. J Med Genet 41:183–186

Geppert M, Südhof TC (1998) RAB3 and synaptotagmin: the yin and yang of synaptic membrane fusion. Annu Rev Neurosci 21:75–95

Giannandrea M, Guarnieri FC, Gehring NH, Monzani E, Benfenati F, Kulozik AE, Valtorta F (2013) Nonsense-mediated mRNA decay and loss-of-function of the protein underlie the X-linked epilepsy associated with the W356x mutation in synapsin I. PLoS One 8:e67724

Giovedi S, Darchen F, Valtorta F, Greengard P, Benfenati F (2004a) Synapsin is a novel Rab3 effector protein on small synaptic vesicles. II. Functional effects of the Rab3A-synapsin I interaction. J Biol Chem 279:43769–43779

Giovedi S, Vaccaro P, Valtorta F, Darchen F, Greengard P, Cesareni G, Benfenati F (2004b) Synapsin is a novel Rab3 effector protein on small synaptic vesicles. I. Identification and characterization of the synapsin I-Rab3 interactions in vitro and in intact nerve terminals. J Biol Chem 279:43760–43768

Gitler D, Takagishi Y, Feng J, Ren Y, Rodriguiz RM, Wetsel WC, Greengard P, Augustine GJ (2004a) Different presynaptic roles of synapsins at excitatory and inhibitory synapses. J Neurosci 24:11368–11380

Gitler D, Xu Y, Kao HT, Lin D, Lim S, Feng J, Greengard P, Augustine GJ (2004b) Molecular determinants of synapsin targeting to presynaptic terminals. J Neurosci 24:3711–3720

Gitler D, Cheng Q, Greengard P, Augustine GJ (2008) Synapsin IIa controls the reserve pool of glutamatergic synaptic vesicles. J Neurosci 28:10835–10843

Gomez-Pinilla F, So V, Kesslak JP (2001) Spatial learning induces neurotrophin receptor and synapsin I in the hippocampus. Brain Res 904:13–19

Greco B, Manago F, Tucci V, Kao HT, Valtorta F, Benfenati F (2013) Autism-related behavioral abnormalities in synapsin knockout mice. Behav Brain Res 251:65–74

Greengard P, Browning MD, McGuinness TL, Llinas R (1987) Synapsin I, a phosphoprotein associated with synaptic vesicles: possible role in regulation of neurotransmitter release. Adv Exp Med Biol 221:135–153

Greengard P, Valtorta F, Czernik AJ, Benfenati F (1993) Synaptic vesicle phosphoproteins and regulation of synaptic function. Science 259:780–785

Gronborg M, Pavlos NJ, Brunk I, Chua JJ, Munster-Wandowski A, Riedel D, Ahnert-Hilger G, Urlaub H, Jahn R (2010) Quantitative comparison of glutamatergic and GABAergic synaptic vesicles unveils selectivity for few proteins including MAL2, a novel synaptic vesicle protein. J Neurosci 30:2–12

Hart AK, Fioravante D, Liu RY, Phares GA, Cleary LJ, Byrne JH (2011) Serotonin-mediated synapsin expression is necessary for long-term facilitation of the Aplysia sensorimotor synapse. J Neurosci 31:18401–18411

Haycock JW, Greengard P, Browning MD (1988) Cholinergic regulation of protein III phosphorylation in bovine adrenal chromaffin cells. J Neurosci 8:3233–3239

Hilfiker S, Schweizer FE, Kao HT, Czernik AJ, Greengard P, Augustine GJ (1998) Two sites of action for synapsin domain E in regulating neurotransmitter release. Nat Neurosci 1:29–35

Hilfiker S, Benfenati F, Doussau F, Nairn AC, Czernik AJ, Augustine GJ, Greengard P (2005) Structural domains involved in the regulation of transmitter release by synapsins. J Neurosci 25:2658–2669

Hoesche C, Bartsch P, Kilimann MW (1995) The CRE consensus sequence in the synapsin I gene promoter region confers constitutive activation but no regulation by cAMP in neuroblastoma cells. Biochim Biophys Acta 1261:249–256

Hosaka M, Südhof TC (1999) Homo- and heterodimerization of synapsins. J Biol Chem 274:16747–16753

Huttner WB, DeGennaro LJ, Greengard P (1981) Differential phosphorylation of multiple sites in purified protein I by cyclic AMP-dependent and calcium-dependent protein kinases. J Biol Chem 256:1482–1488

Huttner WB, Schiebler W, Greengard P, De Camilli P (1983) Synapsin I (protein I), a nerve terminal-specific phosphoprotein. III. Its association with synaptic vesicles studied in a highly purified synaptic vesicle preparation. J Cell Biol 96:1374–1388

James AB, Conway AM, Thiel G, Morris BJ (2004) Egr-1 modulation of synapsin I expression: permissive effect of forskolin via cAMP. Cell Signal 16:1355–1362

Jensen V, Walaas SI, Hilfiker S, Ruiz A, Hvalby O (2007) A delayed response enhancement during hippocampal presynaptic plasticity in mice. J Physiol 583:129–143

John JP, Chen WQ, Pollak A, Lubec G (2007) Mass spectrometric studies on mouse hippocampal synapsins Ia, IIa, and IIb and identification of a novel phosphorylation site at serine-546. J Proteome Res 6:2695–2710

Johnson EM, Ueda T, Maeno H, Greengard P (1972) Adenosine 3',5-monophosphate-dependent phosphorylation of a specific protein in synaptic membrane fractions from rat cerebrum. J Biol Chem 247:5650–5652

Jovanovic JN, Benfenati F, Siow YL, Sihra TS, Sanghera JS, Pelech SL, Greengard P, Czernik AJ (1996) Neurotrophins stimulate phosphorylation of synapsin I by MAP kinase and regulate synapsin I-actin interactions. Proc Natl Acad Sci U S A 93:3679–3683

Jovanovic JN, Czernik AJ, Fienberg AA, Greengard P, Sihra TS (2000) Synapsins as mediators of BDNF-enhanced neurotransmitter release. Nat Neurosci 3:323–329

Jovanovic JN, Sihra TS, Nairn AC, Hemmings HC Jr, Greengard P, Czernik AJ (2001) Opposing changes in phosphorylation of specific sites in synapsin I during Ca2+−dependent glutamate release in isolated nerve terminals. J Neurosci 21:7944–7953

Kao HT, Porton B, Czernik AJ, Feng J, Yiu G, Haring M, Benfenati F, Greengard P (1998) A third member of the synapsin gene family. Proc Natl Acad Sci U S A 95:4667–4672

Kao HT, Porton B, Hilfiker S, Stefani G, Pieribone VA, DeSalle R, Greengard P (1999) Molecular evolution of the synapsin gene family. J Exp Zool 285:360–377

Ketzef M, Gitler D (2014) Epileptic synapsin triple knockout mice exhibit progressive long-term aberrant plasticity in the entorhinal cortex. Cereb Cortex 24(4):996–1008

Ketzef M, Kahn J, Weissberg I, Becker AJ, Friedman A, Gitler D (2011) Compensatory network alterations upon onset of epilepsy in synapsin triple knock-out mice. Neuroscience 189:108–122

Kielland A, Erisir A, Walaas SI, Heggelund P (2006) Synapsin utilization differs among functional classes of synapses on thalamocortical cells. J Neurosci 26:5786–5793

Kim SH, Ryan TA (2010) CDK5 serves as a major control point in neurotransmitter release. Neuron 67:797–809

Krabben L, Fassio A, Bhatia VK, Pechstein A, Onofri F, Fadda M, Messa M, Rao Y, Shupliakov O, Stamou D, Benfenati F, Haucke V (2011) Synapsin I senses membrane curvature by an amphipathic lipid packing sensor motif. J Neurosci 31:18149–18154

Kushner SA, Elgersma Y, Murphy GG, Jaarsma D, van Woerden GM, Hojjati MR, Cui Y, LeBoutillier JC, Marrone DF, Choi ES, De Zeeuw CI, Petit TL, Pozzo-Miller L, Silva AJ (2005) Modulation of presynaptic plasticity and learning by the H-ras/extracellular signal-regulated kinase/synapsin I signaling pathway. J Neurosci 25:9721–9734

Lakhan R, Kalita J, Misra UK, Kumari R, Mittal B (2010) Association of intronic polymorphism rs3773364 A > G in synapsin-2 gene with idiopathic epilepsy. Synapse 64:403–408

Lee HJ, Song JY, Kim JW, Jin SY, Hong MS, Park JK, Chung JH, Shibata H, Fukumaki Y (2005) Association study of polymorphisms in synaptic vesicle-associated genes, SYN2 and CPLX2, with schizophrenia. Behav Brain Funct 1:15

Li L, Suzuki T, Mori N, Greengard P (1993) Identification of a functional silencer element involved in neuron-specific expression of the synapsin I gene. Proc Natl Acad Sci U S A 90:1460–1464

Li L, Chin LS, Shupliakov O, Brodin L, Sihra TS, Hvalby O, Jensen V, Zheng D, McNamara JO, Greengard P et al (1995) Impairment of synaptic vesicle clustering and of synaptic transmission, and increased seizure propensity, in synapsin I-deficient mice. Proc Natl Acad Sci U S A 92:9235–9239

Li J, Han YR, Plummer MR, Herrup K (2009) Cytoplasmic ATM in neurons modulates synaptic function. Curr Biol 19:2091–2096

Lignani G, Raimondi A, Ferrea E, Rocchi A, Paonessa F, Cesca F, Orlando M, Tkatch T, Valtorta F, Cossette P, Baldelli P, Benfenati F (2013) Epileptogenic Q555X SYN1 mutant triggers imbalances in release dynamics and short-term plasticity. Hum Mol Genet 22:2186–2199

Llinas R, McGuinness TL, Leonard CS, Sugimori M, Greengard P (1985) Intraterminal injection of synapsin I or calcium/calmodulin-dependent protein kinase II alters neurotransmitter release at the squid giant synapse. Proc Natl Acad Sci U S A 82:3035–3039

Llinas R, Gruner JA, Sugimori M, McGuinness TL, Greengard P (1991) Regulation by synapsin I and Ca(2+)-calmodulin-dependent protein kinase II of the transmitter release in squid giant synapse. J Physiol 436:257–282

Luthi T, Haltiwanger RS, Greengard P, Bahler M (1991) Synapsins contain O-linked N-acetylglucosamine. J Neurochem 56:1493–1498

Maienschein V, Marxen M, Volknandt W, Zimmermann H (1999) A plethora of presynaptic proteins associated with ATP-storing organelles in cultured astrocytes. Glia 26:233–244

Mandell JW, Townes-Anderson E, Czernik AJ, Cameron R, Greengard P, De Camilli P (1990) Synapsins in the vertebrate retina: absence from ribbon synapses and heterogeneous distribution among conventional synapses. Neuron 5:19–33

Matsubara M, Kusubata M, Ishiguro K, Uchida T, Titani K, Taniguchi H (1996) Site-specific phosphorylation of synapsin I by mitogen-activated protein kinase and Cdk5 and its effects on physiological functions. J Biol Chem 271:21108–21113

Medrihan L, Cesca F, Raimondi A, Lignani G, Baldelli P, Benfenati F (2013) Synapsin II desynchronizes neurotransmitter release at inhibitory synapses by interacting with presynaptic calcium channels. Nat Commun 4:1512

Menegon A, Verderio C, Leoni C, Benfenati F, Czernik AJ, Greengard P, Matteoli M, Valtorta F (2002) Spatial and temporal regulation of Ca2+/calmodulin-dependent protein kinase II activity in developing neurons. J Neurosci 22:7016–7026

Menegon A, Bonanomi D, Albertinazzi C, Lotti F, Ferrari G, Kao HT, Benfenati F, Baldelli P, Valtorta F (2006) Protein kinase A-mediated synapsin I phosphorylation is a central modulator of Ca2+−dependent synaptic activity. J Neurosci 26:11670–11681

Messa M, Congia S, Defranchi E, Valtorta F, Fassio A, Onofri F, Benfenati F (2010) Tyrosine phosphorylation of synapsin I by Src regulates synaptic-vesicle trafficking. J Cell Sci 123:2256–2265

Michels B, Chen YC, Saumweber T, Mishra D, Tanimoto H, Schmid B, Engmann O, Gerber B (2011) Cellular site and molecular mode of synapsin action in associative learning. Learn Mem 18:332–344

Micheva KD, Busse B, Weiler NC, O'Rourke N, Smith SJ (2010) Single-synapse analysis of a diverse synapse population: proteomic imaging methods and markers. Neuron 68:639–653

Monaldi I, Vassalli M, Bachi A, Giovedi S, Millo E, Valtorta F, Raiteri R, Benfenati F, Fassio A (2010) The highly conserved synapsin domain E mediates synapsin dimerization and phospholipid vesicle clustering. Biochem J 426:55–64

Murrey HE, Gama CI, Kalovidouris SA, Luo WI, Driggers EM, Porton B, Hsieh-Wilson LC (2006) Protein fucosylation regulates synapsin Ia/Ib expression and neuronal morphology in primary hippocampal neurons. Proc Natl Acad Sci U S A 103:21–26

Nielander HB, Onofri F, Schaeffer E, Menegon A, Fesce R, Valtorta F, Greengard P, Benfenati F (1997) Phosphorylation-dependent effects of synapsin IIa on actin polymerization and network formation. Eur J Neurosci 9:2712–2722

Nuwal T, Heo S, Lubec G, Buchner E (2011) Mass spectrometric analysis of synapsins in Drosophila melanogaster and identification of novel phosphorylation sites. J Proteome Res 10:541–550

Onofri F, Giovedi S, Vaccaro P, Czernik AJ, Valtorta F, De Camilli P, Greengard P, Benfenati F (1997) Synapsin I interacts with c-Src and stimulates its tyrosine kinase activity. Proc Natl Acad Sci U S A 94:12168–12173

Onofri F, Giovedi S, Kao HT, Valtorta F, Bongiorno Borbone L, De Camilli P, Greengard P, Benfenati F (2000) Specificity of the binding of synapsin I to Src homology 3 domains. J Biol Chem 275:29857–29867

Onofri F, Messa M, Matafora V, Bonanno G, Corradi A, Bachi A, Valtorta F, Benfenati F (2007) Synapsin phosphorylation by SRC tyrosine kinase enhances SRC activity in synaptic vesicles. J Biol Chem 282:15754–15767

Orenbuch A, Shalev L, Marra V, Sinai I, Lavy Y, Kahn J, Burden JJ, Staras K, Gitler D (2012a) Synapsin selectively controls the mobility of resting pool vesicles at hippocampal terminals. J Neurosci 32:3969–3980

Orenbuch A, Shulman Y, Lipstein N, Bechar A, Lavy Y, Brumer E, Vasileva M, Kahn J, Barki-Harrington L, Kuner T, Gitler D (2012b) Inhibition of exocytosis or endocytosis blocks activity-dependent redistribution of synapsin. J Neurochem 120:248–258

Owe SG, Erisir A, Heggelund P (2013) Terminals of the major thalamic input to visual cortex are devoid of synapsin proteins. Neuroscience 243:115–125

Paonessa F, Latifi S, Scarongella H, Cesca F, Benfenati F (2013) Specificity protein 1 (Sp1)-dependent activation of the synapsin I gene (SYN1) is modulated by RE1-silencing transcription factor (REST) and 5'-cytosine-phosphoguanine (CpG) methylation. J Biol Chem 288:3227–3239

Pera I, Stark R, Kappl M, Butt HJ, Benfenati F (2004) Using the atomic force microscope to study the interaction between two solid supported lipid bilayers and the influence of synapsin I. Biophys J 87:2446–2455

Petersohn D, Schoch S, Brinkmann DR, Thiel G (1995) The human synapsin II gene promoter. Possible role for the transcription factor zif268/egr-1, polyoma enhancer activator 3, and AP2. J Biol Chem 270:24361–24369

Pieribone VA, Shupliakov O, Brodin L, Hilfiker-Rothenfluh S, Czernik AJ, Greengard P (1995) Distinct pools of synaptic vesicles in neurotransmitter release. Nature 375:493–497

Pieribone VA, Porton B, Rendon B, Feng J, Greengard P, Kao HT (2002) Expression of synapsin III in nerve terminals and neurogenic regions of the adult brain. J Comp Neurol 454:105–114

Porton B, Kao HT, Greengard P (1999) Characterization of transcripts from the synapsin III gene locus. J Neurochem 73:2266–2271

Qualmann B, Roos J, DiGregorio PJ, Kelly RB (1999) Syndapin I, a synaptic dynamin-binding protein that associates with the neural Wiskott-Aldrich syndrome protein. Mol Biol Cell 10:501–513

Rizzoli SO, Betz WJ (2005) Synaptic vesicle pools. Nat Rev Neurosci 6:57–69

Romano C, Nichols RA, Greengard P, Greene LA (1987) Synapsin I in PC12 cells. I. Characterization of the phosphoprotein and effect of chronic NGF treatment. J Neurosci 7:1294–1299

Rosahl TW, Geppert M, Spillane D, Herz J, Hammer RE, Malenka RC, Südhof TC (1993) Short-term synaptic plasticity is altered in mice lacking synapsin I. Cell 75:661–670

Rosahl TW, Spillane D, Missler M, Herz J, Selig DK, Wolff JR, Hammer RE, Malenka RC, Südhof TC (1995) Essential functions of synapsins I and II in synaptic vesicle regulation. Nature 375:488–493

Ryan TA, Li L, Chin LS, Greengard P, Smith SJ (1996) Synaptic vesicle recycling in synapsin I knock-out mice. J Cell Biol 134:1219–1227

Sadanandappa MK, Blanco Redondo B, Michels B, Rodrigues V, Gerber B, VijayRaghavan K, Buchner E, Ramaswami M (2013) Synapsin function in GABA-ergic interneurons is required for short-term olfactory habituation. J Neurosci 33:16576–16585

Sankaranarayanan S, Atluri PP, Ryan TA (2003) Actin has a molecular scaffolding, not propulsive, role in presynaptic function. Nat Neurosci 6:127–135

Sato K, Morimoto K, Suemaru S, Sato T, Yamada N (2000) Increased synapsin I immunoreactivity during long-term potentiation in rat hippocampus. Brain Res 872:219–222

Sauerwald A, Hoesche C, Oschwald R, Kilimann MW (1990) The 5'-flanking region of the synapsin I gene. A G+C-rich, TATA- and CAAT-less, phylogenetically conserved sequence with cell type-specific promoter function. J Biol Chem 265:14932–14937

Saviouk V, Moreau MP, Tereshchenko IV, Brzustowicz LM (2007) Association of synapsin 2 with schizophrenia in families of Northern European ancestry. Schizophr Res 96:100–111

Schenk U, Menna E, Kim T, Passafaro M, Chang S, De Camilli P, Matteoli M (2005) A novel pathway for presynaptic mitogen-activated kinase activation via AMPA receptors. J Neurosci 25:1654–1663

Schiebler W, Jahn R, Doucet JP, Rothlein J, Greengard P (1986) Characterization of synapsin I binding to small synaptic vesicles. J Biol Chem 261:8383–8390

Schoch S, Cibelli G, Thiel G (1996) Neuron-specific gene expression of synapsin I. Major role of a negative regulatory mechanism. J Biol Chem 271:3317–3323

Shupliakov O, Bloom O, Gustafsson JS, Kjaerulff O, Low P, Tomilin N, Pieribone VA, Greengard P, Brodin L (2002) Impaired recycling of synaptic vesicles after acute perturbation of the presynaptic actin cytoskeleton. Proc Natl Acad Sci U S A 99:14476–14481

Shupliakov O, Haucke V, Pechstein A (2011) How synapsin I may cluster synaptic vesicles. Semin Cell Dev Biol 22:393–399

Sihra TS, Wang JK, Gorelick FS, Greengard P (1989) Translocation of synapsin I in response to depolarization of isolated nerve terminals. Proc Natl Acad Sci U S A 86:8108–8112

Siksou L, Rostaing P, Lechaire JP, Boudier T, Ohtsuka T, Fejtova A, Kao HT, Greengard P, Gundelfinger ED, Triller A, Marty S (2007) Three-dimensional architecture of presynaptic terminal cytomatrix. J Neurosci 27:6868–6877

Silva AJ, Rosahl TW, Chapman PF, Marowitz Z, Friedman E, Frankland PW, Cestari V, Cioffi D, Südhof TC, Bourtchuladze R (1996) Impaired learning in mice with abnormal short-lived plasticity. Curr Biol 6:1509–1518

Skoblenick KJ, Argintaru N, Xu Y, Dyck BA, Basu D, Tan ML, Mazurek MF, Mishra RK (2010) Role of AP-2 alpha transcription factor in the regulation of synapsin II gene expression by dopamine D1 and D2 receptors. J Mol Neurosci 41:267–277

Skorobogatko Y, Landicho A, Chalkley RJ, Kossenkov AV, Gallo G, Vosseller K (2014) O-linked β-N-acetylglucosamine (O-GlcNAc) site thr-87 regulates synapsin I localization to synapses and size of the reserve pool of synaptic vesicles. J Biol Chem 289(6):3602–3612

Spillane DM, Rosahl TW, Südhof TC, Malenka RC (1995) Long-term potentiation in mice lacking synapsins. Neuropharmacology 34:1573–1579

Stone LM, Browning MD, Finger TE (1994) Differential distribution of the synapsins in the rat olfactory bulb. J Neurosci 14:301–309

Südhof TC (1990) The structure of the human synapsin I gene and protein. J Biol Chem 265:7849–7852

Südhof TC, Czernik AJ, Kao HT, Takei K, Johnston PA, Horiuchi A, Kanazir SD, Wagner MA, Perin MS, De Camilli P et al (1989) Synapsins: mosaics of shared and individual domains in a family of synaptic vesicle phosphoproteins. Science 245:1474–1480

Takamori S, Holt M, Stenius K, Lemke EA, Gronborg M, Riedel D, Urlaub H, Schenck S, Brugger B, Ringler P, Muller SA, Rammner B, Grater F, Hub JS, De Groot BL, Mieskes G, Moriyama Y, Klingauf J, Grubmuller H, Heuser J, Wieland F, Jahn R (2006) Molecular anatomy of a trafficking organelle. Cell 127:831–846

Tallent MK, Varghis N, Skorobogatko Y, Hernandez-Cuebas L, Whelan K, Vocadlo DJ, Vosseller K (2009) In vivo modulation of O-GlcNAc levels regulates hippocampal synaptic plasticity through interplay with phosphorylation. J Biol Chem 284:174–181

Tao-Cheng JH, Dosemeci A, Winters CA, Reese TS (2006) Changes in the distribution of calcium calmodulin-dependent protein kinase II at the presynaptic bouton after depolarization. Brain Cell Biol 35:117–124

Thiel G, Südhof TC, Greengard P (1990) Synapsin II. Mapping of a domain in the NH2-terminal region which binds to small synaptic vesicles. J Biol Chem 265:16527–16533

Torri Tarelli F, Bossi M, Fesce R, Greengard P, Valtorta F (1992) Synapsin I partially dissociates from synaptic vesicles during exocytosis induced by electrical stimulation. Neuron 9:1143–1153

Torri-Tarelli F, Villa A, Valtorta F, De Camilli P, Greengard P, Ceccarelli B (1990) Redistribution of synaptophysin and synapsin I during alpha-latrotoxin-induced release of neurotransmitter at the neuromuscular junction. J Cell Biol 110:449–459

Tyler WJ, Alonso M, Bramham CR, Pozzo-Miller LD (2002) From acquisition to consolidation: on the role of brain-derived neurotrophic factor signaling in hippocampal-dependent learning. Learn Mem 9:224–237

Valente P, Casagrande S, Nieus T, Verstegen AM, Valtorta F, Benfenati F, Baldelli P (2012) Site-specific synapsin I phosphorylation participates in the expression of post-tetanic potentiation and its enhancement by BDNF. J Neurosci 32:5868–5879

Valtorta F, Villa A, Jahn R, De Camilli P, Greengard P, Ceccarelli B (1988) Localization of synapsin I at the frog neuromuscular junction. Neuroscience 24:593–603

Valtorta F, Benfenati F, Greengard P (1992a) Structure and function of the synapsins. J Biol Chem 267:7195–7198

Valtorta F, Greengard P, Fesce R, Chieregatti E, Benfenati F (1992b) Effects of the neuronal phosphoprotein synapsin I on actin polymerization. I Evidence for a phosphorylation-dependent nucleating effect. J Biol Chem 267:11281–11288

Vasileva M, Horstmann H, Geumann C, Gitler D, Kuner T (2012) Synapsin-dependent reserve pool of synaptic vesicles supports replenishment of the readily releasable pool under intense synaptic transmission. Eur J Neurosci 36:3005–3020

Vasileva M, Renden R, Horstmann H, Gitler D, Kuner T (2013) Overexpression of synapsin Ia in the rat calyx of Held accelerates short-term plasticity and decreases synaptic vesicle volume and active zone area. Front Cell Neurosci 7:270

Wang S, Cesca F, Loers G, Schweizer M, Buck F, Benfenati F, Schachner M, Kleene R (2011) Synapsin I is an oligomannose-carrying glycoprotein, acts as an oligomannose-binding lectin, and promotes neurite outgrowth and neuronal survival when released via glia-derived exosomes. J Neurosci 31:7275–7290

Zucker RS, Regehr WG (2002) Short-term synaptic plasticity. Annu Rev Physiol 64:355–405

Zurmohle UM, Herms J, Schlingensiepen R, Schlingensiepen KH, Brysch W (1994) Changes of synapsin I messenger RNA expression during rat brain development. Exp Brain Res 99:17–24

Part VI
Synaptic Vesicle Pools

Chapter 14
Synaptic Vesicle Pools: Classical and Emerging Roles

Sven Truckenbrodt and Silvio O. Rizzoli

Abstract Synaptic vesicles display a marked functional specialisation that warrants the definition of the so-called synaptic vesicle pools. In the classical three-pool model, the readily releasable pool (1–2 % of all vesicles) provides fast initial neurotransmitter release, the recycling pool (10–20 %) maintains release during physiological levels of stimulation, and the reserve pool (~80 %) is inert in terms of neurotransmitter release under physiological stimulation. But this classification according to release propensity does not convey the whole range of functional versatility of synaptic vesicles. Recent research has demonstrated that the release-incompetent reserve pool may act as a buffer for soluble proteins essential to synaptic vesicle recycling. Furthermore, new pool concepts have been proposed which illustrate several emerging aspects of synaptic vesicle pool physiology. A super-pool of vesicles is exchanged between synapses, with potential implications for synaptic plasticity. A surface or readily retrievable pool of synaptic vesicles decorates the membrane of synaptic boutons and may be essential for maintaining the recycling pool through rapid compensatory endocytosis. Recent data on the pool of spontaneously releasing vesicles suggests that there may be more functional and molecular heterogeneity among synaptic vesicles than anticipated. Finally, understanding the regulation of pool transitions remains a largely unresolved issue.

Keywords Synaptic vesicles • Synaptic vesicle pools • Synaptic transmission • Neuronal communication • Cell biology

S. Truckenbrodt (✉)
Department of Neuro- and Sensory Physiology, University Medical Centre Göttingen, Göttingen, Germany

International Max Planck Research School for Molecular Biology, Göttingen, Germany
e-mail: strucke@gwdg.de

S.O. Rizzoli
Department of Neuro- and Sensory Physiology, University Medical Centre Göttingen, Göttingen, Germany

S. Mochida (ed.), *Presynaptic Terminals*, DOI 10.1007/978-4-431-55166-9_14

14.1 Synaptic Vesicle Pools: From Observation to Concept and Back

Synaptic vesicles are similar in ultrastructure, subcellular localisation and, presumably, molecular composition. Nonetheless, heterogeneity in their probability to release neurotransmitter has been observed for decades and in an array of preparations (reviewed by Rizzoli and Betz 2005). This has led to the proposal of different 'pools' of synaptic vesicles. The earliest antecedents of the concept that synaptic vesicles may exist in different functional states lie in observations by Birks and MacIntosh (1961) on cat sympathetic ganglia and Elmqvist and Quastel (1965) on human intercostal muscles. Both researcher tandems noted that a fraction of the releasable neurotransmitter in their preparation was more readily available for release than the rest. They concluded that two stores – or pools – of neurotransmitter must exist in the synaptic terminal: one easier and faster to release, but also depleting upon prolonged stimulation, and one that releases with slower kinetics and only upon strong and prolonged stimulation. Birks and MacIntosh (1961) even explicitly suggested that synaptic vesicles might be the organellar correlate for the observed kinetics of neurotransmitter release, despite the fact that the 'vesicle hypothesis' of neurotransmitter release was only just beginning to emerge at the time. Sir Bernard Katz had published the concept of quantal release, which proposes that neurotransmitter comes in distinct packages of uniform size, in the early 1950s (Fatt and Katz 1950, 1952; del Castillo and Katz 1954). The discovery of synaptic vesicles at around the same time (Palade and Palay 1954; de Robertis and Bennet 1955) made it tempting to combine these two observations and deduce that synaptic vesicles must store these 'quanta' of neurotransmitter (Palay 1956; del Castillo and Katz 1956). Evidence that synaptic vesicles are locally recycled (Heuser and Reese 1973; Ceccarelli et al. 1973) and refilled within the synapse (Molenaar et al. 1973a, b) soon lent added weight to the notion of a specialised recycling pool of vesicles maintaining neurotransmitter release and of a complementary reserve pool that is more reluctant in terms of neurotransmitter release and recycling.

Until today, this dualism shapes the consensus on synaptic physiology and communication, amply substantiated by experimental evidence (Rizzoli and Betz 2005; Denker and Rizzoli 2010; Alabi and Tsien 2012). The only known role of synaptic vesicles was, for decades after their discovery, to store and actively release neurotransmitter. The assumption that the cell always works as an economically efficient entity suggested that all vesicles had to participate in active neurotransmitter release in the one or the other physiological situation: the recycling pool during 'normal' activity and the reserve pool during extreme stress situations. In this model, all synaptic vesicles are assigned an intuitive, active and direct role in neuronal communication. This model of the synaptic bouton worked for decades. It is appealing because of its simplicity and apparent efficiency. In recent years, however, experimental evidence has accumulated that suggests that only a small fraction of synaptic vesicles actually participates in neurotransmitter release at

any one time, even under severe physiological stress (e.g. Denker et al. 2011a; Körber et al. 2012). This and other observations of unanticipated versatility of synaptic vesicle function continue to trigger an ongoing rethinking of our general understanding of the cell biology of synaptic boutons and the roles of different pools of synaptic vesicles. We present here the current knowledge on synaptic vesicle pools and their dynamics, integrate emerging roles of synaptic vesicles beyond active neurotransmitter release and discuss possible implications for synaptic physiology, as well as for general cell biology.

14.2 The Three Classical Synaptic Vesicle Pools

Ever since the earliest studies on synaptic physiology (see Sect. 14.1), synaptic vesicles have been classified into distinct pools based on neurotransmitter release kinetics. Indeed, the propensity to release is still the main criterion for defining synaptic vesicle pools. The following pools can be distinguished (see Fig. 14.1): a specialised *recycling pool* which maintains neurotransmitter release under physiological stimulation, a *reserve pool* which is inert in terms of neurotransmitter release under physiological stimulation and a *readily releasable pool* of vesicles, which supports the early, fast and strong response to a bout of stimulation.

The fraction of releasable synaptic vesicles in any given preparation depends heavily on the applied type and frequency of stimulation. Any discussion of

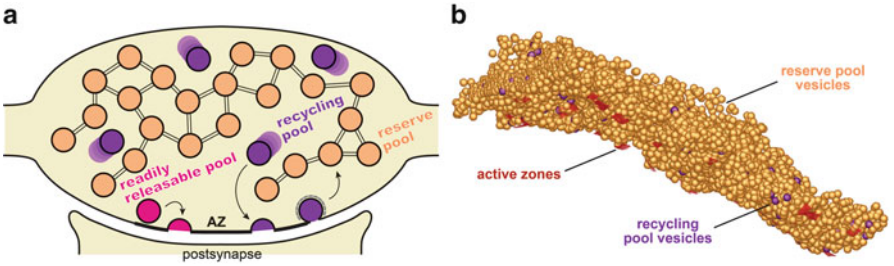

Fig. 14.1 The three-pool model of synaptic vesicle release. (**a**) The readily releasable pool (*pink*) consists of vesicles docked at the active zone, primed for release. The recycling pool (*purple*) consists of vesicles that carry neurotransmitter release under physiological levels of stimulation, and the reserve pool (*orange*) consists of vesicles that are not mobilised under physiological levels of stimulation. While the recycling pool vesicles are mobile, the reserve pool vesicles are immobile and tethered by a meshwork of cytoskeletal elements and synapsin (represented by *double lines*). *AZ* active zone. *Grey* blocks around vesicles represent clathrin molecules. (**b**) The spatial mixing of recycling and reserve pool vesicles can be visualised by FM-dye labelling and photoconversion (see Methods Box). In this reconstruction of a zebrafish neuromuscular junction, vesicles of the recycling pool (*purple*), labelled during 2 h of free behaviour of the living animal, are thoroughly intermixed with the inert reserve pool vesicles that do not contain dye (*orange*). The plasma membrane is omitted from the reconstruction for an unobstructed view of the vesicle cluster; *red* areas indicate active zones (Adapted from Denker et al. 2011a)

synaptic vesicle pools thus has to take into account the stimulation paradigms and experimental conditions under which the evidence for described pool kinetics has been obtained. For example, in cultured hippocampal neurons as many as 50 % of all vesicles can be made available for release during bouts of tetanic stimulation (Opazo et al. 2010), or as little as 15–20 % may sustain prolonged low-frequency stimulation (Harata et al. 2001a, b). In some preparations, like the frog neuromuscular junction, even the entire pool of vesicles can be made available for release upon strong supra-physiological stimulation (Rizzoli and Betz 2005). The stimulation paradigm chosen can thus heavily influence the experimental outcome. To be considered physiological, a stimulation paradigm should mimic what can be observed for the cell type in question in the living and behaving animal, both in frequency and in type (e.g. electric, high potassium or osmolarity shock). Most studies on synaptic vesicle dynamics are, however, dependent on ex situ preparations, which are not lifelike. Most studies have been performed with unphysiologically strong stimulation paradigms and in buffers that might alter the excitability of the preparation. Nonetheless, the three pools mentioned above have, based on their different release kinetics, consistently been identified in a wide range of preparations and under different stimulation protocols, leading to a surprisingly robust three-pool model (Rizzoli and Betz 2005; Denker and Rizzoli 2010; Alabi and Tsien 2012).

14.2.1 The Recycling Pool

The recycling pool consists of those synaptic vesicles that independently maintain a constant rate of transmitter release upon physiological stimulation (Rizzoli and Betz 2005). They fuse with the presynaptic membrane, release neurotransmitter, endocytose, refill and repeat the cycle (Südhof 2004) – thus the term *recycling* pool. Vesicles with such a behaviour have been described in a wide array of preparations, e.g. in hippocampal cultures (Harata et al. 2001a, b), the frog neuromuscular junction (Richards et al. 2003), the *Drosophila* neuromuscular junction (Kuromi and Kidokoro 1998, 2000) and the Calyx of Held (de Lange et al. 2003) (for a detailed discussion, see Rizzoli and Betz 2005).

Remarkably, pool sizes scale very closely with synapse size and the ratios of pools are largely the same in all commonly investigated preparations (Rizzoli and Betz 2005). The recycling pool generally makes up 5–20 % of all vesicles, in synapses with total vesicle numbers differing by several orders of magnitude (e.g. 500 for large hippocampal synapses and 500,000 for the frog neuromuscular junction). However, this might be more flexible at small central synapses. Marra et al. (2012) found, using FM1-43 loading and photoconversion (see Methods Box), on average 15–20 % of all vesicles in hippocampal slices to be part of the recycling pool. In a comparable preparation, Rose et al. (2013) found a still bigger recycling fraction of synaptic vesicles: on average 20–25 %. The variability found in both studies between those small central synapses (mostly 50–500 synaptic vesicles)

also seems to be much larger than between large motor neuron or sensory synapses (which often contain several tens or even hundreds of thousands of synaptic vesicles). Both studies reported a negative correlation of recycling pool size and synapse size (as number of synaptic vesicles): up to 100 % of all vesicles in synapses with 50 or less synaptic vesicles belong to the recycling pool and around 20 % on average in synapses with more than 300 synaptic vesicles (Rose et al. 2013).

Methods Box: Tools for Investigating Synaptic Vesicle Recycling

FM-dye labelling and photoconversion. FM dyes are widely used as probes to study endocytotic processes. They do not cross biological membranes but readily partition into them and become fluorescent upon doing so. There are several variants of FM dyes, with different partitioning kinetics and fluorescence properties; the most commonly used FM dye in neurobiology is FM1-43. When added to the medium of cultured cells, FM1-43 partitions into the outer leaflet of the cell membrane and is thereby taken up into recycling synaptic vesicles. FM1-43 can also be washed out from membranes easily, which allows to monitor exocytosis of previously labelled synaptic vesicles as destaining (loss of dye and fluorescence). FM1-43 can also be used to visualise recycling synaptic vesicles in electron microscopy via a process called photoconversion. Fluorescent dyes such as FM1-43 generate reactive oxygen species under constant illumination. When adding the membrane-permeable compound diaminobenzidine (DAB) to a sample and illuminating it, these reactive oxygen species will lead to the formation of a membrane-impermeable electron-dense precipitate from DAB inside vesicles labelled with FM1-43.

pHluorin. pHluorins are pH-sensitive variants of GFP. They can be targeted to synaptic vesicles by coupling to the transmembrane domain of a synaptic vesicle protein (e.g. synaptobrevin 2). The pHluorin tag resides in the vesicle lumen, where fluorescence is quenched by the acidic pH (~5.5) of synaptic vesicles. Upon stimulation, vesicles fuse with the plasma membrane and thereby expose their lumen to the neutral (pH ~7.4) extracellular medium, which leads to an unquenching of pHluorins and an increase in fluorescence. Synaptic vesicle recycling can be monitored by this change in fluorescence.

Antibody tagging and pH-sensitive dyes. Some synaptic vesicle transmembrane proteins contain a vesicle lumenal domain that is large enough to generate antibodies against it (primarily synaptotagmin I; Kraszewski et al. 1995). Applied to the extracellular medium of neurons, these antibodies bind to recycling vesicles, since the vesicle lumen is exposed to the extracellular space upon exocytosis. Vesicles tagged with antibodies will continue

(continued)

to recycle and can be tracked by using antibodies labelled with fluorescent probes. The pH-sensitive dye cypHer5E can be coupled to antibodies to investigate synaptic vesicle recycling, similar in principle to pHluorins. In contrast to pHluorins, however, cypHer5E is quenched in the neutral pH of extracellular medium and has its peak fluorescence in the pH of acidified synaptic vesicles. The advantage of antibody tagging over the use of pHluorins is that no overexpression is necessary, but endogenous proteins can be tagged.

All of these numbers have been derived from ex situ approaches. These rely on artificial stimulation protocols and artificial buffers. Both may influence the release propensities of synaptic vesicles by changing the excitability of the preparation and the frequency with which it is excited, compared to the situation in situ. This gap in our understanding of synaptic physiology has recently been addressed by Denker et al. (2011a). The authors used an array of model systems (invertebrates, vertebrates, neuromuscular junctions, central synapses, sensory synapses) to assess the number of synaptic vesicles recycling during normal behaviour of an animal. They injected the membrane dye FM1-43 into the body cavity of animals and allowed them to behave freely for 2 h, followed by rapid dissection of the labelled synapses and generation of an electron-dense precipitate inside FM1-43-labelled synaptic vesicles by photoconversion for analysis in electron microscopy (see Methods Box). Denker et al. (2011a) found only 1–5 % of all vesicles in any synaptic bouton to participate in neurotransmitter release. Even under the extreme physiological stress of a locust being hunted by one of its natural predators, the frog, the fraction of recycling vesicles used in the neuromuscular junction of the locust's hind legs (with which it propels itself away from predators as its main escape mechanism) did not exceed 5 % of the total vesicle pool until the locust was caught after about 5–10 min (1 % for not hunted control locusts). This confirms the viability of the concept of a small recycling pool under physiological conditions and demonstrates that it might even be considerably smaller than previously described from ex situ preparations.

14.2.2 The Readily Releasable Pool

Readily releasable vesicles are defined by their immediate availability for release in response to a stimulus. These are the only synaptic vesicles for which a privileged localisation in the synaptic bouton can be pinpointed – they are docked directly at the active zone and are primed for release (Schikorski and Stevens 2001). These synaptic vesicles functionally belong to the pool of recycling vesicles, but they are nonetheless traditionally sorted into their own pool because of repeated

observations of markedly faster and stronger release kinetics in the initial phase of response to a stimulation train (e.g. Elmqvist and Quastel 1965; Neves and Lagnado 1999; Delgado et al. 2000). Since studies addressing the readily releasable pool usually relied on particularly strong stimulation paradigms, the fast and strong initial response phase might, however, be artificially induced and it is unclear what its relevance might be in the living and behaving animal.

The readily releasable vesicles can thus be regarded as a subpopulation of the recycling pool that is ready for immediate release due to being docked at the active zone and being primed for release. It has to be noted, however, that not all docked vesicles are also necessarily readily releasable (Xu-Friedman et al. 2001; Rizzoli and Betz 2004). The readily releasable vesicles are the smallest fraction of all vesicles in a synaptic bouton, often less than 1 % (see Table 14.1). In consequence, the readily releasable pool is exhausted within milliseconds at high-frequency stimulation, in the course of only a few action potentials, often less than 10–20 (see Table 14.1). The readily releasable pool can thus be considered the basis of a fast and strong response mechanism of the neuron, but is too small to maintain neurotransmitter release during prolonged physiological stimulation – that is the role of the recycling pool.

14.2.3 The Reserve Pool

The reserve pool consists of all the synaptic vesicles that do not functionally belong to the two pools described above, i.e. all vesicles that do not release and recycle under physiological stimulation conditions. This is by far the largest of the three classical pools, making up at least ~80 % of all synaptic vesicles in most synaptic preparations. At least some of these vesicles can be forced to release under severe supra-physiological stimulation, such as prolonged high-frequency electrical stimulation (at least 5–10 Hz in frog neuromuscular junctions (Richards et al. 2000; Heuser and Reese 1973), 30 Hz in *Drosophila* larva neuromuscular junctions (Kuromi and Kidokoro 2000)) or application of high potassium (de Lange et al. 2003; Takei et al. 1996). It is very likely, however, that these vesicles are not involved in release and recycling at all under physiological conditions, since the exchange of vesicles with the recycling pool is apparently too low and slow to provide a reservoir for prolonged stimulation (Richards et al. 2000), and since a large fraction of the entire vesicle complement of a synapse remains unreleased even under severe physiological stress (Denker et al. 2011a). Prolonged physiological stimulation is carried exclusively by recycling vesicles. Reserve pool vesicles are immobilised in a meshwork of cytoskeletal elements, the highly abundant (Wilhelm et al. 2014) vesicle- and cytoskeleton-binding protein synapsin (Pechstein and Shupliakov 2010; Cesca et al. 2010) and maybe other less abundant proteins (Siksou et al. 2007).

The majority of the total pool of vesicles does not get mobilised even under extreme physiological stress: ~95 % of all synaptic vesicles in the neuromuscular junction of the locust hind leg do not release when the locust is hunted down and killed by a frog (Denker et al. 2011a). This means that the ‘reserve’ pool vesicles,

Table 14.1 Readily releasable pool (RRP) size and corresponding number of action potentials (AP) to exhaustion of the RRP during high-frequency stimulation in four preparations commonly used in synaptic vesicle research

Preparation	RRP vesicles	% of all vesicles	RRP vesicles released per AP	RRP exhausted after
Hippocampal culture[13]	~5–10[1,2]	~5.35[2]	~0.1–0.5[2,3,4]	~20–50 APs[3,14]
Frog NMJ	~10,000[5,6,7,8,14]	~2[5]	~600–700[5,9,14]	~15 APs[9]
Drosophila larva NMJ	~300[10]	~0.36[10,14]	~75[10,14]	~4 APs[10]
Calyx of Held	~2,400[11]	~1.2 %[11,12,14]	~400[12,14]	~6 APs[12,14]

[1]Schikorski and Stevens (1997); [2]Schikorski and Stevens (2001); [3]Stevens and Williams (2007); [4]Hanse and Gustafsson (2001); [5]Rizzoli and Betz (2004); [6]Heuser and Reese (1973); [7]Ceccarelli et al. (1973); [8]Molgo and Pecot-Dechavassine (1988); [9]Richards et al. (2003); [10]Delgado et al. (2000); [11]Sakaba and Neher (2001); [12]de Lange et al. (2003); [13]Hippocampal cultures display much more inter-bouton variability than the other preparations. They can easily range from 50 to 1,000 vesicles (Marra et al. 2012) and entire boutons can be completely silent in release (Stevens and Wang 1995; Hanse and Gustafsson 2001; Kerchner and Nicoll 2008). These factors may account for the higher numbers derived for percentage of RRP vesicles and the higher number of APs necessary to exhaust the RRP. [14]Numbers derived by the authors of this review, with numbers for variables taken from the publications cited in this table

which have traditionally been perceived as a functional reserve of neurotransmitter for situations of high stimulation and high demand on release during extreme physiological stress, may not serve that purpose at all. However, these vesicles may have other roles essential to synaptic function, such as buffering soluble cofactors for synaptic vesicle recycling into the synapse (see Sect. 14.5). It has been speculated that recycling pool vesicles turn into reserve pool vesicles over time and possibly also the other way around (Kamin et al. 2010; Denker and Rizzoli 2010), but published data on the long-term dynamics of recycling and reserve pool vesicles that could support this hypothesis are still lacking.

14.3 Nonclassical Synaptic Vesicle Pools and Other Functional Concepts

In addition to the three-pool model described above, several more pools of synaptic vesicles have been proposed. The main three of these are the *spontaneous pool* of vesicles releasing without apparent stimulation, the *surface or readily retrievable pool* of vesicles stranded or deposited in a state of fusion with the plasma membrane and available for compensatory retrieval from there in response to exocytosis of other vesicles, and the *super-pool* of vesicles shared between boutons. These pool concepts provide new perspectives on the physiology of synaptic boutons by identifying potential additional layers of functional versatility, but their specific impact and distinct cell biological roles are still heavily debated. The classical concept of defining synaptic vesicle pools by their release behaviour is often not applicable to these new pool concepts; for the surface or readily retrievable pool and the super-pool, release kinetics do not play any direct role. This highlights the necessity to investigate synaptic vesicles not only in terms of active neurotransmitter release but to also consider additional roles in presynaptic function.

14.3.1 The Super-Pool

The ‘super-pool’ of synaptic vesicles was introduced to conceptualise observations of high and rapid rates of exchange of vesicles between neighbouring synaptic boutons (Darcy et al. 2006; Staras et al. 2010; Welzel et al. 2011; see Fig. 14.2a). Inter-bouton mobility was found for recycling pool vesicles (Darcy et al. 2006; Staras et al. 2010) as well as, interestingly, for reserve pool vesicles (Fernández-Alfonso and Ryan 2008; Kamin et al. 2010). It is unclear how the immobilised reserve pool vesicles would participate in such an inter-bouton exchange, but it is possible that individual vesicles peel off from the cluster held together by synapsin and become mobile or that chunks of vesicles break out of the cluster. Interfering with the clustering of reserve pool vesicles by acute injection of synapsin

antibodies (Pieribone et al. 1995), inhibition of phosphatases that have been linked to vesicle clustering via synapsin (Betz and Henkel 1994) or synapsin knockouts (Orenbuch et al. 2012; Fornasiero et al. 2012) increases the inter-bouton mobility of synaptic vesicles; so it is likely that synapsin binding is at least partially restricting reserve pool mobility in the proposed super-pool. The concept of the super-pool forces us to consider the synaptic bouton as a much more dynamic compartment than previously envisioned, with high rates of in- and efflux of synaptic vesicles (Staras and Branco 2010). This may be especially important in synaptic plasticity, where it could be the basis for adjusting synapse strength in neighbouring connections, accompanied by the redistribution of other synaptic elements, like active zone or adhesion proteins (reviewed by Staras and Branco 2010). In line with this interpretation, synapsin, the main factor for organisation and integrity of the synaptic vesicle cluster, has been shown to become more mobile upon stimulation and to be exchanged between neighbouring synapses (Chi et al. 2001; Tsuriel et al. 2006). The redistribution of this essential structural organisation protein in synaptic boutons and the entailing liberation of synaptic vesicles from the meshwork, resulting in increased mobility (Fornasiero et al. 2012; Orenbuch et al. 2012), may well be the basis for long-term plasticity. This model is in line with observations that long-term plasticity can be driven mainly presynaptically, through changes in the frequency and reliability of neurotransmitter release at individual synaptic connections (Zakharenko et al. 2001; Emptage et al. 2003; Enoki et al. 2009), at least under some circumstances (Mayford et al. 2012; Collingridge et al. 2010). It has recently been reported that neuronal activation can trigger elimination of unreliable synapses and, in turn, strengthen other neighbouring synapses in long-term plasticity (Wiegert and Oertner 2013). Altering the vesicle complement of a synaptic bouton by super-pool reorganisation, mediated by synaptic activity, could induce such a strengthening or loss of synaptic connections.

14.3.2 The Surface or Readily Retrievable Pool

A substantial amount of up to 20 % of all synaptic vesicle epitopes has been found to reside on the plasma membrane of synaptic boutons at all times (Sankaranarayanan et al. 2000; Granseth et al 2006; Wienisch and Klingauf 2006; Fernández-Alfonso et al. 2006; Balaji and Ryan 2007; Opazo et al. 2010; Revelo et al. 2014). This surface pool might be available for rapid retrieval, compensating for the increase in cell membrane area upon synaptic vesicle exocytosis (Gandhi and Stevens 2003; Hua et al. 2011a; see Fig. 14.2b).

To release neurotransmitter, synaptic vesicles must fuse with the plasma membrane. Thereby, they become integral parts of the plasma membrane, at least in full-fusion models of synaptic release (see Rizzoli 2014 for a review discussing different modes of vesicle fusion, including full fusion and kiss-and-run, in which a vesicle only makes very brief contact with the cell membrane and is retrieved without collapse into the membrane). Whether the resulting patches of vesicle

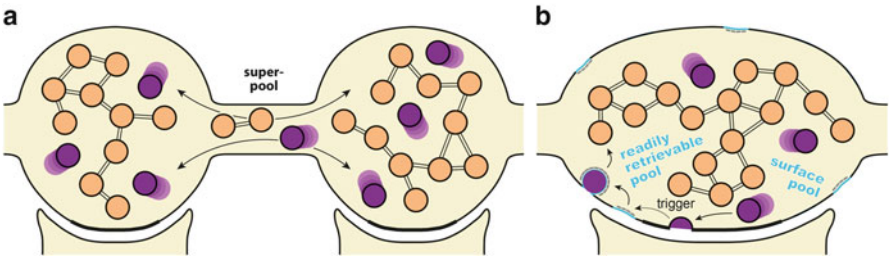

Fig. 14.2 The super-pool and the surface or readily retrievable pool. (**a**) The super-pool consists of synaptic vesicles that can be shared among neighbouring synapses. Many types of neurons contain synapses that are arranged along the axon like 'beads on a string', which allows the exchange of vesicles depicted here. Its functional relevance might lie in altering synaptic strength in long-term plasticity by rearranging the vesicle complement between neighbouring synapses, including complete elimination of synapses. Recycling (*purple*) as well as reserve pool vesicles (*orange*) can be exchanged. (**b**) The surface or readily retrievable pool (*turquoise*) consists of synaptic vesicles deposited on the plasma membrane of the synaptic bouton. At least some of these vesicles are available for rapid compensatory endocytosis, triggered by the exocytosis of synaptic vesicles. *Grey* blocks around vesicles represent clathrin molecules

molecules stay largely intact until retrieval or whether they intermix with plasma membrane contents (i.e. if the vesicles lose integrity on the membrane upon full fusion) has been a matter of debate. Some studies employed constructs of synaptic vesicle proteins (e.g. synaptotagmin, synaptophysin or VAMP2), with the pH-sensitive GFP variant pHluorin tagged to the vesicle lumenal domain (see Methods Box). pHluorin is quenched in the acidic pH inside synaptic vesicles but becomes fluorescent upon exocytosis, due to the rapid equilibration of the vesicle interior with the neutral extracellular environment. Hence, constructs from recently fused vesicles can be followed on the plasma membrane. Several studies, using different constructs, found that proteins rapidly diffuse away from the site of fusion in the plasma membrane (Sankaranarayanan and Ryan 2000; Li and Murthy 2001; Wienisch and Klingauf 2006; Granseth et al. 2006). While these studies did not have the resolution to follow individual proteins or even synaptic vesicle-sized patches, they argued that it seems unlikely that entire patches of complete vesicles could diffuse as fast as observed; but it is not impossible either, as a comparable rate of diffusion has been observed in other studies for whole vesicles (Gaffield et al. 2006; Kamin et al. 2010). However, a second line of evidence from pHluorin studies has been interpreted in support of this hypothesis. By bleaching or enzymatically removing pHluorin on the plasma membrane before stimulating synaptic vesicle exocytosis, one can ascertain whether vesicle recovery after the stimulus is due to endocytosis of recently exocytosed vesicles or endocytosis of vesicles deposited on the cell membrane. Upon stimulation, pHluorin on releasing vesicles is unquenched and, in case of endocytosis of the same vesicles, would quickly be quenched again, while in case of endocytosis of surface pool vesicles, the fluorescence would decline slower, since the pHluorins on the cell surface have been bleached or removed before. Such experiments showed that recently exocytosed synaptic vesicle material is not preferentially endocytosed (Wienisch

and Klingauf 2006; Fernández-Alfonso et al. 2006). The authors interpreted this as a mixing of membrane-resident and vesicular proteins and suggested that vesicles lose integrity upon fusion and are only reassembled upon retrieval or even later, maybe through an endosomal sorting step. However, an alternative interpretation would be that the exocytosed vesicles stayed intact and that other intact vesicles, stranded on the cell surface, were available for compensatory endocytosis (Wienisch and Klingauf 2006).

There are several caveats associated with the use of pHluorin constructs (Granseth et al. 2006; Opazo et al. 2010). They all require overexpression, which might alter their distribution and behaviour after fusion, compared to endogenous proteins (Granseth et al. 2006). Furthermore, the pHluorin studies were all restricted by the resolution barrier of classical light microscopy, i.e. the cohesiveness of vesicle protein clusters on the cell membrane and their proposed loss of integrity could not be observed directly. The only other studies addressing this issue used antibodies to tag the vesicle lumenal domain of endogenous synaptotagmin epitopes on the plasma membrane and recycling vesicles and visualised them via super-resolution STED microscopy (Willig et al. 2006; Opazo et al. 2010), which provides a resolution in the range of the diameter of single fused synaptic vesicles on the plasma membrane (~60–80 nm). These studies found the complete opposite of what the pHluorin studies suggested. Synaptotagmin protein patches seemed to remain largely intact and clustered, indicating that synaptic vesicles can maintain integrity after fusion. Supporting this view, clustering of different synaptic vesicle proteins with each other and with cholesterol, which is enriched in synaptic vesicles compared to the cell membrane, is a well-documented factor in synaptic vesicle and synapse biogenesis (e.g. Mitter et al. 2003; Stevens et al. 2012; Tarsa and Goda 2002; Hannah et al. 1999); this would also help synaptic vesicles to maintain integrity on the plasma membrane. It can be concluded that the evidence for maintenance of synaptic vesicle integrity on the plasma membrane currently outweighs the evidence for loss of integrity.

A functional participation of the surface pool in synaptic vesicle recycling has first been suggested by Gandhi and Stevens (2003). They expressed a construct based on the vesicular protein VAMP2, coupled to pHluorin. With this construct, Gandhi and Stephens (2003) observed the retrieval of apparently stranded vesicles to be triggered by stimulation. This could be explained as a form of compensatory endocytosis to replenish the recycling pool and keep the cell surface area constant. This interpretation has since received supported from other independent studies using similar tools (Wienisch and Klingauf 2006; Fernández-Alfonso et al. 2006; see above) and alternative approaches (Hua et al. 2011a). Hua et al. (2011a) used the pH-sensitive cyanine dye cypHer5E coupled to antibodies (see Methods Box) to demonstrate rapid compensatory endocytosis of endogenous cell surface resident synaptotagmin in response to stimulated synaptic vesicle release. According to these results, readily retrievable vesicles on the bouton membrane are preferentially endocytosed, and recently exocytosed vesicles remain stranded on the membrane in turn. presumably, until they themselves can serve as reservoir for compensatory endocytosis upon release of other vesicles. Taking into account the fraction of

synaptic vesicle epitopes present on the neuronal cell surface in hippocampal cultures (Sankaranarayanan et al. 2000; Granseth et al 2006; Wienisch and Klingauf 2006; Fernández-Alfonso et al. 2006; Balaji and Ryan 2007; Opazo et al. 2010; Revelo et al. 2014), the surface area of an average synaptic bouton in hippocampal cultures (derived from Schikorski and Stevens 1997; Wilhelm et al. 2014) and the area of a fused synaptic vesicle, up to 23 % of the surface area of a synaptic bouton could be occupied by fused synaptic vesicles (assuming all surface epitopes represent stranded synaptic vesicles). This large membrane fraction of stranded vesicles might be essential for the replenishment of the recycling pool. Providing a large enough reservoir of readily retrievable vesicles on the cell membrane may be necessary to expedite the restoration of recycling-competent vesicles. Just the assembly of the clathrin coat required for clathrin-mediated endocytosis can take 20–30 s (Cocucci et al. 2012). The assembly of the machinery of clathrin-mediated endocytosis might thus be a time limiting factor in synaptic vesicle recycling, and adding the time to find one single specific recently fused vesicle on the plasma membrane of a synaptic bouton may simply take too long to recycle enough vesicles in time before the whole recycling pool is depleted. Assuming 0.5 fusion events per action potential in an average hippocampal bouton (Schikorski and Stevens 1997; Stevens and Williams 2007; Hanse and Gustafsson 2001), the entire recycling pool could be depleted within 20 s of a moderate 5 Hz stimulation, so before the clathrin coat of the first vesicle fused at the beginning of the stimulation period has even been fully assembled. An observed time constant of 10–20 s for compensatory vesicle endocytosis after stimulation (Granseth et al. 2006) indicates that at least some of the surface pool vesicles are readily retrievable, since it reflects an endocytosis that is considerably faster than what would be expected if the clathrin coat had to assemble on a recently exocytosed vesicle (Cocucci et al. 2012). Decorating a substantial fraction of the membrane of a synaptic bouton with patches of vesicle, any one of which could be retrieved to replenish the recycling pool in compensation for vesicle exocytosis, would thus presumably help to maintain a pool of recycling-competent vesicles.

14.3.3 The Spontaneous Pool

Fatt and Katz already observed spontaneous quantal release of neurotransmitter in the absence of any stimulation in 1950/1952. Single spontaneous quanta roughly corresponded to single quanta of evoked release in amplitude and kinetics of the postsynaptic response they elicited. The spontaneous vesicles apparently released randomly and without any trigger (see Fig. 14.3), which is at odds with the tight and reliable coupling of stimulation and neurotransmitter release necessary in faithful synaptic communication. This release would thus have to be regarded as a mistake, an accidental fusion of synaptic vesicles. However, it could serve another purpose. Spontaneous release is especially prevalent in developing neurons, but severely decreases as they mature and as evoked release increases (Andreae et al. 2012).

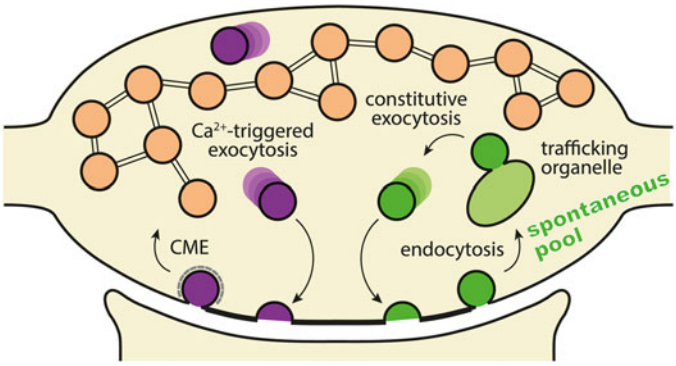

Fig. 14.3 The spontaneous pool. While the recycling pool (*purple*) is dependent on a Ca^{2+} trigger for exocytosis, mainly relies on clathrin-mediated endocytosis (CME) and is not enriched in constitutive or other trafficking proteins (*left*), the spontaneous pool (*green*) releases in constitutive exocytosis without any apparent trigger, does not seem to depend on CME and is enriched in markers of constitutive and other trafficking pathways (*right*). *Grey* blocks around vesicles represent clathrin molecules

Acutely abolishing spontaneous release can induce compensatory receptor synthesis at dendritic spines (Sutton et al. 2006) and can ultimately lead to loss of postsynaptic glutamate receptor clusters (Saitoe et al. 2001) and dendritic spines (McKinney et al. 1999) when maintained over a prolonged period of time. Spontaneous release might thus play a role in the establishment and maintenance of synaptic contacts, serving as signal flags for the postsynapse. A constitutive spontaneous neurotransmitter secretion may be the synaptic analogue of a 'dead man's switch' by providing continuous input in the absence of active use of a synaptic connection (Verhage et al. 2000); if this constant signal ceases, the synaptic connection would be lost or not be properly established in the first place. This hypothesis has recently received further experimental confirmation: Choi et al. (2014) investigated the formation of neuromuscular junctions in *Drosophila* larvae, using genetic tools to separately suppress evoked and spontaneous transmission. They found that suppression of evoked release had no detrimental effect on synapse formation and maturation, while additionally abolishing spontaneous release largely arrested synapse development. Increasing spontaneous release, on the other hand, resulted in an expansion of synaptic boutons. This data strongly supports the view that spontaneous neurotransmission has an essential role in synapse development and maintenance.

In recent years, a debate ensued on the question whether spontaneous release is carried out by vesicles from the recycling pool or from a distinct pool of synaptic vesicles. The opinions on the matter are still wildly divergent – as are the experimental data. A common way to study spontaneous release is to suppress evoked release by applying the sodium channel blocker TTX. Sara et al. (2005) used this approach to study spontaneous release in hippocampal cultures. They loaded vesicles with the membrane dye FM2-10 either without stimulation in the presence

of TTX (spontaneous release) or during stimulation with high potassium (evoked release). They then tried to destain the vesicles labelled under these conditions by evoking release via stimulation (see Methods Box). Upon destaining stimulation, spontaneously labelled vesicles destained much more reluctantly than vesicles labelled during stimulation. In support of this, Mathew et al. (2008) found that vesicles loaded with FM1-43 under spontaneous conditions destained markedly slower under a stimulation of 10 Hz than vesicles loaded by evoking release via electrical stimulation, with kinetics comparable to what Sara et al. (2005) had observed. Taken together, this supports the notion that spontaneous vesicles are 'deaf' to stimulation, but are more likely to release during rest. In a different approach, Fredj and Burrone (2009) used a VAMP2-based construct with vesicle lumenal biotin to investigate the spontaneous pool. They tagged the biotin with labelled streptavidin on vesicles releasing under spontaneous conditions and under stimulation. They found, like Sara et al. (2005), that the vesicles belong to two different populations. These seem to be clear indications that the spontaneously releasing vesicles are specialised for spontaneous release and can thus be thought of as an independent pool.

Groemer and Klingauf (2007) disputed this view. They also used FM dyes to stain vesicles, but they used a much milder stimulation paradigm (30 Hz for 4 s) to load vesicles than Sara et al. (2005) and Mathew et al. (2008). They sequentially loaded neurons with two FM dyes with different spectral properties under spontaneous and evoked labelling conditions and then simultaneously imaged unloading of dye from these two pools – and found completely identical kinetics. Wilhelm et al. (2010) addressed the question in several model systems, using both biotin-tagged antibodies and FM dyes and a combination of mild release paradigms. They also found that vesicles releasing spontaneously and upon stimulation belong to the same pool. Groemer and Klingauf (2007) had suggested that normalisation could have distorted data interpretation in earlier studies, but Mathew et al. (2008) took this into account and still found differences between vesicles loaded under spontaneous and evoked conditions. The controversy on the spontaneous pool thus remains unresolved from functional studies.

Several studies sought to investigate the molecular composition of vesicles of the proposed spontaneous pool. Hua et al. (2011b) and Bal et al. (2013) found that VAMP7 is enriched in spontaneously releasing vesicles, while Ramirez et al. (2012) found the same for Vti1a. Both of these SNAREs are, however, so scarce on synaptic vesicles in general – 2 copies or less, on average (Takamori et al. 2006) – that they might even be considered contaminants or leftovers from development, as synaptic vesicles pass through compartments enriched in these SNAREs during biogenesis (see Rizzoli 2014). Furthermore, they have both primarily been implicated in trafficking distinct from synaptic vesicle recycling: Vti1a in *cis*- and *trans*-Golgi trafficking (Fischer von Mollard and Stevens 1998; Ganley et al. 2008) and VAMP7 in growth cone development and late endosome/lysosome trafficking (Wang and Tang 2006; Burgo et al. 2012). While assigning molecular markers to distinct trafficking pathways can be deceiving, this calls into question the identity of spontaneously releasing vesicles as bona fide synaptic vesicles. This concern is fortified by the finding that a Vti1a/VAMP7 SNARE

complex seems to facilitate non-canonical constitutive trafficking of neuronal voltage-gated potassium channels to the cell surface in non-neuronal as well as neuronal cells (Flowerdew and Burgoyne 2009). Furthermore, recent findings suggest that the spontaneously releasing vesicles differ from recycling pool vesicles in their relative enrichment of several other endosomal or constitutive trafficking proteins (syntaxin 13, VAMP4) and relative lack of several canonical synaptic vesicle proteins (synaptotagmin I, VAMP2) (Revelo et al. 2014). Interestingly, Sara et al. (2005) found that vesicles labelled under spontaneous conditions destained much faster during a 20 min resting period without any stimulation than vesicles labelled under conditions of evoked synaptic vesicle release. This also seems to indicate a constitutive trafficking pathway. Taken together, these findings lead to the question if spontaneously releasing vesicles can be considered synaptic vesicles at all or if they are some other type of trafficking organelle in the synaptic bouton.

Pointing to yet another fundamental departure of spontaneously releasing vesicles from the canonical synaptic vesicle recycling machinery is the observation that spontaneous release seems to be completely unaffected by dynamin inhibition (Chung et al. 2010). Since dynamin is indispensable for clathrin-mediated endocytosis, regarded as the main way of synaptic vesicle recycling (Granseth et al. 2006; Rizzoli and Jahn 2007), this can either mean that spontaneously releasing vesicles only release via dynamin-independent kiss-and-run or that they are not bona fide synaptic vesicles. To view the spontaneously releasing vesicles not as a pool of synaptic vesicles but as a set of distinct organelles (see Fig. 14.3) might help to resolve some of the conundrum surrounding the wildly divergent experimental evidence on the 'spontaneous pool' in the future. It is likely that organelles do not solely have the clear-cut properties and functions traditionally assigned to them. Organelles we sort into distinct categories may rather fall on a continuous spectrum. Cells may even frequently make – and tolerate – mistakes. In this case, this might result in vesicles that contain neurotransmitter (thus the evoked postsynaptic potentials already observed by Fatt and Katz 1950 and Fatt and Katz 1952), but do not respond to triggers of synaptic vesicle release and instead constitutively traffic to the cell surface.

14.4 Pool Identity and Pool Transitions

The main remaining questions regarding the maintenance and alteration of pool identity are: What distinguishes synaptic vesicles with different functional roles? What triggers a vesicle to change this role? How can this process be regulated and adapted to enable the neuron to respond to different demands? It is surprisingly difficult to pinpoint the cause for the functional distinction of synaptic vesicles into actively releasing and recycling ones or inert ones of the reserve pool. Recycling vesicles do not have a privileged localisation in the bouton. The ultrastructure is no indication; a recent study strove to elucidate the structure of the lumenal assembly of synaptic vesicles and found it to be surprisingly similar between vesicles docked

at the active zone and undocked vesicles (Harlow et al. 2013). There is only very scarce data linking synaptic vesicle composition to their functionality.

14.4.1 Subcellular Localisation

Only the relatively small fraction of readily releasable vesicles can claim a privileged localisation in the synaptic bouton linked to their release propensity: they are docked and primed for release at the active zone. However, not even this is always an indication of function: not all vesicles docked at the active zone appear to be readily releasable (Xu-Friedman et al. 2001; Rizzoli and Betz 2004). The recycling pool and the reserve pool are thoroughly intermixed throughout the synaptic bouton (e.g. Denker et al. 2011a, 2009; see Sect. 14.2 and Fig. 14.1a). This has been observed in a variety of preparations in independent studies, from invertebrates to vertebrates and from neuro-motor and sensory synapses to central synapses: in the Drosophila larva neuromuscular junction (Denker et al. 2009); frog neuromuscular junction (Rizzoli and Betz 2004); snake neuromuscular junction (Teng and Wilkinson 2000); in the neuromuscular junctions of locust, zebrafish, *C. elegans*, mouse and developing chicken embryos (Denker et al. 2011a); goldfish bipolar nerve terminal (Paillart et al. 2003); mammalian hippocampal synapses (Harata et al. 2001a, b); the mammalian Calyx of Held (de Lange et al. 2003); and Drosophila larva central synapses and the optic lobe of the cricket (Denker et al. 2011a). This body of evidence suggests that access to the active zone and release propensity are not dependent on the physical distance of the recycling pool vesicles to release sites. This means that other factors must be considered as determinants of synaptic vesicle functionality.

14.4.2 Mobility

As contact to the active zone is indispensable for membrane fusion and neurotransmitter release and recycling pool vesicles can be located far away from the active zone (see Sects 14.4.1 and 14.2.1), mobility is essential for the availability of recycling pool vesicles for release at the active zone on demand. Considering this, the mobility of synaptic vesicles may be the defining factor for release propensity. It has been reported that synaptic vesicles of the recycling pool exhibit a higher degree of mobility than those of the reserve pool (Gaffield et al. 2006), which also allows their rapid exchange between neighbouring synapses (Staras et al. 2010; Welzel et al. 2011).

These observations lead to the question of how reserve pool vesicles are immobilised and how mobilisation might be controlled. One protein family in particular has emerged as the main factor in vesicle immobilisation – synapsin (as reviewed by Pechstein and Shupliakov 2010 and Cesca et al. 2010). This protein

can bind actin (Greengard et al. 1993; Pieribone et al. 1995) as well as protein and phospholipid components of synaptic vesicle membranes (Benfenati et al. 1989) at the same time, with a selective preference for membranes with the curvature of synaptic vesicles (Krabben et al. 2011). This led to the formulation of the 'synapsin hypothesis' by Paul Greengard in 1993, according to which an immobilisation of synaptic vesicles by tethering them to each other as well as to a cytoskeletal scaffold restricts their mobility and thereby controls their release propensity. Triple knockout mice for all three mammalian synapsin variants are viable (with a severe epileptic phenotype), but their synapses show an increased mobility of synaptic vesicles, with vesicles drifting into the axonal inter-bouton space (Fornasiero et al. 2012; Orenbuch et al. 2012). The tethering of synaptic vesicles in the cytomatrix formed by synapsin and other structural elements is not irreversible but dynamically regulated by calcium-dependent phosphorylation (Fornasiero et al. 2009; Cesca et al. 2010; Valtorta et al. 2011; Ho et al. 2011). This may contribute to explaining the observed differences in the fraction of vesicles available for release under different stimulation paradigms (see Sects. 14.2 and 14.2.1). Additionally, recycling synaptic vesicles have been noted to turn immobile within tens of minutes (Kamin et al. 2010), likely marking their transition into the reserve pool.

These results indicate that immobilisation defines the reserve pool, while mobility functionally defines the recycling pool. It remains unclear, however, what the trigger for the integration of synaptic vesicles into the immobilising synapsin meshwork might be.

14.4.3 Molecular Tags

It could be assumed that a molecular tag triggers the transition of synaptic vesicles between the recycling and the reserve pool – one or more proteins or post-translational modifications or a combination of those. But despite the fact that the synaptic vesicle is an organelle that has been exceptionally well characterised on a molecular level, qualitatively (Jahn and Südhof 1994; Blondeau et al. 2004; Morciano et al. 2005) as well as quantitatively (Takamori et al. 2006), a molecular tag governing pool transitions is still elusive. While there is substantial experimental evidence that synapsin maintains synaptic vesicles in an immobilised state (e.g. Orenbuch et al. 2012; Fornasiero et al. 2012), there is virtually no evidence on how integration into the synapsin meshwork is triggered. While phosphorylation of synapsin can globally alter the release propensity of synaptic vesicles in one bouton (see previous and following section), there is, to our knowledge, no published data on post-translational modifications of synaptic vesicle proteins selectively regulating the functional state of individual vesicles in distinction from other synaptic vesicles.

For a tag protein to enter (or leave) a synaptic vesicle, there would have to be an exchange of material of the vesicle with other membrane compartments.

The only membrane compartments with which a synaptic vesicle regularly comes into contact after biogenesis are the plasma membrane and possibly endosomes. The full-fusion model of synaptic vesicle recycling (reviewed by Rizzoli and Jahn 2007) suggests that vesicles completely collapse into the plasma membrane, with subsequent clathrin-mediated retrieval of fully functional vesicles directly from the plasma membrane or retrieval of larger membrane patches (bulk endocytosis) or disassembled vesicles which require a step of endosomal sorting before functional vesicles can be restored. The role of endosomes during physiological recycling processes remains highly controversial, and it is possible that endosomes only play a role during recycling after strong supra-physiological stimulation and thus have little or no bearing on functional regulation in vivo. A strong argument for this view is the finding by Murthy and Stevens (1998) that FM1-43 dye taken up by vesicles during a short and mild stimulation of 5 s at 1 Hz does not get diluted, i.e. the released amount of FM1-43 upon a destaining stimulation equals that of FM1-43 originally taken up. Since FM1-43 indiscriminately labels membranes, recycling through endosomal structures would lead to dilution of dye into the endosome and out of the vesicle membrane, reducing the amount of dye available for release via synaptic vesicles during subsequent destaining stimulation. In a recent review, Morgan et al. (2013) argue against this dilution scenario, citing observations by Richards et al. (2000, 2003) of large FM1-43-labelled endosomal cisternae budding off from the plasma membrane after stimulation. If these represent endosomal structures involved in synaptic vesicle recycling, dilution of dye from synaptic vesicles would not occur, since the endosomes themselves are equally labelled. However, Richards et al. (2000, 2003) used prolonged high-frequency stimulation protocols (30 Hz for 1 min) which likely induce the formation of cisternae as a recycling artefact (Ceccarelli and Hurlbut 1980; Rizzoli and Betz 2005). These likely represent a compensatory mechanism in response to supra-physiological levels of stimulation, which does not occur in the synapse in situ: recent findings suggest that the clathrin-mediated endocytosis machinery is only sufficient for simultaneous recycling of ~7–11 % of all synaptic vesicles at a synaptic bouton (Wilhelm et al. 2014), which would be sufficient for the 1–5 % of vesicles in use over several minutes to hours in situ (Denker et al. 2011a), but insufficient to deal with the ~20 % of vesicles released within seconds by artificial stimulation. On the other hand, analyses of vesicles of the readily releasable pool have shown that association with endosomal markers increases after stimulating release (Hoopmann et al. 2010) and endosomal sorting may have a role in eliminating dysfunctional synaptic vesicle components (Uytterhoeven et al. 2011). Endosomes could thus be involved in synaptic vesicle recycling. But it remains uncertain if they are a site for maintenance of vesicle integrity or if they could also serve to compromise that integrity.

AP180 and stonin 2 have been identified as regulators for maintaining synaptic vesicle composition upon recycling from the plasma membrane, and their deletion can cause severe missorting upon endocytosis (Diril et al. 2006; Koo et al. 2011; Kononekno et al. 2013). This indicates that measures for maintenance of synaptic vesicle integrity exist. But it remains controversial if synaptic vesicles maintain

their identity during recycling. Strong evidence argues for it (also see Sect. 14.3.2), but even minute changes during a single recycling event might sum up to severe changes over time, with repeated rounds of exo- and endocytosis. Hoopmann et al. (2010) found differences in the composition of readily releasable vesicles after exocytosis in comparison to the total vesicle pool. They labelled vesicles by stimulating synaptosomes (connected presynaptic and postsynaptic compartments sheared off from intact neurons) in the presence of antibodies against the lumenal domain of synaptotagmin (see Methods Box) and co-immunostained for several proteins involved in synaptic vesicle recycling. Apart from significant increases in association with several soluble endocytotic proteins (e.g. endophilin, dynamin, Rab5), they found increases in association of readily releasable vesicles with the cell membrane SNAREs SNAP25 and syntaxin 1. It is unclear, however, if these changes were permanent or only transient (and could have been reversed, e.g. through endosomal sorting), since the investigated time scale was on the order of seconds to minutes. It also has to be cautioned that the fragility of the preparation (synaptosomes) and the used stimulation paradigm might be prone to induce bulk-endocytosis artefacts.

Hua et al. (2011b) found that a transfected VAMP7-pHluorin construct targets mainly to vesicles that are release incompetent in response to stimulation and concluded that those vesicles are reserve pool vesicles and that thus VAMP7 might be an integral molecular marker for the reserve pool. Since VAMP7 has also been reported to target to spontaneously releasing vesicles that are incompetent to respond to stimulation (Hua et al. 2011b; Bal et al. 2013), it remains an open question, however, if the VAMP7-positive vesicles were, in fact, reserve pool vesicles or bona fide synaptic vesicles at all (see Sect. 14.3.3). The question of the existence of a specific molecular pool tag thus remains unresolved, to date.

14.4.4 Modulation of Pool Transitions

Recycling pool vesicles may regularly turn into release-incompetent reserve pool vesicles (see Sect. 14.2.3), a process dubbed ‘maturation’ (Denker and Rizzoli 2010). The main evidence for this is the observation that synaptic vesicles that have been released once become reluctant to release after a resting period (Richards et al. 2000, 2003; Rizzoli and Betz 2004). This is in line with the observation that recently recycling vesicles become immobile after a while (Kamin et al. 2010), presumably due to integration into the largely immobile reserve pool cluster of synapsin, synaptic vesicles and cytoskeletal elements. All this suggests a linear unidirectional model for pool transitions of vesicles from the recycling pool to the reserve pool, albeit this cannot be ascertained without further experimental confirmation.

The fraction of recycling vesicles is, however, not fixed. It can be dynamically modulated to suit the requirements of the neuron by a plethora of pathways (see de Jong and Verhage 2009 for a review) – probably also as a means of achieving

plasticity in neuronal communication. The kinase CDK5 and the phosphatase calcineurin emerged as important antagonistic co-regulators of synaptic vesicle function. Inhibition of CDK5 increases the fraction of vesicles available for release, while suppression of calcineurin decreases the size of the recycling pool (Kim and Ryan 2010). MAP kinases and CaM kinases have also been implicated in regulating synaptic function via phosphorylation of synapsin (see Cesca et al. 2010 for a review). These phosphorylations can have different effects, but especially Ca^{2+}-dependent phosphorylation by CaM kinases I/IV and II can decrease the binding of synapsin to actin and synaptic vesicles (Cesca et al. 2010). This most likely helps to mobilise synaptic vesicles during high-frequency stimulation. To couple this regulation to Ca^{2+} influx, which is a direct consequence of stimulation, provides a direct link between demand and adjustment of supply. These examples highlight the importance of post-translational modifications of soluble regulators in determining the release propensity of synaptic vesicles. They cannot, however, be interpreted as the elusive molecular pool tag (see Sect. 14.4.3). Kinases and phosphatases cannot in themselves be specific 'switches' of pool identity for individual synaptic vesicles. If kinases or phosphatases in the synaptic space get activated, e.g. by Ca^{2+} influx, they will act on all available targets. So unless some synaptic vesicles are selectively shielded from post-translational modifications or the enzymes directed to specific vesicles, all will be affected equally. This would shift the probability for pool transitions, but equally for all vesicles, and would thus represent a regulator of pool transitions rather than a determinant. A pool tag would have to specifically alter the probability of a pool transition for an individual vesicle or subset of vesicles.

14.5 Roles of Synaptic Vesicles in Synaptic Communication Beyond Neurotransmitter Release

It has originally been believed that the reserve pool would constitute a strategic reserve of vesicles for phases of severe synaptic stress – thus the name *reserve* pool – and that all vesicles would regularly participate in synaptic transmission (Rizzoli and Betz 2005). This does not seem to be the case (see Sect. 14.2.3). While several ex situ studies found that the reserve pool could be mobilised when the recycling pool was artificially blocked (Kuromi and Kidokoro 1998) or depleted by supra-physiological stimulation (Richards et al. 2000, 2003), there is no evidence for this from experiments in the behaving animal. Indeed, the opposite seems to be the case. In a wide array of preparations, the vast majority of synaptic vesicles do not participate in release even under severe physiological stress – in excess of 90–95 % (Denker et al. 2011a; Körber et al. 2012; also see Sect. 14.2.1).

Why then does the cell maintain such a large reserve pool of synaptic vesicles that do not actively participate in neurotransmitter release under any physiological circumstances? It has been suggested that the main role of the large reserve pool

would be to act as a buffer for soluble synaptic proteins (Shupliakov 2009; Denker et al. 2011b). For synapses, this would solve the problem of protein targeting and sequestration with very low incremental cost to the cell and very low evolutionary demands. Synaptic proteins are trafficked to the synapse on several different carriers (Bonanomi et al. 2006) and have vastly differing lifetimes (Cohen et al. 2013) and thus, presumably, production rates. Simply sequestering them into synaptic boutons by affinity to the vesicle cluster seems to be evolutionary much more favourable than evolving individual protein targeting mechanisms or synchronising production and transport of proteins with divergent individual roles in synaptic vesicle release and recycling. Most proteins involved in synaptic vesicle release and recycling display a certain degree of association with the vesicle cluster (Denker et al. 2011b), probably because they all have some moderate affinity for synaptic vesicles (conceivable, since most proteins involved in synaptic vesicle release and recycling directly interact with synaptic vesicles at some point). Even a low-affinity interaction with synaptic vesicles could buffer a substantial amount of protein into the synaptic vesicle cluster of a bouton, simply by avidity. Biochemical evidence for this hypothesis has recently been obtained for complexin (Wragg et al. 2013), a key regulator of synaptic vesicle exocytosis that restricts spontaneous fusion by blocking spontaneous SNARE complex assembly. In agreement with previous observations (Denker et al. 2011b), Wragg et al. (2013) found a high co-localisation of complexin with synaptic vesicles. Co-localisation of the effector (complexin) and the target (synaptic vesicle SNAREs) is essential; accordingly, a certain affinity of complexin to synaptic vesicles can be expected. However, Wragg et al. (2013) found only a relatively low-affinity interaction of complexin with synaptic vesicles but could not identify an alternative targeting mechanism to the synapse. They concluded that the observed low affinity could nonetheless explain how complexin gets trapped in the synaptic vesicle cluster because of the avidity effects provided by the tightly organised cluster of hundreds to thousands of synaptic vesicles found in synaptic boutons. Note that a high affinity is not essential in this scenario; it could even interfere with the normal function of soluble regulators of synaptic function. They need to be present at the synapse, but should only be recruited to perform their function on specific vesicles upon specific cues. A high affinity of many cofactors for synaptic vesicles would preclude this, since synaptic vesicles would be covered with cofactors of various (even opposing) trafficking steps at all times.

Furthermore, the protein buffering hypothesis might provide explanations for some phenomena observed in synapse formation and plasticity. While the contribution of pre- and postsynaptic factors in synapse formation and the possible dominating role of the one or the other remain controversial (Jin and Garner 2008; Mayford et al. 2012), in several scenarios the presynapse is favoured as the initiating part (e.g. Friedman et al. 2000; Sabo et al. 2006). To form a synapse, active zone protein assemblies, adhesion complexes and cytoskeletal and structural elements all have to be recruited, assembled and maintained. Providing a large buffer for soluble proteins involved in synaptic vesicle recycling and active zone maintenance (e.g. Muncs), as well as an anchor point for structural elements and

cargo vesicles loaded with those essential structural elements, will ensure the maintenance of the presynapse. Pausing and clustering of synaptic vesicles or synaptic vesicle precursors is the inductive factor for synapse formation (Friedman et al. 2000), at least under some circumstances, by providing an anchor point for trafficking of other synaptic components (Sabo et al. 2006). Such nascent clusters recruit presynaptic proteins as well as structural elements, like actin (Sabo et al. 2006), which are required to shape the forming synaptic bouton. As disruption of normal actin dynamics by application of latrunculin A abolishes actin recruitment, but not vesicle pausing (Sabo et al. 2006), it is clear that the assembly of vesicles precedes recruitment of structural elements and thus appears to be causative for recruitment of synaptic factors – perfectly in agreement with the proposed buffering hypothesis. Small synapses, with a larger number of vesicles participating in active recycling (Marra et al. 2012; Rose et al. 2013) and a potentially less reliable release rate (due to their lower buffer capacity), may furthermore more easily be eliminated in long-term plasticity processes (Wiegert and Oertner 2013). This may be due to the fact that recycling vesicles cannot act as a protein buffer as efficiently as reserve pool vesicles, because they repeatedly collapse into the membrane, shedding soluble cofactors in the process (e.g. synapsin: Tarelli et al. 1992), and are much more mobile. It should be noted in this context that the especially large motoneuron synapses contain a much larger fraction of reserve pool vesicles and display much less plasticity, compared to small central synapses. On the contrary: their stability has to be maintained at all costs to preclude fatal neuro-motor failure, since one effector neuron forms much fewer contacts with its target muscle than central neurons form with each other, and additionally, lost contacts are extremely difficult to re-establish for developmental reasons. If a synaptic terminal is lost, axon guidance and outgrowth to re-establish the connection to the muscle has to be initiated and can take weeks to months. A large reserve pool acting as protein buffer will help to ensure synapse maintenance and additionally provide added redundancy in cofactor availability for ensuring reliable release of the recycling pool vesicles.

14.6 New Perspectives on Cell Biological Problems from the Study of Synaptic Vesicle Pools

Synaptic vesicles are clearly divided into functionally different subpopulations, despite the fact that all of them are (so far) largely indistinguishable in terms of ultrastructure, molecular composition and subcellular localisation. This functional heterogeneity within the same type of organelle might not represent the cell biological exception, but rather the rule. For example, it has been reported that mitochondria may be selected on the basis of their ability to produce ATP during germ cell development (Zhang et al. 2008; Zhou et al. 2010; Poulton et al. 2010). The mitochondria that produce more ATP can move faster along the cytoskeletal

tracks leading to the primary oocyte, while less productive mitochondria fall behind and get diverted to the secondary oocytes, which do not participate in reproduction. While further experimental confirmation of this phenomenon would be desirable, it is currently one of the only cues as to how dysfunctional mitochondria are eliminated from the germ line (Poulton et al. 2010). It is conceivable that synaptic vesicle pool transitions might follow the same principle of 'organelle competition'. This process might thus be interpreted as 'elimination', on the basis of deteriorating organellar functionality. As the vesicles age, their protein complement and functionality might undergo deterioration, which reduces their release probability, which leads to their gradual integration into the synapsin meshwork of the reserve pool cluster.

Another lesson from the investigation of synaptic vesicle pools is that one organelle might fulfil more than its traditionally assigned function. Synaptic vesicles very likely do not only take care of neurotransmitter release itself but also serve as a buffer for protein cofactors of exo- and endocytosis (Denker et al. 2011b; Shupliakov 2009; see Sect. 14.5). Connected to this observation is the interesting notion that the same organelle can adopt different roles in the cell over time. Not only do synaptic vesicles switch between a release-competent and a release-incompetent state – they assume a new role in the framework of cellular function at the same time. This illustrates the potential for functional versatility in organelles and that the cell is an economic entity that cannot afford waste – notwithstanding the possibility of errors or mistakes in the execution of cellular processes (Saka and Rizzoli 2012). The potential for errors and the apparent economic nature of the cell can be reconciled by bearing in mind that the occurrence of mistakes is an evolutionary necessity. The occurrence of mistakes in one process might be useful in another process. For example, should spontaneously releasing vesicles prove to be fundamentally different from synaptic vesicles in molecular composition, they might represent a form of rejects from synaptic vesicle biogenesis that are deaf to the usual triggers of synaptic vesicle release but nonetheless serve an essential role in maintaining synaptic contacts by facilitating a constant rate of constitutive release of neurotransmitter even in the absence of such triggers (see Sect. 14.3.3). Furthermore, spontaneous vesicles behave like synaptic vesicles in some respects (release of neurotransmitter), but like constitutive trafficking organelles in others ('deafness' to stimulation, SNARE complement, constant constitutive release). The case of the spontaneous pool thus also illustrates the possibility that there might often not be a clear distinction between related classes of organelles, but rather a functional continuum.

The unanticipated functional heterogeneity of synaptic vesicles (and maybe of organelles in general) highlighted here offers a new perspective for cell biologists. Potential for plasticity and ability to adapt must not always be looked for solely on the microscale molecular level or the macroscale level of cell-cell interactions but on the mesoscale of organelles. Future research will have to focus on the possibility of functional heterogeneity among organelles that have previously been treated as completely identical. Not only might there be an unanticipated heterogeneity between organelles of the same class, but there might even be faulty ones.

How the cell deals with those faulty organelles (elimination or integration into evolutionarily new roles) is an important question for future research, and the awareness that the cell has to deal with this at all might provide a fruitful framework for interpretation of experimental data.

References

Alabi ARA, Tsien RW (2012) Synaptic vesicle pools and dynamics. Cold Spring Harb Perspect Biol 4:a013680

Andreae LC, Fredj NB, Burrone J (2012) Independent vesicle pools underlie different modes of release during neuronal development. J Neurosci 32(5):1867–1874

Bal M, Leitz J, Reese AL, Ramirez DMO, Durakoglugil M, Herz J, Monteggia LM, Kavalali ET (2013) Reelin mobilizes a VAMP7-dependent synaptic vesicle pool and selectively augments spontaneous neurotransmission. Neuron 80:934–946

Balaji J, Ryan TA (2007) Single-vesicle imaging reveals that synaptic vesicle exocytosis and endocytosis are coupled by a single stochastic mode. Proc Natl Acad Sci U S A 104:20576–20581

Benfenati F, Bähler M, Jahn R, Greengard P (1989) Interaction of synapsin I with small synaptic vesicles: distinct sites in synapsin I bind to vesicle phospholipids and vesicle proteins. J Cell Biol 108:1863–1872

Betz WJ, Henkel AW (1994) Okadaic acid disrupts clusters of synaptic vesicles in frog motor nerve terminals. J Cell Biol 124(5):843–854

Birks R, MacIntosh FC (1961) Acetylcholine metabolism of a sympathetic ganglion. Can J Biochem Physiol 39:787–827

Blondeau F, Ritter B, Allaire PD, Wasiak S, Girard M, Hussain NK, Angers A, Legendre-Guillemin V, Roy L, Boismenu D, Kearney RE, Bell AW, Bergeron JJ, McPherson PS (2004) Tandem MS analysis of brain clathrin-coated vesicles reveals their critical involvement in synaptic vesicle recycling. Proc Natl Acad Sci U S A 101(11):3833–3838

Bonanomi D, Benfenati F, Valtorta F (2006) Protein sorting in the synaptic vesicle life cycle. Prog Neurobiol 80:177–217

Burgo A, Proux-Gillardeaux V, Sotirakis E, Bun P, Casano A, Veraes A, Liem RKH, Formstecher E, Coppey-Moisan M, Galli T (2012) A molecular network for the transport of the TI-VAMP/VAMP7 vesicles from cell center to periphery. Dev Cell 23:166–180

Ceccarelli B, Hurlbut WP (1980) Vesicle hypothesis of the release of quanta of acetylcholine. Phys Rev 60(2):396–440

Ceccarelli B, Hurlbut WP, Mauro A (1973) Turnover of transmitter and synaptic vesicles at the frog neuromuscular junction. J Cell Biol 57:499–524

Cesca F, Baldelli P, Valtorta F, Benfenati F (2010) The synapsins: key actors of synapse function and plasticity. Prog Neurobiol 91:313–348

Chi P, Greengard P, Ryan TA (2001) Synapsin dispersion and reclustering during synaptic activity. Nat Neurosci 4(12):1187–1193

Choi BJ, Imlach WL, Jiao W, Wolfram V, Wu Y, Grbic M, Cela C, Baines RA, Nitabach MN, McCabe BD (2014) Miniature neurotransmission regulates *Drosophila* synaptic structural maturation. Neuron 82:618–634

Chung C, Barylko B, Leitz J, Liu X, Kavalali ET (2010) Acute dynamin inhibition dissects synaptic vesicle recycling pathways that drive spontaneous and evoked neurotransmission. J Neurosci 30(4):1363–1376

Cocucci E, Aguet F, Boulant S, Kirchausen T (2012) The first five seconds in the life of a clathrin-coated pit. Cell 150:495–507

Cohen LD, Zuchman R, Sorokina O, Müller A, Dietrich DC, Armstrong JD, Ziv T, Ziv NE (2013) Metabolic turnover of synaptic proteins: kinetics, interdependencies and implications for synaptic maintenance. PLoS One 8(5):e63191

Collingridge GL, Peineau S, Howland JG, Wang YT (2010) Long-term depression in the CNS. Nat Rev Neurosci 11:459–473

Darcy KJ, Staras K, Collinson LM, Goda Y (2006) Constitutive sharing of recycling synaptic vesicles between presynaptic boutons. Nat Neurosci 9(3):315–321

de Jong APH, Verhage M (2009) Presynaptic signal transduction pathways that modulate synaptic transmission. Curr Opin Neurobiol 19:245–253

de Lange RP, De Roos AD, Borst JG (2003) Two modes of vesicle recycling in the rat calyx of Held. J Neurosci 23:10164–10173

de Robertis EDP, Bennet HS (1955) Some features of the submicroscopic morphology of synapses in frog and earthworm. J Biophys Biochem Cytol 1(1):47–58

del Castillo JB, Katz B (1954) Quantal components of the end-plate potential. J Physiol 124:560–573

del Castillo JB, Katz B (1956) Biophysical aspects of neuromuscular transmission. Prog Biophys Biophys Chem 6:121–170

Delgado R, Maureira C, Oliva C, Kidokoro Y, Labarca P (2000) Size of vesicle pools, rates of mobilization, and recycling at neuromuscular synapses of a drosophila mutant, *shibire*. Neuron 28:941–953

Denker A, Rizzoli SO (2010) Synaptic vesicle pools: an update. Front Synaptic Neurosci 2:135

Denker A, Kröhnert K, Rizzoli SO (2009) Revisiting synaptic vesicle pool localization in the Drosophila neuromuscular junction. J Physiol 587(12):2919–2926

Denker A, Bethani I, Kröhnert K, Körber C, Horstmann H, Wilhelm BG, Barysch SV, Kuner T, Neher E, Rizzoli SO (2011a) A small pool of vesicles maintains synaptic activity in vivo. Proc Natl Acad Sci U S A 108(41):17177–17182

Denker A, Kröhnert K, Bückers J, Neher E, Rizzoli SO (2011b) The reserve pool of synaptic vesicles acts as a buffer for proteins involved in synaptic vesicle recycling. Proc Natl Acad Sci U S A 108(41):17183–17188

Diril MK, Wienisch M, Jung N, Klingauf J, Haucke V (2006) Stonin 2 is an AP-2-dependent endocytic sorting adaptor for synaptotagmin internalization and recycling. Dev Cell 10(2):233–244

Elmqvist D, Quastel DMJ (1965) A quantitative study of endplate potentials in isolated human muscle. J Physiol Lond 178:505–529

Emptage NJ, Reid CA, Fine A, Bliss TVP (2003) Optical quantal analysis reveals a presynaptic component of LTP at hippocampal Schaffer-associational synapses. Neuron 38:797–804

Enoki R, Hu Y, Hamilton D, Fine A (2009) Expression of long-term plasticity at individual synapses in hippocampus is graded, bidirectional, and mainly presynaptic: optical quantal analysis. Neuron 62:242–253

Fatt P, Katz B (1950) Some observations on biological noise. Nature 166:597–598

Fatt P, Katz B (1952) Spontaneous subthreshold activity at motor nerve endings. J Physiol 117:109–128

Fernández-Alfonso T, Ryan TA (2008) A heterogeneous “resting” pool of synaptic vesicles is dynamically interchanged across boutons in mammalian CNS synapses. Brain Cell Biol 36:87–100

Fernández-Alfonso T, Kwan R, Ryan TA (2006) Synaptic vesicles interchange their membrane proteins with a large surface reservoir during recycling. Neuron 51(2):179–186

Fischer von Mollard G, Stevens TH (1998) A human homolog can functionally replace the yeast vesicle-associated SNARE Vti1p in two vesicle transport pathways. J Biol Chem 273:2624–2630

Flowerdew SE, Burgoyne RD (2009) A VAMP7/Vti1a SNARE complex distinguishes a non-conventional traffic route to the cell surface used by KChIP1 and Kv4 potassium channels. Biochem J 418:529–540

Fornasiero EF, Bonanomi D, Benfenati F, Valtorta F (2009) The role of synapsins in neuronal development. Cell Mol Life Sci 67:1383–1396
Fornasiero EF, Raimondi A, Guarnieri FC, Orlando M, Fesce R, Benfenati F, Valtorta F (2012) Synapsins contribute to the dynamic spatial organization of synaptic vesicles in an activity-dependent manner. J Neurosci 32(35):12214–12227
Fredj NB, Burrone J (2009) A resting pool of vesicles is responsible for spontaneous vesicle fusion at the synapse. Nat Neurosci 12:751–758
Friedman HV, Bresler T, Garner CC, Ziv NE (2000) Assembly of new individual excitatory synapses: time course and temporal order of synaptic molecule recruitment. Neuron 27:57–59
Gaffield MA, Rizzoli SO, Betz WJ (2006) Mobility of synaptic vesicles in different pools in resting and stimulated frog motor nerve terminals. Neuron 51(3):317–325
Gandhi SP, Stevens CF (2003) Three modes of synaptic vesicular recycling by single-vesicle imaging. Nature 423:607–613
Ganley IG, Espinosa E, Pfeffer SR (2008) A syntaxin 10-SNARE complex distinguishes two distinct transport routes from endosomes to the trans-Golgi in human cells. J Cell Biol 180(1):159–172
Granseth B, Odermatt B, Royle SJ, Lagnado L (2006) Clathrin-mediated endocytosis is the dominant mechanism of vesicle retrieval at hippocampal synapses. Neuron 51:773–786
Greengard P, Valtorta F, Czernik AJ, Benfenati F (1993) Synaptic vesicle phosphoproteins and regulation of synaptic function. Science 259:780–785
Groemer TW, Klingauf J (2007) Synaptic vesicles recycling spontaneously and during activity belong to the same vesicle pool. Nat Neurosci 10:145–147
Hannah MJ, Schmidt AA, Huttner WB (1999) Synaptic vesicle biogenesis. Annu Rev Cell Dev Biol 15:733–798
Hanse E, Gustafsson B (2001) Vesicle release probability and pre-primed pool at glutamatergic synapses in area CA1 of the rat neonatal hippocampus. J Physiol 531(2):481–493
Harata N, Pyle JL, Aravanis AM, Mozhayeva M, Kavalali ET, Tsien RW (2001a) Limited Numbers of recycling vesicles in small CNS nerve terminals: implications for neural signaling and vesicular cycling. Trends Neurosci 24:637–643
Harata N, Ryan TA, Smith SJ, Buchanan J, Tsien RW (2001b) Visualizing recycling synaptic vesicles in hippocampal neurons by FM 1–43 photoconversion. Proc Natl Acad Sci U S A 98:12748–12753
Harlow ML, Szule JA, Xu J, Jung JH, Marshall RM, McMahan UJ (2013) Alignment of synaptic vesicle macromolecules with the macromolecules in active zone material that direct vesicle docking. PLoS One 8(7):e69410
Heuser JE, Reese TS (1973) Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. J Cell Biol 57:315–344
Ho VM, Lee JA, Martin KC (2011) The cell biology of synaptic plasticity. Science 334:623–628
Hoopmann P, Punge A, Barysch SV, Westphal V, Bückers J, Opazo F, Bethani I, Lauterbach MA, Hell SW, Rizzoli SO (2010) Endosomal sorting of readily releasable synaptic vesicles. Proc Natl Acad Sci U S A 107(44):19055–19060
Hua Y, Sinha R, Thiel CS, Schmidt R, Hüve J, Martens H, Hell SW, Egner A, Klingauf J (2011a) A readily retrievable pool of synaptic vesicles. Nat Neurosci 14(7):833–839
Hua Z, Leal-Ortiz S, Foss SM, Waites CL, Garner CC, Voglmaier SM, Edwards RH (2011b) v-SNARE composition distinguishes synaptic vesicle pools. Neuron 71:474–487
Jahn R, Südhof T (1994) Synaptic vesicles and exocytosis. Annu Rev Neurosci 17:219–246
Jin Y, Garner CC (2008) Molecular mechanisms of presynaptic differentiation. Annu Rev Cell Dev Biol 24:237–262
Kamin D, Lauterbach MA, Westphal V, Keller J, Schönle A, Hell SW, Rizzoli SO (2010) High- and low-mobility stages in the synaptic vesicle cycle. Biophys J 99:675–684
Kerchner GA, Nicoll RA (2008) Silent synapses and the emergence of a postsynaptic mechanism for LTP. Nat Rev Neurosci 9:813–825

Kim SH, Ryan TA (2010) CDK5 serves as a major control point in neurotransmitter release. Neuron 67(5):797–809

Kononekno NL, Diril MK, Puchkov D, Kintscher M, Koo JS, Pfuhl G, Winter Y, Wienisch M, Klingauf J, Breustedt J, Schmitz D, Maritzen T, Haucke V (2013) Compromised fidelity of endocytic synaptic vesicle protein sorting in the absence of stonin 2. Proc Natl Acad Sci U S A 110(6):E526–E535

Koo JS, Markovic S, Puchkov D, Mahrenholz CC, Beceren-Braun F, Maritzen T, Dernedde J, Volkmer R, Oschkinat H, Haucke V (2011) SNARE motif-mediated sorting of synaptobrevin by the endocytic adaptors clathrin assembly lymphoid myeloid leukemia (CALM) and AP180 at synapses. Proc Natl Acad Sci U S A 108(33):13540–13545

Körber C, Horstmann H, Sätzler K, Kuner T (2012) Endocytic structures and synaptic vesicle recycling at a central synapse in awake rats. Traffic 13(12):1601–1611

Krabben L, Fassio A, Bhati VK, Pechstein A, Onofri F, Fadda M, Messa M, Rao Y, Shupliakov O, Stamou D, Benfenati F, Haucke V (2011) Synapsin I senses membrane curvature by an amphipathic lipid packing sensor motif. J Neurosci 31(49):18149–18154

Kraszewski K, Mundigl O, Daniell L, Verderio C, Matteoli M, De Camilli P (1995) Synaptic vesicle dynamics in living cultured hippocampal neurons visualized with CY3-conjugated antibodies directed against the lumenal domain of synaptotagmin. J Neurosci 15(6):4328–4342

Kuromi H, Kidokoro Y (1998) Two distinct pools of synaptic vesicles in single presynaptic boutons in a temperature-sensitive *Drosophila* mutant, *shibire*. Neuron 20:917–925

Kuromi H, Kidokoro Y (2000) Tetanic stimulation recruits vesicles from reserve pool via a cAMP-mediated process in drosophila synapses. Neuron 27:133–143

Li Z, Murthy VN (2001) Visualizing postendocytic traffic of synaptic vesicles at hippocampal synapses. Neuron 31:593–605

Marra V, Burden JJ, Thorpe JR, Smith IT, Smith SL, Häusser M, Branco T, Staras K (2012) A preferentially segregated recycling vesicle pool of limited size supports neurotransmission in native central synapses. Neuron 76(3):579–589

Mathew SS, Pozzo-Miller L, Hablitz JJ (2008) Kainate modulates presynaptic GABA release from two vesicle pools. J Neurosci 28(3):725–773

Mayford M, Siegelbaum SA, Kandel ER (2012) Synapses and memory storage. Cold Spring Harb Perspect Biol 4:a00575

McKinney RA, Capogna M, Dürr R, Gähweiler BH, Thompson SM (1999) Miniature synaptic events maintain dendritic spines via AMPA receptor activation. Nat Neurosci 2(1):44–49

Mitter D, Reisinger C, Hinz B, Hollmann S, Yelmanchili SV, Treiber-Held S, Ohm TG, Herrmann A, Ahnert-Hilger G (2003) The synaptophysin/synaptobrevin interaction critically depends on cholesterol content. J Neurochem 84:35–42

Molenaar PC, Nickolson VJ, Polak RL (1973a) Preferential release of newly synthesized ^{3}H-acetylcholine from rat cerebral cortex slices in vitro. Br J Pharm 47:97–108

Molenaar PC, Polak RL, Nickolson VJ (1973b) Subcellular localization of newly-formed [^{3}H] acetylcholine in rat cerebral cortex in vitro. J Neurochem 21:667–678

Molgo J, Pecot-Dechavassine M (1988) Effects of carbonyl cyanide m-chlorophenylhydrazone on quantal transmitter release and ultrastructure of frog motor nerve terminals. Neuroscience 24:695–708

Morciano M, Burre J, Corvey C, Karas M, Zimmermann H, Volknandt W (2005) Immunoisolation of two synaptic vesicle pools from synaptosomes: a proteomics analysis. J Neurochem 95(6):1732–1745

Morgan JR, Comstra HS, Cohen M, Faundez V (2013) Presynaptic membrane retrieval and endosome biology: defining molecularly heterogeneous synaptic vesicles. Cold Spring Harb Perspect Biol 5:a016915

Murthy VN, Stevens CF (1998) Synaptic vesicles retain their identity through the endocytic cycle. Nature 392:497–501

Neves G, Lagnado L (1999) The kinetics of exocytosis and endocytosis in the synaptic terminal of goldfish retinal bipolar cells. J Physiol 515(1):181–202

Opazo F, Punge A, Bückers J, Hoopmann P, Kastrup L, Hell SW, Rizzoli SO (2010) Limited intermixing of synaptic vesicle components upon vesicle recycling. Traffic 11:800–812
Orenbuch A, Shalev L, Marra V, Sinai I, Lavy Y, Kahn J, Burden JJ, Staras K, Gitler D (2012) Synapsin selectively controls the mobility of resting pool vesicles at hippocampal terminals. J Neurosci 32(12):3969–3980
Paillart C, Li J, Matthews G, Sterling P (2003) Endocytosis and vesicle recycling at a ribbon synapse. J Neurosci 23:4092–4099
Palade GE, Palay SL (1954) Electron microscope observations of interneuronal and neuromuscular synapses. Anat Rec 118:335–336
Palay SL (1956) Synapses in the central nervous system. J Biophys Biochem Cytol 2(4):193–202
Pechstein A, Shupliakov O (2010) Taking a back seat: synaptic vesicle clustering in presynaptic terminals. Front Synaptic Neurosci 2:143
Pieribone VA, Shupliakov O, Brodin L, Hilfiker-Rothenfluh S, Czernik AJ, Greengard P (1995) Distinct pools of synaptic vesicles in neurotransmitter release. Nature 375:493–497
Poulton J, Chiaratti MR, Meirelles FV, Kennedy S, Wells D, Holt IJ (2010) Transmission of mitochondrial DNA diseases and ways to prevent them. PLoS Genet 6(8):e1001066
Ramirez DMO, Khvotchev M, Trauterman B, Kavalali ET (2012) Vti1a identifies a vesicle pool that preferentially recycles at rest and maintains spontaneous neurotransmission. Neuron 73:121–134
Revelo NH, Kamin D, Truckenbrodt S, Wong AB, Reuter-Jessen K, Reisinger E, Moser T, Rizzoli SO (2014) A new probe for super-resolution imaging of membranes elucidates trafficking pathways. J Cell Biol 205(4):591–606
Richards DA, Guatimosim C, Betz WJ (2000) Two endocytic recycling routes selectively fill two vesicle pools in frog motor nerve terminals. Neuron 27:551–559
Richards DA, Guatimosim C, Rizzoli SO, Betz WJ (2003) Synaptic vesicle pools at the frog neuromuscular junction. Neuron 39:529–541
Rizzoli SO (2014) Synaptic vesicle recycling: steps and principles. EMBO J 33(8):788–822
Rizzoli SO, Betz WJ (2004) The structural organization of the readily releasable pool of synaptic vesicles. Science 303:2037–2039
Rizzoli SO, Betz WJ (2005) Synaptic vesicle pools. Nat Rev Neurosci 6:57–69
Rizzoli SO, Jahn R (2007) Kiss-and-run, collapse and 'readily retrievable' vesicles. Traffic 8 (9):1137–1144
Rose T, Schoenenberger P, Jezek K, Oertner TG (2013) Developmental refinement of vesicle cycling at Schaffer collateral synapses. Neuron 6:1109–1121
Sabo SL, Gomes RA, McAllister AK (2006) Formation of presynaptic terminals at predefined sites along axons. J Neurosci 26:10813–10825
Saitoe M, Schwarz TL, Umbach JA, Gundersen CB, Kidokoro Y (2001) Absence of junctional glutamate receptor clusters in drosophila mutants lacking spontaneous transmitter release. Science 293:514–517
Saka S, Rizzoli SO (2012) Super-resolution imaging prompts re-thinking of cell biology mechanisms. Bioessays 34:386–395
Sakaba T, Neher E (2001) Calmodulin mediates rapid recruitment of fast-releasing synaptic vesicles at a calyx-type synapse. Neuron 23:821–832
Sankaranarayanan S, Ryan TA (2000) Real-time measurements of vesicle-SNARE recycling in synapses of the central nervous system. Nat Cell Biol 2:197–204
Sankaranarayanan S, de Angelis D, Rothman JE, Ryan TA (2000) The use of pHluorins for optical measurements of presynaptic activity. Biophys J 79:2199–2208
Sara Y, Virmani T, Deák F, Liu X, Kavalali ET (2005) An isolated pool of vesicles recycles at rest and drives spontaneous neurotransmission. Neuron 45(4):563–573
Schikorski T, Stevens CF (1997) Quantitative ultrastructural analysis of hippocampal excitatory synapses. J Neurosci 17:5858–5867
Schikorski T, Stevens CF (2001) Morphological correlates of functionally defined synaptic vesicle populations. Nat Neurosci 4(4):391–395

Shupliakov O (2009) The synaptic vesicle cluster: a source of endocytic proteins during neurotransmitter release. Neuroscience 158:204–210
Siksou L, Rostaing P, Lechaire JP, Boudier T, Ohtsuka T, Fejtova A, Kao HT, Greengard P, Gundelfinger ED, Triller A, Marty S (2007) Three-dimensional architecture of presynaptic terminal cytomatrix. J Neurosci 27:6868–6877
Staras K, Branco T (2010) Sharing vesicles between central presynaptic terminals: implications for synaptic function. Front Synaptic Neurosci 2(20)
Staras K, Branco T, Burden JJ, Pozo K, Darcy K, Marra V, Ratnayaka A, Goda Y (2010) A vesicle superpool spans multiple presynaptic terminals in hippocampal neurons. Neuron 66:37–44
Stevens CF, Wang Y (1995) Facilitation and depression at single central synapses. Neuron 14:795–802
Stevens CF, Williams JH (2007) Discharge of the readily releasable pool with action potentials at hippocampal synapses. J Neurophysiol 98:3221–3229
Stevens RJ, Akbergenova Y, Jorquera RA, Littleton JT (2012) Abnormal synaptic vesicle biogenesis in drosophila synaptogyrin mutants. J Neurosci 35(50):18054–18067
Südhof TC (2004) The synaptic vesicle cycle. Annu Rev Neurosci 27:509–547
Sutton AM, Ito HT, Cressy P, Kempf C, Woo JC, Schuman EM (2006) Miniature neurotransmission stabilizes synaptic function via tonic suppression of local dendritic protein synthesis. Cell 125:785–799
Takamori S, Holt M, Stenius K, Lemke EA, Grønborg M, Riedel D, Urlaub H, Schenck S, Brügger B, Ringler P, Müller SA, Rammner B, Gräter F, Hub JS, De Groot BL, Mieskes G, Moriyama Y, Klingauf J, Grubmüller H, Heuser J, Wieland F, Jahn R (2006) Molecular anatomy of a trafficking organelle. Cell 127:831–846
Takei K, Mundigl O, Daniell L, De Camilli P (1996) The synaptic vesicle cycle: a single vesicle budding step involving clathrin and dynamin. J Cell Biol 133(6):1237–1250
Tarelli FT, Bossi M, Fesce R, Greengard P, Valtorta F (1992) Synapsin I partially dissociates from synaptic vesicles during exocytosis induced by electrical stimulation. Neuron 9:1143–1153
Tarsa L, Goda Y (2002) Synaptophysin regulates activity-dependent synapse formation in cultured hippocampal neurons. Proc Natl Acad Sci U S A 99(2):1012–1016
Teng H, Wilkinson RS (2000) Clathrin-mediated endocytosis near active zones in snake motor boutons. J Neurosci 20:7986–7993
Tsuriel S, Geva R, Zamorano P, Dresbach T, Boecker T, Gundelfinger ED, Garner CC, Ziv NE (2006) Local sharing as a predominant determinant of synaptic vesicle matrix molecular dynamics. PLoS Biol 4(9):e271
Uytterhoeven V, Kuenen S, Kasprowicz J, Miskiewicz K, Verstreken P (2011) Loss of skywalker reveals synaptic endosomes as sorting stations for synaptic vesicle proteins. Cell 145:117–132
Valtorta F, Pozzi D, Benfenati F, Fornasiero EF (2011) The synapsins: multitask modulators of neuronal development. Semin Cell Dev Biol 22(4):378–386
Verhage M, Maia AS, Plomp JJ, Brussard AB, Heeroma JH, Vermeer H, Toonen RF, Hammer RE, van den Berg TK, Missler M, Geuze HJ, Südhof TC (2000) Synaptic assembly of the brain in the absence of neurotransmitter secretion. Science 287:864–869
Wang Y, Tang BL (2006) SNAREs in neurons – beyond synaptic vesicle exocytosis. Mol Membr Biol 23(5):377–384
Welzel O, Knörr J, Stroebel AM, Kornhuber J, Groemer TW (2011) A fast and robust method for automated analysis of axonal transport. Eur Biophys J 40(9):1061–1069
Wiegert JS, Oertner TG (2013) Long-term depression triggers the selective elimination of weakly integrated synapses. Proc Natl Acad Sci U S A 110(47):E4510–E4519
Wienisch M, Klingauf J (2006) Vesicular proteins exocytosed and subsequently retrieved by compensatory endocytosis are nonidentical. Nat Neurosci 9:1019–1027
Wilhelm BG, Groemer TW, Rizzoli SO (2010) The same synaptic vesicles drive active and spontaneous release. Nat Neurosci 13(12):1454–1456

Wilhelm BG, Mandad S, Truckenbrodt S, Kröhnert K, Schäfer C, Rammner B, Koo SJ, Claßen GA, Krauss M, Haucke V, Urlaub H, Rizzoli SO (2014) Composition of isolated synaptic boutons reveals the amounts of vesicle trafficking proteins. Science 344:1023–1028

Willig KI, Rizzoli SO, Westphal V, Jahn R, Hell SW (2006) STED microscopy reveals that synaptotagmin remains clustered after synaptic vesicle exocytosis. Nature 440:935–939

Wragg RT, Snead D, Dong Y, Ramlall TF, Menon I, Bai J, Eliezer D, Dittman JS (2013) Synaptic vesicles position complexin to block spontaneous fusion. Neuron 77:323–334

Xu-Friedman MA, Harris KM, Regehr WG (2001) Three-dimensional comparison of ultrastructural characteristics at depressing and facilitating synapses onto cerebellar purkinje cells. J Neurosci 21(17):6666–6672

Zakharenko SS, Zablow L, Siegelbaum SA (2001) Visualization of changes in presynaptic function during long-term synaptic plasticity. Nat Neurosci 4(7):711–717

Zhang YZ, Ouyang YC, Hou Y, Schatten H, Chen DY, Sun QY (2008) Mitochondrial behavior during oogenesis in zebrafish: a confocal microscopy analysis. Dev Growth Differ 50(3):189–201

Zhou RR, Wang B, Wang J, Schatten H, Zhang YZ (2010) Is the mitochondrial cloud the selection machinery for preferentially transmitting wild-type mtDNA between generations? Rewinding Müller's ratchet efficiently. Curr Genet 56(2):101–107

Index

A
Acidification, 279
Actin, 23, 28, 237–238, 303–304
Action potential, 8, 257
Active zone, 7, 48, 212
Active zone proteins, 7, 12–13
Adaptor protein 2 (AP2), 226, 232
Adaptor protein 180 (AP180), 232–233
Adenosine triphosphate (ATP), 184
Alternative splicing, 212
Amphipathic lipid packing sensor (ALPS), 303
Amphiphysin, 235, 262, 311
Antiport, 287
AP-2 adapter complex, 177
AP firing, 264
Asynchronous release, 173, 314
ATP, 278
ATPase NSF, 106
Autaptic neurons, 313
Autism, 312
Auxilin, 240

B
Bassoon, 7, 52, 70
Bin/Amphiphysin/RVS (BAR), 234
Brain-derived neurotrophic factor (BDNF), 317
Bruchpilot, 8, 78, 79
Bulk endocytosis, 17, 225, 245–247

C
Ca^{2+}, 259
Ca^{2+}/CaM-dependent-kinase II inhibitor protein (CaMKIIN), 208
Ca^{2+}/CaM-dependent protein kinase II (CaMKII), 6, 16, 27, 204, 299
Ca^{2+} channel modulation, 6
Ca^{2+} channels, 12–16, 175
Ca^{2+} currents, 206
Ca^{2+}-dependent inactivation, 209
Ca^{2+}-dependent neurotransmitter release, 172–177
C_2A-domain, 70
Ca^{2+} influx, 174, 212
Ca^{2+}-influx dependent facilitation, 208
Ca^{2+} ion, 171
Calcineurin, 18, 311
Calcium-binding protein 1 (CaBP1), 15
Calcium channels, 4
Calcium ions, 4
Calmodulin (CaM), 6, 14, 15, 196
Calmodulin binding domain (CBD), 209
Calyx of Held, 259, 288
Cargo selection, 228
Ca^{2+} sensor, 172–173
Ca^{2+} sensor proteins (CaS), 4, 6, 208, 268
Ca^{2+} signal, 8
CaS proteins calcium binding protein 1 (CaBP1), 211
CAST, 8, 74
CAST/ERC2, 7, 70
$Ca_v2.1$, 16
  and $Ca_v2.2$, 13
  channels, 14, 202
$Ca_v2.2$ channels, 202
$Ca_v2.3$ channels, 199
C_2B-domain, 72
C_2B-domain of synaptotagmin-1, 14
Cdc42, 238

S. Mochida (ed.), *Presynaptic Terminals*, DOI 10.1007/978-4-431-55166-9

C_2 domain, 169
Cell biology, 331
Central nervous system (CNS), 258–260
Channel-like pore, 160
Chloride (Cl^-), 276
Chloride channel, 283
Clathrin, 17, 230–231
  adaptors, 21
  independent pathways, 244–247
  uncoating, 22
Clathrin-coated pits, 21, 228
Clathrin-mediated endocytosis, 20, 176–177, 225, 226, 341
ClC-3, 283
Clearance of the active zone, 261
Coat assembly, 228
Compensatory endocytosis, 225
Complexin, 10, 141, 175, 183
Constitutive trafficking, 343–344
Co-transporter, 286
Cultured SCG neurons, 10
Cytomatrix at the active zone-associated structural protein (CAST), 75

D
Delayed response enhancement (DRE), 316
Dephosphins, 311
Depression, 312
Diacylglycerol (DAG), 109
Dileucine motif, 289
Dimerization, 104, 304
DOC2α, 74
Docking, 174
Drosophila, 317
Drosophila shibire, 19, 261
Dynamin, 17–19, 22, 23, 238–239, 259, 262, 267, 268, 311
Dynamin 1, 259, 260
Dynamin 2, 260
Dynamin 3, 260
3 Dynamins (Dynamins 1, 2 and 3), 18, 258, 263–265

E
Electrical gradient, 26
Electron tomography, 310
ELKS, 7, 70, 74
Endocytic pathways, 267
Endocytosis, 16–23, 258, 259, 311, 339
Endophilin, 22, 234–235, 290
Endosomal recycling, 23
Endosome, 343
Energy barrier for membrane fusion, 102
Epilepsy, 312
Epileptogenesis, 316
Eps15, 237
Epsin, 233
Excitability, 315
Excitatory, 312–316
Excitatory postsynaptic currents (EPSCs), 288, 314
Exocytic-endocytic coupling, 268
Exocytosis, 152, 203, 223, 275, 339
Exocytotic efficacy, 136
Extracellular signal-regulated kinase (ERK), 299, 317

F
Fatigue, 313
FCHo, 234
FM1-43, 332
Frog neuromuscular junction, 306
Fucosylation, 309
Functional heterogeneity, 352
Functional versatility, 352
Fusion clamp, 175
Fusion pore, 153, 176

G
Gamma-aminobutyric acid (GABA), 275, 314
Glutamatergic, 313
Glutamic acid dehydrogenase (GAD), 282–283
Glycine-specific transporters (GlyTs), 26
Glycine transporters (GlyT2), 26, 283
G protein, 196

H
H_{abc}-domain, 96
Held, 313
Hemifused state, 101
H^+ gradient, 25
Hsc70 ATPase, 22, 240–241

I
Induced pluripotent stem cells (iPSC), 314
Inhibitory, 312–316
Inhibitory transmission, 314
Inositol polyphosphate, 178

Intersectin, 237, 311
Intramolecular FRET, 100
In vitro fusion, 183
In vitro reconstituted fusion, 182
IQ-like motif (IM), 209

K
Kinesin-3, 53
Kiss-and-run, 17, 225, 244–245
Kiss and run fusion mode, 154

L
Lamprey, 309
Learning, 317
Lethal giant larvae(Lgl), 129
Leucine-rich repeat transmembrane neuronal proteins 4 (LRRTMs 4), 59–60
Leukocyte common antigen related (LAR), 75
Lipid binding, 302
Lipid stalk, 101
Liprin, 75
α-Liprin, 7, 73
Local recycling, 24
Long-term plasticity, 338
Long-term potentiation (LTP), 317

M
Maturation, 57
Membrane fission, 22–23
Membrane potential ($\Delta\Psi$), 277
Memory, 317
Mobility, 345–346
Molecular tags, 346–348
Mover, 54
Munc13, 7, 54
Munc13-1, 70
Munc18-1, 9
Munc13/CAPS, 95–97
Munc18-1/nsec-1, 93
Mun domain, 76, 96
Myosin II, 28
Myosin IIB, 23

N
Na^+/H^+ exchanger (NHE), 284
Neurexins, 59
Neurite elongation, 301
Neurotransmitter release, 3–4, 203
Neurotransmitters, 155, 224, 275–290
Noncanonical SNAREs, 110–113
Nonclassical synaptic vesicle pools, 337–344
NRSF/REST, 297
N-terminal peptide of syntaxin-1, 94
N-to C-terminal assembly, 98
N-type Ca^{2+}, 79

O
Oligomerization, 299
O-linked N-acetylglucosamine (O-GlcNAc), 309
Opening syntaxin, 95
Organelle competition, 352

P
Paired-pulse depression (PPD), 316
Paired-pulse facilitation (PPF), 135, 316
Pause sites, 51
Peripheral nervous system, 258
pH gradient (ΔpH), 26, 276, 277
Phosphatidic acid (PA), 110
Phosphatidylinositides (PIs), 108, 242
Phosphatidyl-inositol-4,5 bisphosphate (PIP_2), 206
Phosphatidylinositol 4,5-bisphosphate (PtdIns (4,5)P2), 178
Phosphatidylinositol-3,4-bisphosphate [PI(3,4) P2], 243
Phosphatidylinositol-4,5-bisphosphate [PI(4,5) P2], 228
Phosphatidylserine (PS), 110, 177
Phosphorylation, 296, 306, 308
Photoconversion, 332
Piccolo/Aczonin, 7, 70
Piccolo-Bassoon transport vesicles (PTVs), 51
$PI(4,5)P_2$, 22, 108, 243
Plasticity, 311–317
Pool transitions, 348–349
Post-docking, 313
Postsynaptic density (PSD), 70
Post-tetanic potentiation (PTP), 316
P/Q-type Ca^{2+} channels, 73
Pre-docking, 313
Presynaptic, 49, 307
Presynaptic $Ca_V2.1$ channels, 15
Priming/fusion, 311
Promoter, 297
Protein buffering, 350
Protein kinase A (PKA), 298
Protein kinase C (PKC), 203
Proteins, 234
Proton electrochemical gradient, 276

Q
Quantal size, 287–288

R
Rab3A, 302, 310
Rab-interacting molecule 1 (RIM1), 7, 70
Rab-interacting molecule (RIM), 12, 201
Readily releasable pool (RRP), 27–28, 334–336
  refilling, 266
  replenishment, 267
  size, 135
Receptor tyrosine phosphatase proteins (RPTPs), 58
Reconstitution, 285–287
Recycling, 257, 305, 311
Recycling pool, 28, 313, 332–334
Release kinetics, 310
Reserve pool (RP), 27, 335, 337
Residual Ca^{2+}, 8
Resting pool, 313
Reuse pathway, 24
RIM-binding proteins (RIM-BPs), 7, 13, 74

S
Scaffolding proteins, 62
SCG neurons, 15, 18, 79
Scotopic electroretinogram, 79
Sec9, 137
SH3 domain, 302
Short-term plasticity, 200
Short-term potentiation, 135
Siah1, 55
siRNA, 263
SNAP-25, 9, 13, 89, 141, 181, 201
α-/β-/γ-SNAP, 106
cis-SNARE complex, 106, 182
SNX9, 235–236
Soluble NSF attachment protein receptors (SNAREs), 10, 74, 88, 131, 141, 150, 179
  complex, 199
  hypothesis, 4, 6
  proteins, 9
Speed of endocytosis, 20
Splayed lipids, 103
Splicing, 298
Spontaneous pool, 341–344
Spontaneous release, 172–173
Squid giant synapse, 305
Src, 299, 302
Sro7, 137
Stonin, 233–234
Stonin 2, 177
Subcellular localisation, 345
α1 subunit, 207
$\alpha_2\delta$ subunit, 198
β subunit, 197
βγ subunit, 204
Superior cervical ganglion neurons (SCGN), 206, 258, 262, 265
Super-pool, 337–338
Surface/readily retrievable pool, 338–341
SV recycling, 225
SYD-1, 60
SYD-2, 75
Syd-2/liprin-á, 53
Sympathetic superior cervical ganglion (SCG) neurons, 6
Synapsin, 27, 337
Synaptic communication, 349–351
Synaptic depression, 137
Synaptic transmission, 311–317
Synaptic vesicles, 152, 169, 183, 257, 275–290
  exocytosis, 131
  pools, 305–310, 330–331
  recycling, 16
  recycling pathways, 23–24
  refilling, 25–26
  trafficking, 28
Synaptobrevin-2, 141
Synaptobrevin/VAMP, 91
Synaptogenesis, 301
Synaptojanin 1, 239–240
Synaptotagmin, 10, 18, 141, 168
Synaptotagmin-1, 12, 133, 199
Synaptotagmin-2, 12
Synaptotagmin-10, 12
Synaptotagmin-1-tomosyn complex, 134
Synaptotagmin-1-tomosyn-SNARE complex, 134
Synchronous release, 173, 314
Syndapin, 236
Synprint, 196
Synprint (synaptic protein interaction) sites, 13, 14
Syntabulin, 56
Syntaxin, 180–181
Syntaxin-1, 13, 90, 141
Syntaxin-1A, 201
Syntaxin-1VAMP, 9

T
Tail domain, 134
Target SNAREs (t-SNAREs), 7, 180, 212
Tethering, 50
Time course of endocytosis, 19
Toca1/FBP17/CIP4, 236
Tomosyn, 10, 129
Tomosyn-SNARE complex, 131
Trafficking, 288–290
Transporters, 25
Trans-SNARE complex, 175

U
Ultrafast endocytosis, 20, 260–261
Ultrafast mode of endocytosis, 263
Unc-13, 74
Unc-47, 282

V
Vacuolar proton pump (V-ATPase), 11, 150, 154, 155, 275–276
$V0_a$ subunit, 158
V0c, 158
Vesicle associated membrane proteins (VAMP), 9
  VAMP2, 141, 159
  VAMP4, 112
Vesicle docking, 93
Vesicle filling, 155
Vesicle priming, 184
Vesicle scission, 228
Vesicle uncoating, 229
Vesicular acetylcholine transporters (VAChT), 25
Vesicular GABA transporter (VGAT), 25, 282–283, 314
Vesicular glutamate transporters (VGLUTs), 25, 280–282
Vesicular glutamate transporters (VGLUTs)
  VGLUT1, 26, 281, 314
  VGLUT2, 281
  VGLUT3, 281
Vesicular monoamine transporter (VMAT), 25
Visinin-like protein-2 (VILIP-2), 15, 211
VMAT1, 25
Voltage-gated Ca_V2 channels, 195
V0 sector, 159
v-SNAREs, 7, 183
Vti1a, 112

W
WASP, 238
WD40 repeats, 133

Z
Zipper hypothesis, 97