CHAPTER TWO

Cellular Mechanisms for the Biogenesis and Transport of Synaptic and Dense-Core Vesicles

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Abstract

The release of intercellular messengers from synaptic (SVs) and dense-core vesicles (DCVs) constitutes the primary mechanism for communication between neighboring or distant cells and organs in response to stimuli. Here we review the life span of SVs and DCVs found in the brain, neuroendocrine and exocrine tissues. These vesicles must be formed, trafficked, and their contents secreted; processes which require orchestrated actions of a great repertoire of lipids, proteins, and enzymes. For biogenesis and vesicular budding, lipids that influence curvature and aggregation of cargo are necessary for pinching off of vesicles. Vesicles travel on cytoskeletal filaments powered by motors that control the dynamics: location, speed, and directionality of movement. Regardless of mechanisms of traffic, vesicles arrive at sites of release and are docked for exocytosis, followed by membrane fusion, and release of vesicular content to exert physiological responses. Neurological disorders with pathology involving abnormal vesicular budding, trafficking, or secretion are discussed.

1. INTRODUCTION

All eukaryotic cells contain a constitutive secretory pathway (CSP), which is used to deliver soluble (e.g. albumin, growth factors) and membrane

proteins (e.g. receptors) to the plasma membrane (PM) to maintain growth, survival and differentiation of cells (Kelly, 1985). Proteins transported in vesicles of the CSP to the PM are secreted continuously and do not require an external stimulus (Gumbiner and Kelly, 1982; Dumermuth and Moore, 1998). Endocrine cells, neurons and exocrine cells, in addition to the CSP, have a regulated secretory pathway (RSP) which transports peptide hormones, neuropeptides and digestive enzymes, respectively, in large dense-core vesicles/granules (DCVs/DCGs) to the cell surface for secretion to mediate higher physiological function. Secretion of content from these DCVs is dependent upon external stimulation of the cell (Gumbiner and Kelly, 1982; Kelly, 1985; Dumermuth and Moore, 1998). Characteristic budding and trafficking behavior of the CSP and RSP are demonstrated in Fig. 2.1 and contrasting properties are listed in Table 2.1. For neurons, in addition to DCV function within the RSP, regulated release of neurotransmitters packaged in synaptic vesicles (SVs) is key for communication. Thus, neurons use both DCVs and SVs to mediate and modulate neurotransmission (De Camilli and Jahn, 1990).

This review is organized into four main segments. First, we introduce the types of vesicles found in professional secretory cells with an emphasis on neurons, where behavior and function of DCVs and SVs can be well contrasted. We examine non-neuronal professional secretory cells where regulated secretion is prominent, and discuss the components of the neuroendocrine system, with an emphasis on the stress response axis (Sections 2.2–2.4). Secondly, we present the current state of research for DCV biogenesis inclusive of sorting mechanisms at the trans-Golgi network (TGN) and genetic, posttranscriptional and posttranslational regulation of DCV biogenesis (Section 2.5). This is followed by an in-depth analysis of SV biogenesis at the synaptic terminal where protein and lipid constituents involved in the process of sorting and assembly are paramount (Section 2.6). In the third part, mechanisms of transport used by immature and mature DCVs, Piccolo–Bassoon transport vesicles (PTVs), and synaptic protein precursors/transport vesicles are discussed (Section 2.7). The involvement of DCV-specific and SV-specific mechanisms of exocytosis is briefly reviewed. Throughout this third segment (sections 2.5–2.8), several knockout (KO), mutant or dominant-negative cellular or animal models of members of the molecular machinery involved during vesicular biogenesis, trafficking and exocytosis illustrate the necessity of each component in these processes. Defective vesicle biogenesis and trafficking give rise to or is associated with various neuroendocrine disorders in humans. In the last segment

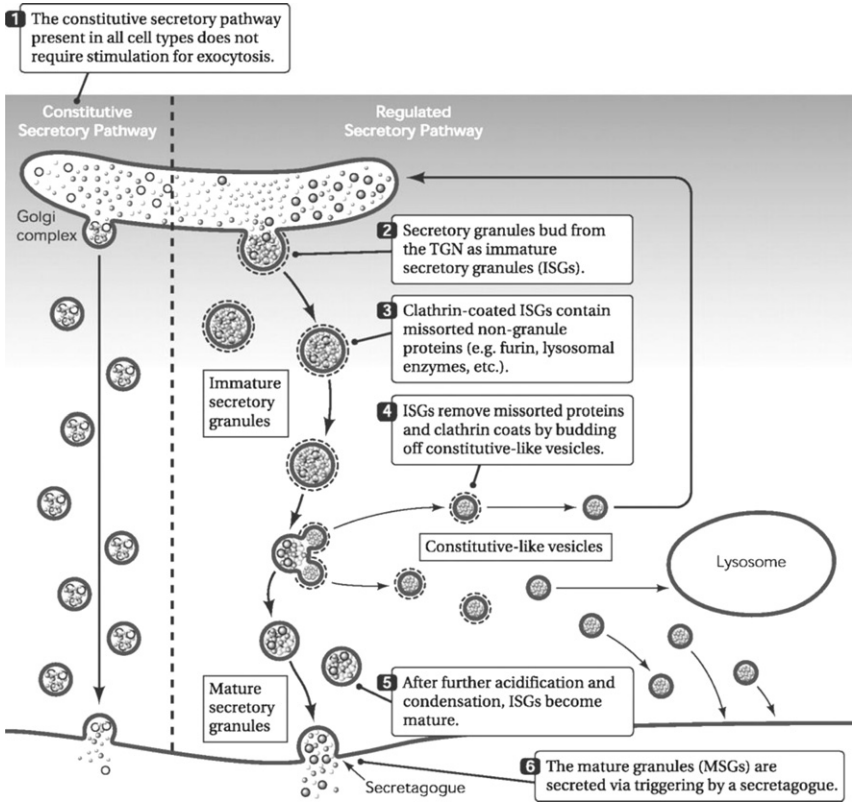

Figure 2.1 ***Formation and exocytosis of CVs and DCVs.*** Two distinctive secretory pathways are present in neurons, neuroendocrine and endocrine and exocrine cells, for constitutive and regulated secretion of proteins and peptides. Constitutive vesicles (CVs), release their content rapidly, while DCVs in the RSP undergo several maturation steps and storage prior to releasing their content.

(Section 2.9), although scientific information is sparse and slowly emerging, we discuss examples of neurodevelopmental, psychiatric and neurodegenerative disorders where defects in DCV and SV biology and function contribute to the pathogenesis of disease.

2. SVs AND DCVs IN NEUROTRANSMISSION

The most prevalent way that one neuron communicates with another is by releasing an excitatory, inhibitory or modulatory factor into the synapse, and eliciting a response from the second neuron. With the

Table 2.1 Comparison of constitutive versus RSP vesicles

	CVs	DCVs
Properties	Small, clear Diameter: 80−100 nm	Large, dense core Diameter: 100−300 nm
Assembly	No sorting signal required for cargo entry into CVs No aggregation of proteins before vesiculation Assembly not dependent on acidic pH at TGN	Sorting signal/motif generally required for entry into DCVs Aggregation is a prerequisite before vesiculation Clathrin coat and APs necessary for budding Assembly dependent on acidic pH at TGN and for DCV maturation
Secretion	Transported along microtubules from TGN to PM Independent of an external signal Non-calcium triggered Rapid transit and release from Golgi ~10 min; No storage pool Membrane components not recycled	Transported along microtubules from TGN to proximity of PM and actin-based transport to release site where they are docked Exocytosis requires external signal and intracellular calcium Exocytosis requires external signal and intracellular calcium Long residence time 7−10 h, stored in cytoplasm Membrane components recycled back to TGN after exocytosis

exception of constitutive inputs whereby neurons are constantly exposed to a particular environment, most neurotransmission occurs as a result of sufficient presynaptic stimulation to induce release of vesicular content into extracellular space—i.e. via a RSP. The vesicles that transport neurotransmitters or neuromodulators are classified as either SVs or DCVs, respectively. Whereas both SVs and DCVs are prominent in brain (Salio et al., 2007; Dieni et al., 2012), neuroendocrine tissues such as the pituitary and adrenal glands, endocrine pancreas, and gonads are primarily enriched with DCVs (Gorr et al., 2001). These neuroendocrine organs regulate communication, growth, food intake, mood, stress and cognition (Guan et al., 1997; Pacak and Palkovits, 2001; Stanley et al., 2005; Kenna et al., 2009). Because of these important functions, the abnormal biogenesis, transport and secretion of SVs and DCVs could lead to severe, debilitating disorders. Our focus is on the shared mechanisms used by secretory cells to generate and traffic DCVs and SVs to sites of regulated exocytosis.

2.1. SVs and Classical Neurotransmitters

SVs predominantly carry classical neurotransmitters classified as amino acids [glutamate, γ-aminobutyric acid (GABA), acetylcholine (ACh) or monoamines (dopamine, serotonin, epinephrine, norepinephrine, etc.)] and are specifically found in neurons. At the electron microscopic level, these vesicles have a translucent, circular appearance and are often found clustered and docked at the active zone of an axonal terminal, near a synapse demarcated by an electron dense region (Fig. 2.2E). Upon sufficient depolarization of the presynaptic neuron, the resultant increase in intracellular calcium causes SVs to fuse with the axon terminal membrane, assisted by anchoring soluble *N*-ethylmaleimide-sensitive factor attachment receptor (SNARE) proteins to exocytose their content into the synapse (Sollner, 2003; Malsam et al., 2008). SVs are recycled in the axon terminal, interact with synaptic protein transport vesicle (SPTVs), and are refilled locally with neurotransmitter in preparation for a subsequent depolarization (Parsons et al., 1999; Fei et al., 2008). Mechanisms of fusion and regulated exocytosis are only briefly discussed, and are more extensively reviewed elsewhere (Lin and Scheller, 2000; Sollner, 2003). The closely apposed dendritic spines of the postsynaptic cell are competent to respond to this signal if they express the appropriate neurotransmitter receptor to induce postsynaptic signaling. Activation of postsynaptic receptors can either result in the postsynaptic cell's depolarization or hyperpolarization depending on the receptors expressed at the dendritic membrane.

2.2. DCVs and Neuropeptides

In general, in secretory tissues, DCVs, also referred to as DCGs, have a characteristic opaque core, which appears as an electron-dense area encased within the vesicular lipid bilayer when observed with an electron microscope (Fig. 2.2A–D). The DCV sizes vary between 100 and 300 nm in diameter in different cells types. DCVs in exocrine cells (e.g. in the pancreas) are larger than those in endocrine cells, while the neurotrophin-containing vesicles in neurons, e.g. brain-derived neurotrophic factor (BDNF), are smaller and less electron dense (Fig. 2.2) (Sadakata et al., 2004; Gondre-Lewis et al., 2006; Kim et al., 2006). Contents of DCVs are highly condensed and concentrated, and DCVs abundantly express at least one member of the chromogranin or secretogranin class of acidic Ca^{2+}-binding proteins. At the light level, these DCVs have a heavily granulated appearance when stained for protein content, hence the reference as granules (Table 2.1).

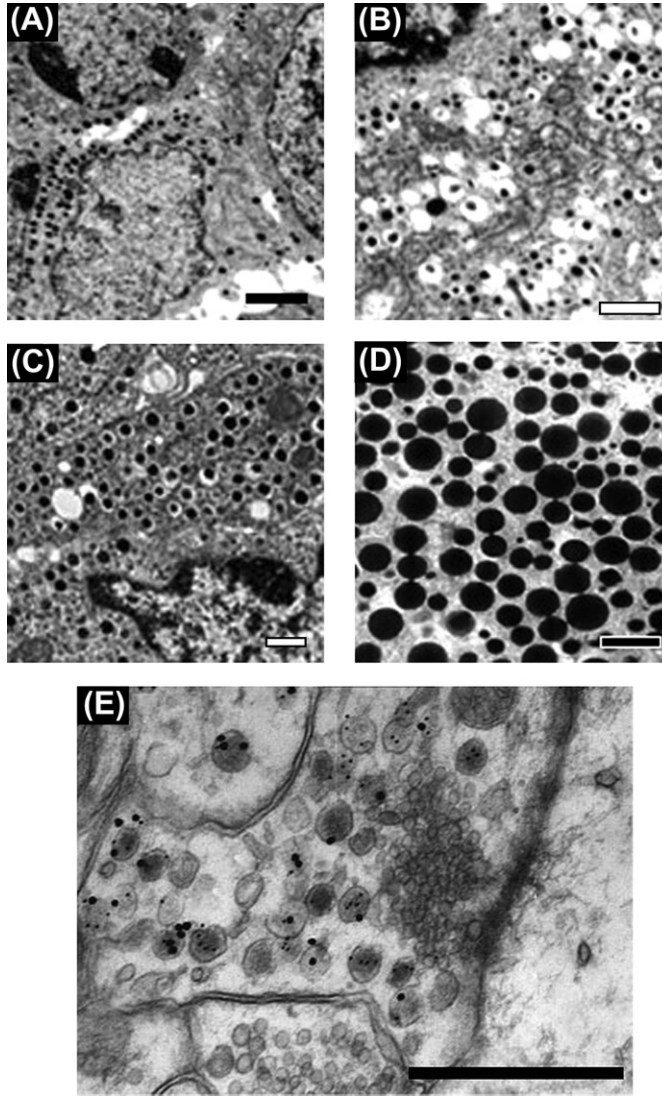

Figure 2.2 ***The many faces of DCVs in neurons, endocrine and exocrine tissues.*** Electron micrographs of DCVs in newborn mouse anterior pituitary (A), adrenal medulla (B), endocrine pancreatic islet cells (C), and exocrine pancreas packaging zymogen granules (D). (E) is a synaptic terminal with both SVs—small, clear, clustered—and DCVs, clearly larger than SVs with a dense-core and co-packaging BDNF and substance P (SP) as revealed with immuno-EM labels; BDNF 10 nm particles, SP, 20 nM. Note the size, morphology and distribution across tissues, with neurons and the pituitary having the smallest DCVs. *Panel E is from Salio et al., Devel Neurobiol., 67:3, 326–338, 2007, and is reproduced with the permission of John Wiley & Sons, Inc. Bars: A, 1 μm; B,D, 2 μm; C,E, 500 nm.*

DCVs in the nervous system package small neuropeptides and peptide hormones such as endorphins, neurotrophin family of growth factors, inclusive of BDNF, tachykinins, and somatostatin, to name a few. DCVs in the nervous system are said to be peptidergic vesicles. Unlike SVs, DCVs generally package their cargo in the soma and not at the synaptic terminal. Similarly, both SVs and DCVs bud from the TGN by specific molecular and physical mechanisms that will be discussed in the next sections. DCVs travel to the periphery using cellular motors, and once in the periphery, release their cargo into the extracellular space. Fusion with the PM and release of content represents their final action although some of the DCV membrane can be recycled and reutilized (Arnaoutova et al., 2003).

Neuropeptides are the most diverse, most numerous and most common signaling molecules in the brain with more than 100 neuropeptides having been identified thus far. Neuropeptides are synthesized in the nervous system and can function as neurotransmitters, neuromodulators and neurohormones (Hokfelt et al., 1980; Ogren et al., 2010). When released from neurons, those that function as neurotransmitters have receptors at the synapse, likely at extrasynaptic sites, whereas those that function as neuromodulators may have receptors distributed all along the target cell in addition to extrasynaptic sites. Neuropeptides packaged in DCVs respond to much stronger calcium signals than SVs, and thus for DCVs to release their content, they require robust, high frequency stimulation and not just a single action potential (Tallent, 2008).

Neuropeptides range in approximate size from 2 to 80 amino acids, and as mentioned above, are packaged and secreted via the RSP. Similar to peptides and hormones secreted by other cells in the body, they are synthesized as precursors (pre-pro-neuropeptide) and are cleaved by proteolytic enzymes to generate specific signal peptides. One precursor can produce different sets of mature peptides depending on tissue expression of proprotein convertases and other enzymes necessary for processing (Burbach, 2011). Neuropeptides constitute a very diverse group of neurotransmitters and constitute the majority of signaling in the brain (Ogren et al., 2010). In humans, there are approximately 90 different genes encoding 100s of peptides. Pro-opiomelanocortin (POMC) is the prototypical proneuropeptide, encoded by one gene, but depending on which cleavage site is targeted by processing enzymes and the neuron type in which it is expressed, it can give rise to several mature biologically active peptides.

2.3. DCVs in Astrocytes for Modulating Neuron Function

It is widely accepted that glia, especially astrocytes, secrete neurotransmitters such as glutamate in response to changes in calcium dynamics in order to modulate neuron function (Parpura and Haydon, 2000; Jourdain et al., 2007). Both the presence of DCVs and mechanisms of regulated secretion in astrocytes and other glial cell lines have recently been investigated. Secretory granules positive for the DCV markers, chromogranin B (CgB), secretogranin II (SgII), and Secretogranin III (SgIII), were detected in primary astrocytes (Calegari et al., 1999; Hur et al., 2010; Paco et al., 2010). In hippocampal astrocytes, SgII was demonstrated to undergo stimulated release in the presence of various secretagogues (Calegari et al., 1999), and in cortical astrocytes an unprocessed form of SgIII was found to be robustly expressed and continually secreted (Paco et al., 2010). These DCVs are proposed to function similarly to DCVs in other cells as internal inositol 1,4,5-trisphosphate (IP_3)-sensitive calcium stores. They respond to low levels of IP_3 via three different IP_3 receptors (IP_3Rs)expressed on their DCV membrane (Hur et al., 2010). Furthermore, the precursor of glia-derived neurotrophic factor (GDNF), (beta) pro-GDNF but not the constitutively secreted (alpha) pro-GDNF, was co-localized in secretory granules with the DCV marker, SgII, in these glial cell lines. The beta precursor protein was found to undergo enzymatic processing to produce mature GDNF and both precursor and mature forms were secreted in a regulated fashion in response to depolarization of the cell with KCl (Lonka-Nevalaita et al., 2010). It has been reported that astrocytes also synthesize and secrete neuropeptides and the processing enzyme carboxypeptidase E (CPE), but in a constitutive manner (Klein et al., 1992).

3. DCVs AND SVs IN TISSUES

Both neurotransmitters and small peptides act as signaling molecules that mediate synaptic transmission in the brain. These signaling molecules traffic to their site of release as cargo inside clear SV or large DCV. They are subsequently released into the synaptic cleft where they exert their function. Unlike SVs which are locally filled with neurotransmitters at the synapse, DCVs travel long distances from the cell body, along microtubule-based motors (Burbach, 1982; Yajima et al., 2008), to axon terminals and dendrites to release their cargo. Furthermore, at the Golgi, immature DCV cargos are packaged along with processing enzymes which cleave and generate the mature, active form of the protein inside the DCVs. Similar to SVs, DCVs

release their cargo upon stimulation. In the past decade, it has been shown that neurons and other professional secretory cells like the acinar cells use "porosomes"—previously termed depressions—as a cup-like lipoprotein portal to the extracellular space that facilitates emptying of DCV content without expanding the PM (Anderson, 2006; Jena, 2009a, 2009b; Trikha et al., 2010). This is especially important in polarized secretory cells like the acinar cells of the pancreas or in neurons. The limited fusion of the DCVs with porosomes is mediated by the same SNARE complex mechanism described for SVs (Sutton et al., 1998).

3.1. Brain

3.1.1. Hippocampus

This interesting brain region is intensely studied for its role in acquisition of new memories, and its function is influenced by exercise, emotion, hormones, and social interactions. In addition to its own highly organized synaptic connections, the hippocampus receives and integrates afferents from the basal forebrain, subcortical regions, the amygdaloid nuclei, and the thalamus. Thus, neurons in the hippocampus express receptors for a great many neuromodulators and neurotransmitters—not just glutamate and GABA. Perforant path projections from the entorhinal cortex and mossy fibers from the dentate gyrus co-express glutamate and the opioids enkephalin and dynorphin, and modulate function of neurons in the dentate gyrus and CA3 neurons, respectively, via their receptors (Schwarzer, 2009). Dynorphin is produced in the RSP by actions of endopeptidases PC1, PC2 and CPE (Dupuy et al., 1994; Ogren et al., 2010). Somatostatin and neuropeptide Y (NPY) expressing GABA-ergic neurons regulate hippocampal glutamate release via their pre- and postsynaptically localized receptors (Mancillas et al., 1986; Boehm and Betz, 1997); a mechanism that can aid in the control of epileptic seizures. In response to stress, the hippocampus abundantly expresses receptors for cortisol and its releasing hormone, and under healthy conditions, provides cortisol-induced inhibitory feedback to the hypothalamic–pituitary–adrenal axis (HPA) axis (Stokes, 1995; Meaney et al., 1996).

The interplay of SV- and DCV-mediated responses of the RSP in the hippocampus, which serves as a model system for studying enhancement or repression of synaptic inputs, is still unclear and is being intensively studied. Although small SVs have been localized and can be induced to exocytose at dendrites (Ovsepian and Dolly, 2011), traditionally, SV neurotransmitter release and vesicle recycling takes place in synaptic and extrasynaptic sites of

axonal terminals (Staras et al., 2010; Ratnayaka et al., 2011) whereas peptidergic or BDNF-containing DCVs are trafficked bidirectionally toward both axons and dendrites (Washburn et al., 2002; Lochner et al., 2008). This has even been found for DCV-localized vesicular monoamine transporter-2 (VMAT2), a monoamine transporter, which would implicate monoamines as being packaged and released to the somatodendritic domain (Li et al., 2005) as has been reported for NPY (Danger et al., 1990; Ramamoorthy et al., 2011). Later, we discuss the bidirectional dynein and kinesin motors responsible for DCV transport to both axons and dendrites (Kwinter et al., 2009; Lo et al., 2011) in hippocampal neurons.

3.1.2. Hypothalamus

The hypothalamus is in the brain but considered a neuroendocrine organ because like other endocrine organs, it secretes hormones into the bloodstream. Axons from the nuclei of the hypothalamus travel via the median eminence located ventral to the third ventricle, and when activated, secrete peptide hormones and dopamine into small capillaries that are connected to the pituitary via the infundibulum. Visceral organs in the periphery, circadian rhythm, sleep, temperature regulation, fluid homeostasis, stress, food intake, growth, spermatogenesis and ovulation are all regulated by the hypothalamus in collaboration with the autonomic nervous system. Thus, the hypothalamus integrates input from almost all brain regions as well as the circumventricular organs to produce a response in the form of secretion of neuropeptides.

3.2. The Neuroendocrine System

The neuroendocrine system is tripartite, consisting of the hypothalamus, which surrounds the third ventricle, the pituitary gland which sits just outside the brain, but protected in the sella turcica of the sphenoid bone in the interior of the cranium, and the target organ located within the abdominal cavity in the body. These target organs include the adrenal, mammary and thyroid glands, the gonads, and the liver. The specialized neurons clustered in nuclei within the hypothalamus control hormone release from the anterior pituitary by secreting hypothalamic-releasing or inhibiting hormones packaged in DCVs. The stimulated pituitary subsequently releases hormones from its DCVs into the bloodstream. These hormones travel long distances to reach their target organs and stimulate or repress their activity. In this manner, the hypothalamus works as the master regulator of appetite, growth, sex drive, reproduction, stressful situations, and other physiological behaviors.

To exert its function, the hypothalamus integrates afferent input from other parts of the central nervous system (CNS) that may also respond to circulating hormones released by target organs.

3.2.1. The Hypothalamic–Pituitary–Adrenal Axis

The manner in which an individual responds to stress can influence homeostatic regulation of a number of related physiological processes. The HPA axis is responsible for the neuroendocrine stress response by regulating gene expression and transcription in the hypothalamus, pituitary and adrenals to induce corticosteroid release from the adrenals (Elkabes and Loh, 1988; O'Connor et al., 2000, 2004). Upon stressful stimulation, corticotropin-releasing hormone from the paraventricular nucleus of the hypothalamus alone or in synergistic action with arginine vasopressin activates corticotrophs in the anterior pituitary to secrete adrenal corticotropic hormone (ACTH), a product of alternative cleavage of POMC precursor protein, into the peripheral circulation. ACTH binds to its receptor on the adrenals and induces glucocorticoid production and release. The effects of the HPA stress response is not limited to those three regions because for each of the hormones released, there are receptors on other brain areas or organs with the capacity to respond. For example, corticotropin releasing hormone (CRH) receptors are also localized throughout the CNS and abundantly expressed in the cerebral cortex, striatum, and cerebellum (Potter et al., 1994). Glucocorticoid receptors are widely distributed in conjunction with the high-affinity mineralocorticoid receptors (Reul and de Kloet, 1985; Han et al., 2005). Importantly, they are localized locally in the hypothalamus for self-regulation of CRH release. However, they are abundant in nearly all areas of the brain (Morimoto et al., 1996), especially in the hippocampus, amygdala and prefrontal cortex, regions intimately involved in mediating the stress response (Dedovic et al., 2009; Mora et al., 2012) as well as many organs in the periphery such as the heart, liver, etc. Therefore, in many ways the HPA axis regulates not only stress but also learning and memory and pleasure centers of the brain. It also regulates other H–P axes' functions such as growth and reproduction.

3.2.2. Insulin-Secreting Islet Cells

Islets are interspersed within a sea of exocrine acinar cells in the pancreas. They function as endocrine cells that secrete the hormone insulin from DCVs into the bloodstream in response to circulating blood glucose. Insulin secretion is also modulated by neuronal inputs or circulating hormones, and

the secretory response occurs in the same manner described for other neuroendocrine tissues and neurons (Wheeler et al., 1996; Anderson, 2006; MacDonald and Rorsman, 2007). Islet endocrine cells also contain synaptic-like microvesicles (SLMVs) similar to SVs in neurons, and these SLMVs have been shown to store and secrete the neurotransmitter GABA by regulated exocytosis in response to stimulation (Anhert-Hilger et al., 1996). SLMVs are similar to SVs in membrane composition, biogenesis and life cycle. The neuron-specific cytosolic adaptor protein (AP), AP-3B complex, associated with GABA-ergic vesicle biogenesis has recently been found to mediate SLMV biogenesis in pancreatic β-cells, in a mechanism similar to neurons (Suckow et al., 2010). The β-cells of the endocrine pancreas abundantly express glutamic acid decarboxylase and have a proton pump-dependent GABA transporter (GAT) supporting the idea that GABA can be synthesized, trafficked and released in these cells which use the SNARE proteins, SNAP25, synaptobrevin and syntaxin to aid in exocytosis (Thomas-Reetz et al., 1993; Anhert-Hilger et al., 1996). These SLMVs are present in anterior pituitary and adrenals, and have been well studied in the PC12 tumor cell line derived from adrenal chromaffin cells (Park et al., 2011). PC12 cells package and store ACh in addition to DCV cargo, and use synaptophysin, synaptotagmin and the same trafficking and exocytosis machinery used by neurons (Thomas-Reetz and De Camilli, 1994; Park et al., 2011).

3.3. Exocrine Tissues

Whereas endocrine tissues secrete hormones and peptides that act to regulate physiological homeostasis via the bloodstream, exocrine tissues secrete enzymes and fluid via ducts, and these are eventually excreted into the external environment. Zymogen granules from acinar cells of the exocrine pancreas secrete alpha amylase, trypsinogen, chymotrypsinogen, elastase, and lipase, and other digestive enzymes into the small intestine to aid in digestion (Bendayan and Ito, 1979). Likewise, in addition to saliva, the salivary glands produce alpha amylase which helps in the onset of digestion to breakdown complex carbohydrates to maltose and glucose (Jacobsen et al., 1972). Similar to the endocrine system, secretion of enzymes from pancreatic acinar and other exocrine cells occurs in a regulated manner in response to secretagogues (Jamieson and Palade, 1971). Contents of the DCGs of exocrine pancreas are processed and packaged at the Golgi (Fig. 2.2D) similarly to the endocrine pancreas, but the DCVs have a circular, black appearance at the electron microscopic level and are large

compared to endocrine granules, with areas that measure up to 2 μm^2 (Gondré-Lewis et al., 2006). Because of the ease of identification, these exocrine cells are often used to study stimulated secretion.

4. IMPORTANT CARGO IN DCVs ACTING ON BRAIN FUNCTION

4.1. Neurotrophins—BDNF

Neuronal birth, function and survival are dependent on the proper trafficking, processing, and release of neuropeptides packaged inside of DCVs. Neurotrophins constitute a class of growth factors necessary for neuronal survival, growth, and plasticity during development. They are modulators of synaptic transmission and influence synaptic efficacy during learning and in response to stressful stimuli in adults (Patterson et al., 1996; Poo, 2001). Deficiency in neurotrophin signaling due to either reduced growth factor or their tyrosine receptor kinases has been cited in the pathogenesis of neuropsychiatric, neurodegenerative and neurodevelopmental disorders. Neurotrophins are packaged in DCVs (Fig. 2.2E) and are trafficked to and secreted at release sites in response to calcium signaling, in a regulated manner, similar to other neuropeptides packaged within DCVs. We pay special attention to BDNF because it is critical for synaptic mechanisms related to learning and in fact is necessary for long-term synaptic plasticity in CA1 neurons in the hippocampus (Patterson et al., 1996; Matsumoto et al., 2008), cerebral cortex and throughout the brain. Similar to other DCVs that do not necessarily release contents at the synapse, BDNF has been shown to release its contents presynaptically from axons, postsynaptically from dendrites, or perisynaptically, thus endowing it with great versatility of action. Because this DCV growth factor is such a potent modulator of synaptic signaling, we will address its DCV biogenesis at the TGN, its retrograde and anterograde trafficking on microtubule motors (section 7.1.2) and its release at synaptic and non-synaptic sites.

Co-packaging and release of neuromodulators is an efficient means of regulating synaptic functions and plasticity in brain. For example, the growth factor BDNF is co-stored with calcitonin gene-related peptide (CGRP) and with substance P in the visual system and amygdala, as well as in the trigeminal ganglion and dorsal root ganglia of the peripheral nervous system (Berg et al., 2000; Buldyrev et al., 2006; Salio et al., 2007). Its co-release with CGRP has also been demonstrated. When co-packaged with

these neuropeptides, BDNF DCVs were more granular in appearance and BDNF was present at 36 times the rate of more agranular vesicles in the amygdala (Salio et al., 2007). Thus, the influential power of this growth factor at the synapse is amplified. In hippocampal neurons, plasminogen, tissue plasminogen activator which converts plasminogen to plasmin, and pro-BDNF for which the cleavage to BDNF is facilitated by plasmin, are co-packaged and co-released at dendritic spines to influence synaptic efficacy (Lochner et al., 2008). Although not necessarily co-packaged with neurotransmitters, BDNF has been shown to increase SV docking and enhance quantal neurotransmitter release (Tyler and Pozzo-Miller, 2001; Tyler et al., 2006).

It is now well established that neuropeptides including BDNF and NPY are often co-expressed and can be co-released from a given neuron along with catecholamines, GABA, other neurotransmitters, or other neuropeptides in order to facilitate neurotransmission (Hokfelt et al., 1980; Danger et al., 1990).

4.2. Granins in Brain

A recent review extensively characterizes the structure, processing, packaging, function and most other aspects of the granin family members (Bartolomucci et al., 2011), and therefore, our discussions here will be limited. Granins are traditionally considered markers for DCVs and the RSP, and although they are peptides that can be synthesized in the brain, they are not classified as neuropeptides primarily because their specific function in neuronal signaling is not clear. More recently, however, three hormones derived from proteolytic processing of chromogranin A (CgA), vasostatin (CgA 1–76), catestatin (CgA 344–364), and serpinin (bCgA 403–428) were shown to be regulators of not only cardiac function but were also important for cell survival and neuroprotection (Helle, 2010; Loh et al., 2012). Whether oxidative stress or low K^+ induced apoptosis in CNS neurons or in the AtT-20 pituitary cell line, exposure to serpinin effectively rescued neurons and neuroendocrine cells from the apoptotic cascade, possibly by regulating the transcription factors involved in its induction (Koshimizu et al., 2011a, 2011b).

SgII is a precursor for the neuropeptide manserin, expressed in the hypothalamus, cerebral cortex, pituitary, adrenal glands and pancreatic islets (Yajima et al., 2004; Tano et al., 2010). Because of Manserin's expression profile, it has been postulated that it may be involved in stress responses.

Manserin was recently identified in neuronal terminals of the organ of Corti and type II spiral ganglion cells in the auditory system, and in the synapses associated with the vestibular system (Yajima et al., 2004; Ida-Eto et al., 2012). That SgII itself is localized in secretory vesicles throughout the nervous system and endocrine tissues implies that its dysregulation can influence a number of functions. Nerve growth factor (NGF)-mediated differentiation of neuroblastoma cells requires SgII expression, and this expression is also protective against nitric oxide (NO)-induced apoptosis (Li et al., 2008). The functional role of granins in neurons and CNS function is a still evolving field.

5. OVERVIEW OF SECRETORY VESICLE BIOGENESIS

Secretory proteins upon synthesis at the rough endoplasmic reticulum (RER) are directed via the signal peptide into the RER cisternae and transported in COP II-coated vesicles to the Golgi apparatus. The proteins traverse from the cis through medial Golgi stacks to the TGN. Within the Golgi apparatus, secretory proteins are posttranslationally modified with carbohydrate modifications and proteolytic cleavage carried out by enzymes concentrated in distinct cisternae in the Golgi stack. The proteins are then sorted and packaged into constitutive or regulated secretory vesicles (Fig. 2.1). RSP vesicles are formed at the TGN while CSP vesicles can bud off from medial Golgi stacks as well as the TGN (Emr et al., 2009). Newly formed CSP and RSP vesicles are transported by a microtubule-based motor system to the proximity of the cell surface (Park and Loh, 2008). CSP vesicles are constantly transported and fuse with the PM without forming a storage pool. In contrast, RSP vesicles are stored in the actin-rich cortex just underneath the PM. Some of these vesicles are tethered or docked at the PM and poised for activity-dependent secretion.

5.1. Biogenesis and Properties of Constitutive Secretory Vesicles

Constitutively secreted proteins travel from the site of synthesis in the RER through the Golgi complex and is packaged into constitutive vesicles (CVs) formed by budding at medial Golgi stacks or the TGN. Additionally, AP-1/clathrin-coated constitutive-like vesicles are formed by budding from immature RSP vesicles to selectively remove cargo that does not belong to the RSP vesicles, but entered the RSP inadvertently during sorting

(Kuliawat et al., 1997; Klumperman et al., 1998). These missorted proteins such as lysosomal enzymes are delivered to endosomes. There are no specific sorting signals on constitutively secreted proteins that direct them into the CSP, rather they enter the CVs by "default". There is no concentration of constitutive vesicle (CV) proteins at the TGN prior to vesiculation, and moreover, the assembly of CVs is not associated with a clathrin coat or acidification (Orci et al., 1987). CVs transport both soluble secretory proteins, e.g. growth factors, as well as membrane proteins such as neurotransmitter transporters and receptors to the cell surface for secretion or incorporation into the PM, respectively. They are clear vesicles with a diameter of 80–100 nm (Walworth and Novick, 1987). CV membranes do not contain a proton pump and the internal pH of CVs is presumed to be neutral, since in yeast, pro-α-mating factor processing is carried out in CVs by *kex2*, an enzyme that functions at neutral pH (Julius et al., 1984). Secretion of CV content is independent of an external signal and is non-calcium triggered. The secretion rate of CV proteins depends on the rate at which they are synthesized. Since they do not have a storage pool, the transit time between the ER and the Golgi complex is about 20 min and from there to the cell surface is ~10 min except in cells that have long processes, such as neurons. The CV membrane contains all the exocytotic machinery necessary for fusion to the PM and for secretion of its contents. In a live-cell imaging study of PC12 cells, it was observed that membrane vesicular acetylcholine transporter (VAChT) moves from the Golgi compartment into a distinct Golgi subcompartment together with DCV proteins briefly, before being incorporated into constitutive-like vesicles, known as SPTVs that bud off this compartment (Park et al., 2011). It was proposed that in this sorting compartment, SPTVs acquire the exocytic machinery which is enriched in this subcompartment, for fusion and delivery of VAChT to the PM. The membrane containing VAChT is then recycled to form the SV.

5.2. Biogenesis and Characteristics of RSP Vesicles

Formation of DCVs requires a number of steps that are common to both endocrine and exocrine cells, and neurons (Kim et al., 2006). Proteins destined for the RSP are posttranslationally modified by glycosylation, sulfation and phosphorylation. They are sorted away from other proteins by aggregation in the TGN at an acidic pH in the presence of calcium, and are engulfed in budding vesicles to form immature DCVs (Orci et al., 1987). Maturation of DCVs involve further acidification to ~pH 5.5, and

processing of precursor hormones in endocrine cells and neurons, followed by storage of mature DCVs until release upon stimulation of the cell (Molinete et al., 2000; Kim et al., 2006). Storage time of DCVs within the cytoplasm is long, 7–10 h, compared to the short transient times of CVs, and depends on an external signal followed by entry of Ca^{2+} to trigger release (Orci et al., 1987). Differences in assembly and secretion of DCVs and CVs are illustrated in Table 2.1. The various steps and the lipids involved in DCV biogenesis is discussed below and summarized in Table 2.2.

5.2.1. Mechanisms of Sorting of DCV Proteins at the TGN

For some time, there has been a debate as to whether sorting of DCV proteins at the TGN to the RSP is a "passive" or an "active" process (Kelly, 1985; Chanat, 1993). The passive process involves aggregation of proteins to form a condensing aggregate, which is then trapped and packaged into the DCV. The active process involves a sorting signal on the protein binding to a sorting receptor located in the lumen of the TGN membrane, which then facilitates entry into the budding immature DCV (Dikeakos and Reudelhuber, 2007). Current evidence indicates that aggregation alone may not be sufficient to sort proteins into DCVs (Gorr et al., 2001; Garcia et al., 2005), but that both aggregation and sorting receptors/binding proteins are necessary for efficient sorting to the RSP.

5.2.1.1. Aggregation and Calcium-Dependent Sorting

Electron microscopic studies by Orci and his colleagues (Orci et al., 1987) have shown that DCV proteins aggregate at the TGN in endocrine cells (Dannies, 2012). They and others (Hutton, 1982; Orci, 1986; Demaurex et al., 1998; Rindler, 1998; Machen et al., 2003) have also demonstrated that the pH at the TGN is mildly acidic (~6.0–6.5). Numerous in vitro studies have shown that DCV proteins such as prohormones and granins aggregate at an acidic pH, with either itself or other DCV proteins (Rindler, 1998; Sobota et al., 2009). Acidification is achieved by membrane proton pumps (V/H-ATPase) present at the TGN and DCV membranes (Wu et al., 2001). Treatment of PC12 cells with bafilomycin A1, a specific inhibitor for H-ATPase, caused a decrease both in numbers of DCVs and secretion of CgA, supporting the importance of acidification at the TGN for granin aggregation and DCV biogenesis (Taupenot et al., 2005). In addition to an acidic environment, in general, calcium present in DCVs is required for DCV protein aggregation as has been demonstrated for prohormones, PCs, CPE and granins (Shennan et al., 1994; Rindler, 1998; Dikeakos et al., 2009).

Table 2.2 Protein and lipid components involved in different steps of DCV biogenesis

Step	Function	Proteins	Lipids	Discussed in section
Cargo sorting at TGN	Cargo aggregation★, sorting "receptors", acidification	Granins, CPE, SgIII, muclin, V-ATPase	Cholesterol, lipid rafts†	2.5.2.1
TGN budding	Membrane curvature	PLD, DAG kinase, protein kinase D, PI kinases, GTPases (Arf1, Rabs), FAPPs, clathrin, APs (AP-1, GGA)	PAs, phosphoinositides, DAG, cholesterol, lipid rafts	2.5.2.3
Vesicle fission at TGN	Vesicle release	Dynamin, actin, γ-adducin, myosin II, GTPases (Rabs)		2.5.2.4
Vesicle maturation	Removal of missorted cargo, constitutive-like vesicle budding, acidification, condensation Coat shedding	GTP-ase (Rab-3D), myosin Va, V-ATPase proton pump, AQPs 1 and 5		2.5.2.2, 2.5.2.4

†Lipid rafts consist of platforms of lipids rich in cholesterol and sphingolipids.
★Soluble cargo such as prohormones and proneuropeptides co-aggregate with chromogranins.

However, not all DCV proteins require calcium for self-aggregation (Cawley et al., 2000).

Ca^{2+} exists in the free and bound form in DCVs. The mechanism of uptake of Ca^{2+} into acidic Ca^{2+} stores which include DCVs and the Golgi complex is unclear but likely driven by mammalian homologues of Ca^{2+} ATPases and Ca^{2+}/H^{+} exchangers since such activities have been detected, but the molecules have yet to be identified (Patel and Muallem, 2011). By contrast, Ca^{2+} release from DCVs is mediated by members of the TRP (transient receptor potential) channel superfamily and the inositol trisphosphate receptors IP_3Rs/calcium channels (Patel and Muallem, 2011; Yoo, 2011; Yoo and Hur, 2012). CgA and CgB both bind the IP_3Rs and activate the IP_3Rs/calcium channels at the intragranular pH of 5.5. This coupling is proposed to play key roles in the IP_3-mediated Ca^{2+} signaling mechanisms in the cytoplasm. Thus, Ca^{2+} in the Golgi complex and DCVs are important sources of intracellular Ca^{2+} for DCV protein aggregation and perhaps also cell signaling.

5.2.1.2. Sorting Signal Motifs

Over the last 20 years, a large number of sorting signals have been proposed for sorting DCV proteins at the TGN into DCVs (Dikeakos and Reudelhuber, 2007). The signals are complex and vary with different proteins and with cell type. In our discussion here, we will divide DCV proteins into two classes: soluble proteins and membrane proteins that are either tethered to or traversing the membrane, respectively. The first group of proteins includes prohormones in endocrine cells, BDNF in neurons, granins in both cell types and in exocrine cells. The second group includes proprotein/hormone convertases (PCs), CPE, phogrin and muclin.

Sorting signals within prohormones, proneuropeptides and granins have been the most studied. Site-directed mutagenesis studies have identified a three-dimensional conformation-dependent consensus sorting motif consisting of two acidic residues, 12–15 Å apart from each other, exposed on the surface of the molecule, and two hydrophobic residues, 5–7 Å away from the acidic residues which is necessary for sorting of POMC, proinsulin and proenkephalin to the RSP (Cool et al., 1997; Dhanvantari et al., 2003). This sorting motif was found to interact with a sorting receptor, membrane CPE (see next section) to mediate sorting to the RSP. For POMC, this motif resides in the N-terminus disulfide loop (Cool et al., 1995). For proenkephalin, the motif lies within the N-terminal residues 1–32 (Loh et al., 2002). In the case of proinsulin sorting, while such a motif is present in

the monomeric form contributed by residues from the A and B chain, the motif was also found in the hexameric aggregated form of insulin in two adjacent A chains (Dhanvantari et al., 2003). In pancreatic cells, proinsulin appears not to be sorted at the TGN, but enters immature vesicles along with lysosomal enzymes and constitutive proteins and is processed. Insulin is retained in the immature vesicle while constitutive-like vesicles bud off from the vesicle to remove non-DCV proteins (Tooze and Tooze, 1986). In this case, the motif acts as a retention signal by binding to a sorting receptor, membrane CPE, in the vesicle (see next section, and Dhanvantari et al., 2003). Such a three-dimensional sorting motif has also been found to direct BDNF (Lou et al., 2005) to the RSP at the TGN. The conformation of BDNF and NGF are very similar, except that NGF is missing one amino acid residue to complete such a motif. Introduction of the missing residue by mutagenesis (Val20Glu) redirected NGF to the RSP, indicating the importance of the identified sorting motif in targeting BDNF to the RSP. This sorting motif can only be recognized in molecules that have a conformation model or a crystal structure available, and hence have been identified in only a limited number of prohormones and proneuropeptides.

Paired basic residues have also been suggested to act as sorting signals for targeting some neuropeptides to the RSP. These include pro-renin (Brechler et al., 1996), proneurotensin (Feliciangeli et al., 2001), progastrin (Bundgaard et al., 2004) and prothyrotropin-releasing hormone (Mulcahy et al., 2005). However, the mechanism is unclear. It is postulated that a certain pair of basic residues must be cleaved by one of the prohormone convertases (PCs), which usually acts in DCVs at an acidic pH, before it can be correctly sorted to/retained in the DCVs since mutation of the pair of basic residues to a furin (which cleaves proproteins early in the Golgi complex) cleavage site redirects them to the CSP (Brechler et al., 1996).

Various sorting domains have been identified for targeting granins, such as CgA, CgB, and secretogranins to the RSP. These domains have been extensively reviewed by Bartolomucci et al. (2011), and a few will be highlighted here. Taupenot et al. (2002) found that the N-terminal domain of CgA (bovine/human residues 40–115) is necessary for targeting CgA into the secretory granules of PC12 cells. This region concurred with the findings of Hosaka et al. (2002) who showed that the N-terminal domain (rat/bovine residues 48–111/48–95) of CgA was essential for binding to secretogranin III (SgIII), a membrane-associated sorting receptor for CgA (see next section). In contrast, studies on CgB identified a disulfide bonded loop structure located within the first 37 amino acids of the N-terminal of the

molecule that was essential for sorting to the RSP in PC12 cells (Kromer et al., 1998). Two putative α-helix-containing domains, hSgII 25–41 and hSgII 334–348, that can act independently, have been shown to target SgII to the RSP in PC12 cells (Courel et al., 2008). SgII has been shown to interact with SgIII in yeast two-hybrid studies, raising the possibility that SgII sorting may be through interaction with SgIII similar to CgA.

For membrane-associated DCV proteins, sorting to the RSP occurs by interaction with TGN membranes. The TGN has cholesterol–sphingolipid-rich microdomains known as "lipid rafts" where budding is thought to occur to form immature DCVs (see Section 2.5.2.3) consistent with these organelles being highly enriched with cholesterol in their membranes (Dhanvantari and Loh, 2000). SgIII has a domain at the N-terminus (residues 23–186 in the rat sequence) of the molecule that binds cholesterol and this domain is essential for sorting of SgIII to the RSP (Hosaka et al., 2004). Membrane-binding domains containing short α-helical structures residing in the C-terminus of CPE (Zhang et al., 2003) and proprotein convertases PC1/3 (Jutras et al., 2000; Lou et al., 2007) and PC2 (Assadi et al., 2004) have been demonstrated to be essential for targeting these prohormone-processing enzymes to the RSP. The membrane-binding domains interact with lipid rafts of the TGN membrane to effect sorting to the RSP. In the case of membrane CPE, the membrane-binding domain appears to traverse the DCV membrane (Dhanvantari et al., 2002; Zhang et al., 2003) despite the lack of a typical transmembrane amino acid sequence. This has led to some debate about the transmembrane orientation of CPE, but the evidence supporting the existence of a cytoplasmic tail in CPE is strong (Cawley et al., 2012). Phogrin, an integral membrane DCV protein, also has a sorting domain located at the C-terminal cytoplasmic tail necessary for targeting to the RSP (Wasmeier et al., 2002; Torii et al., 2005). These membrane-associated DCV proteins are ideal candidate sorting receptors for soluble DCV proteins (see next section).

5.2.1.3. Membrane Sorting Receptors

It is clear that sorting of soluble aggregates of DCV proteins such as prohormones, proneuropeptides or granins to the RSP requires tethering/binding to a membrane receptor at the TGN. It has been shown that SgIII is a sorting receptor for targeting CgA to the RSP. The CgA sorting domain binds strongly to the SgIII domain comprising of residues 214–373 at pH 5.5 in the presence of 10 nM calcium (Hosaka et al., 2002).

An RSP sorting receptor that binds to the conformation-dependent sorting signal described above for POMC, proinsulin, proenkephalin and BDNF was identified as the transmembrane form of CPE. The two acidic residues in the prohormone/pro-BDNF sorting motif specifically interact with two basic residues, R255 and K260, of the sorting receptor, CPE, to effect sorting to the RSP (Cool et al., 1997). In vivo studies using a CPE KO mouse model showed that BDNF was not sorted to the RSP (Lou et al., 2005). Instead it was secreted constitutively in cortical and hippocampal neurons of the CPE KO mice. Constitutive secretion of proinsulin from isolated pancreatic islets was elevated and plasma levels of proinsulin were significantly higher in these mice, indicating a role of CPE in sorting of proinsulin to the RSP (Naggert et al., 1995; Irminger et al., 1997; Cawley et al., 2004). Recent studies of POMC sorting in primary anterior pituitary cultures from CPE KO mice showed defective regulated secretion of ACTH, consistent with CPE acting as a sorting receptor in vivo. However, some stimulated secretion of POMC was observed. Further investigation of pituitaries revealed elevated expression of SgIII (Cawley, N. X., and Loh, Y.P., unpublished data). an RSP sorting receptor, for CgA at the TGN. Previous work reported in the literature (Hosaka et al., 2005) has shown that SgIII binds to POMC, albeit with lower affinity than CPE, and can potentially act as a sorting receptor for POMC. Thus higher expression of SgIII in the pituitary of CPE KO mice may compensate as a sorting receptor for POMC in these mice.

Peptidyl-α-amidating monooxygenase (PAM), another type 1 membrane spanning DCV protein which function to amidate peptide hormones, has been suggested to play a role in sorting of atrial natriuretic peptide to the RSP since it binds membrane PAM very tightly in myocytes (O'Donnell et al., 2003). Muclin, a DCV membrane protein in exocrine pancreatic acinar cells, has been shown to mediate sorting of zymogens including amylase, prolipase, procarboxypeptidase A1, pro-elastase II, and chymotrypsinogen B to the RSP (Boulatnikov and De Lisle, 2004). It acts by binding to these zymogen aggregates in a mildly acidic pH present at the TGN through its sulfated, *O*-linked oligosaccharide groups.

5.2.1.4. Glycosylation, Sulfation and Phosphorylation in DCV Protein Sorting

Secretory glycoproteins undergo glycosylation during transit through the cis-medial-trans Golgi cisterni. At the TGN, some glycosylated proteins are further modified by sulfation. Protein glycosylation or sulfation could affect protein–protein interactions required for sorting some proteins to the RSP.

For example, in pancreatic acinar cells, sulfated, *O*-glycosylated Muclin, a type 1 membrane spanning protein, binds aggregated zymogens at the TGN to facilitate sorting to the RSP (Boulatnikov and De Lisle, 2004). Blockage of sulfation and glycosylation of Muclin inhibited sorting of the zymogens to the RSP. Another molecule, VMAT2, has an *N*-glycosylated luminal loop domain necessary for sorting to the RSP (Yao and Hersh, 2007). Inhibition of glycosylation of the luminal loop with 1-deoxymannojirimycin, a specific alpha-mannosidase 1 inhibitor, resulted in missorting of VMAT2 to CVs in PC12 cells.

However, protein glycosylation does not always affect protein sorting to the RSP. Sorting of cathepsin G to granules for its processing, activation, and secretion does not depend on its glycosylation (Garwicz et al., 1995). The mutant form of cathepsin G that lacks a functional glycosylation site can be processed to an enzymatically active form and released in a regulated manner in neutrophil granulocytes. Sorting of POMC/ACTH to the RSP was also not affected by inhibition of its glycosylation or sulfation (Moore, 1987). In addition, sorting of cerebral dopamine neurotrophic factor (Sun et al., 2011), pancreatic lysosomal enzymes (Chu et al., 1990), and renin (Kuliawat and Arvan, 1994) to the RSP were not inhibited by prevention of glycosylation. Thus, glycosylation-based sorting appears to be limited for some RSP proteins.

Evidence for phosphorylation of secretory proteins as a signal for guiding sorting to the RSP is sparse. In classical cholinergic neurons, chromaffin, and PC12 cells, VAChT is transported constitutively to the PM and recycled into SLMVs while VMATs are found mostly in DCVs (Fei et al., 2008). In protein kinase A (PKA)-deficient PC12 cells, VMATs are found in SLMVs (Yao et al., 2004). Treatment of wild-type (WT) PC12 cells with a PKA inhibitor, H89, also caused accumulation of VMATs in SLMVs. While VMATs undergo phosphorylation of the cytoplasmic tail (Krantz et al., 1997), it does not appear that it is carried out by PKA but rather by casein kinase II. It is not clear that this phosphorylation is important for sorting VMATs to the RSP. Instead, PKA facilitates sorting of VMAT to DCV indirectly. On the other hand, PKC-mediated phosphorylation of a serine residue in the cytoplasmic domain (R<u>S</u>ERDVLL) of VAChT prevents sorting of VAChT to SLMVs (Cho et al., 2000; Krantz et al., 2000). A phospho-mimicking mutation of serine to glutamate in the VAChT cytoplasmic tail caused sorting of VAChT to DCVs, maybe because the mutation renders the VAChT tail similar to the VMAT2 tail (KEEKMAIL). Despite these studies, there is no clear evidence indicating that phosphorylation coordinates sorting of VAChT and VMATs to SV/SLMVs and DCVs, respectively.

In another example, furin, a Golgi-resident protein that has inadvertently entered immature DCVs can be removed from these DCVs via clathrin-mediated constitutive-like secretion (Fig. 2.1). The cytoplasmic tail of furin interacts with AP-1 or Golgi-localized, gamma-ear-containing, ADP-ribosylation factor-binding proteins (GGA) that, in turn, recruits clathrin coat for removal of furin from immature granules (Park and Loh, 2008). Casein kinase II mediates phosphorylation of the cytoplasmic tail of furin, which is a prerequisite step for the interaction of AP-1 or GGA with furin (Dittie et al., 1997). Hence phosphorylation of the furin tail is important for its sorting and removal from immature granules.

In summary, all the findings reviewed above indicate that sorting of various proteins from the Golgi complex to DCVs in the RSP is mediated by a mechanism involving aggregation and a sorting signal/domain within the protein, interacting with a membrane-associated sorting receptor/binding protein, in endocrine, exocrine cells and neurons. Posttranslational modifications such as glycosylation, sulfation and phosphorylation do play a role in facilitating sorting of some DCV proteins.

5.2.2. Granins and Membrane Proteins in DCV Assembly

Granins have long been proposed to be important in DCV assembly since they are generally present in a high molar ratio relative to other proteins in the granule. Indeed, it has been demonstrated that overexpression of granins such as CgA, CgB and SgII induced DCG-like structures in fibroblast cells that have no RSP (Kim et al., 2001; Huh et al., 2003; Beuret et al., 2004). These structures are not likely to be "bona fide" DCVs with all the components necessary for regulated secretion, although addition of calcium ionophores has resulted in secretion of their contents in some studies (Beuret et al., 2004). Furthermore, antisense RNA downregulation of CgA expression inhibited granule structures in PC12 cells (Kim et al., 2001), and depletion of SgII by small interfering RNA (siRNA) in these cells resulted in a decrease in size and number of DCVs (Courel et al., 2010). In WT neuroendocrine PC12 cells, downregulation of CgA impaired both DCV formation and activity-dependent secretion of an exogenous cargo molecule, the prohormone POMC (Kim and Loh, 2006). Additionally, in 6T3 cells, a variant of the AtT-20 pituitary cell line, which lacks CgA and DCVs, upon exogenous expression of bovine CgA, DCV biogenesis was induced and regulated secretion was restored (Kim et al., 2001). Antisense RNA downregulation of CgA expression in transgenic mice and CgA KO mice showed significant decrease in the size and numbers of DCVs in adrenal

chromaffin cells (Mahapatra et al., 2005; Kim and Loh, 2006). However, a CgA KO mouse generated by Hendy et al. (2006) showed no difference in numbers of chromaffin granules compared to WT mice. Other granins such as CgB and SgII–VI were increased 2- to 3-fold in these mice, suggesting that they may compensate for the lack of CgA. All these studies taken together demonstrate that the granins are granulogenic proteins and play a very important role in providing the "driving force" for vesicle budding at the TGN to form immature DCVs.

DCVs also contain various membrane components necessary for their function and here we highlight the key ones. These include integral membrane proteins such as aquaporins (AQPs) and various ion-gated channels. AQPs are a family of water channels expressed in epithelial cells to transport water bidirectionally to mediate fluid absorption and secretion. AQP1 has been found in DCVs in pituitary, chromaffin granules from adrenal medulla and pancreatic zymogen granules, while AQP5 has been found in parotid gland DCVs (Cho et al., 2002; Arnaoutova et al., 2008; Francone et al., 2010). AQP1 has also been found in the TGN (Francone et al., 2010). Its function in TGN and granules is to remove water to facilitate condensation of aggregated DCV proteins at the TGN and during DCV maturation. Studies have shown that downregulation of AQP1 expression in AtT-20 cells, an anterior pituitary cell line, resulted in a significant decrease of ACTH-containing DCVs. Pulse-chase labeling studies showed that while POMC synthesis was unaffected, in these AQP1-deficient cells there was a major decrease in newly synthesized DCV proteins 1 h after synthesis, indicating that in the absence of DCV biogenesis these proteins were degraded. These findings corroborated with decreased POMC and its processing enzymes in anterior pituitary of AQP1 KO mice. Hence AQP1 appears to be a key component in condensation of DCV proteins and maintaining DCV biogenesis.

DCV membranes also contain a number of ion channels. These include calcium channels (see Section 2.5.2.1.1) for calcium uptake and sequestration, and a calcium-independent K+ selective channel found in chromaffin granules that may play a role in ion movement during granule assembly (Arispe et al., 1992). There are also proton pumps (H-ATPase) in the DCV membrane required for acidification during DCV maturation (Apps et al., 1989; Saroussi and Nelson, 2009). In addition there are components of the exocytic machinery such as vesicle associate membrane proteins (VAMPs), GGAs and SNAP25 present in immature DCVs (Eaton et al., 2000). These components have recently been found in a post-TGN-Golgi subcompartment from which DCVs and SPTVs bud (Park et al., 2011) in PC12 cells,

and are presumably assembled in microdomains where DCVs bud. However, the distribution of these components along the TGN membrane is unclear and it remains to be determined if they are randomly distributed or concentrated in lipid rafts (see Section 2.5.2.3).

5.2.3. Role of Membrane Lipids and Proteins in DCV Budding

Subsequent to assembly of DCV cargo and membrane components at the TGN, the next step is vesicular budding of the membrane to form the immature DCVs. Lipids play a significant role in this budding event, facilitated by proteins. Most studies on the role of lipids in vesicle budding at the TGN and membrane curvature pertain to CV budding in mammalian cells and yeast (Corda et al., 2002; Roth, 2004; van Meer and Sprong, 2004; Kim et al., 2006), and only a few relate specifically to DCV budding in endocrine/exocrine cells (Tooze and Tooze, 1986; Kim et al., 2006). Nevertheless, the lipid-mediated mechanisms for vesicle budding for both these two types of vesicles are likely to be similar.

For both constitutive and DCV vesicle budding at the TGN, two types of lipids are necessary, phosphatidic acid (PA) (Siddhanta and Shields, 1998; Siddhanta et al., 2000) and cholesterol (Wang et al., 2000). Inhibition of PA synthesis altered the structure of Golgi apparatus and quantitatively inhibited secretion of growth hormone (Siddhanta et al., 2000). In in vitro studies, when PA levels were increased by diacylglycerol (DAG) kinase or phospholipase D (PLD) 1 in GH3 cells, there was significant increase in secretory vesicle budding (Siddhanta and Shields, 1998).

PA can be derived from DAG or phosphatidylcholine by DAG kinase or phosphatidylcholine-specific PLD1, respectively. DAG is present in the TGN membranes and participates in CV budding (Yeaman et al., 2004), but its role in DCV budding is not known. PA has a cylindrical shape at neutral pH but assumes a cone structure under acidic pH and low calcium concentration, conditions present within the lumen of the TGN (Kooijman et al., 2003). This conical molecular structure apparently provides enough force to induce negative curvature in the Golgi membrane, leading to pearling of the bilayer and then fission (Corda et al., 2002; Shemesh et al., 2003).

Phosphatidylinositol (PI) is another lipid that promotes membrane curvature (Bryan and Hagen, 1991). In its phosphorylated form, phosphatidylinositol 4-phosphate (PI4P) has been shown to mediate budding of CVs at the TGN (Santiago-Tirado and Bretscher, 2011). PI4P is enriched in the TGN and recruits four-phosphate-adaptor protein (FAPP) 1 and FAPP2, which bind the small GTPase [ADP-ribosylation factor (Arf)] Arf1-

guanidine triphosphate (GTP). Through interaction of the pleckstrin homology (PH) domain of FAPPs with PI4P and Arf1-GTP, vesicle formation and transport of cargo to the PM is regulated (Godi et al., 2004; Santiago-Tirado and Bretscher, 2011). Phosphoinositol kinases are differentially localized in the Golgi complex to control local production of PI4P. This allows for additional control of cargo selection and vesicular subtype specification during vesicle transport (Weixel et al., 2005). While the role of PI4P in DCV budding has not been studied, it is likely also involved.

Cholesterol is a lipid that is of major importance in DCV budding. Pituitary DCV membranes are reportedly highly enriched in cholesterol (Dhanvantari and Loh, 2000). It has also been shown that the TGN is enriched with cholesterol, glycosphingolipids and sphingomyelin, and together they are packed in microdomains known as "lipid rafts" (Orci et al., 1981; van Meer, 1998). Because of the high cholesterol content of the DCV membranes, these microdomains are proposed to be the site of DCV budding in endocrine cells. Consistent with this is the finding that when cholesterol synthesis was blocked in AtT-20 cells, a pituitary endocrine cell line, DCV formation was halted, and condensed dense cores were found accumulated at the TGN. However, upon addition of cholesterol, DCV biogenesis resumed (Wang et al., 2000). Cholesterol also assumes a conical-shaped structure that promotes a negative curvature and therefore facilitates vesicle budding (Orci et al., 1980; Corda et al., 2002; Bacia et al., 2005). It would appear that cholesterol cannot be substituted by other sterols (e.g. precursors of cholesterol) in this function. In mouse models of human genetic diseases, Smith–Lemli–Opitz syndrome (SLOS) and lathosterolosis, where cholesterol synthesis is impaired due to defective synthesizing enzymes, DCV numbers were decreased in the pancreas, pituitary and adrenal gland. Moreover, the DCVs in the exocrine pancreas were malformed due to decreased membrane curvature as a result of increased rigidity of the cholesterol precursors in these mice (Gondré-Lewis et al., 2006). From the above studies reviewed, it is evident that lipids, especially cholesterol, are critical for secretory vesicle budding and biogenesis.

5.2.4. GTPases, Clathrin and Cytoskeletal Proteins in DCV Biogenesis

Small GTPases such as the Arf and Rab families play a role in vesicle budding while the large GTPase, dynamin, which assembles at the neck of budding vesicles, functions in membrane fission and vesicle release. These GTPases cycle between an inactive guanidine diphosphate (GDP)-bound form and the active GTP-bound form. As mentioned above, small GTPases such as Arf1

interact with FAPP-PI4P to mediate constitutive secretory vesicle budding at the Golgi complex (Santiago-Tirado and Bretscher, 2011). Arfs (Arf1 and Arf6) function to induce membrane curvature by insertion of an amphipathic helix into the cytosolic leaflet of the membrane (Lundmark et al., 2008). Moreover, GTP-induced dimerization of Arf1 appears to be an essential step for inducing Golgi membrane curvature during the formation of coated vesicles (Beck et al., 2008).In addition, two members of the Rab family, Rab2 and Rab6 are involved in vesicle biogenesis at the Golgi complex. In *Caenorhabditis elegans*, the unc-108 (equivalent of mammalian Rab2) is present in the soma of neurons, but not at the synapses, and it co-localizes with Golgi but not ER markers. *Unc108*/Rab2 mutants showed deficits in locomotion and abnormalities in the size and content of the DCVs in peptidergic neurons. Their DCVs were slightly larger and more variable in size, but the numbers, the dense-core diameter, and ability to secrete the contents were no different from the WT worms. While the DCVs in these mutants contained normal neuropeptide cargo, labeling studies indicate that two thirds of the cargo in immature DCVs, both soluble and membrane components, are moved into endosomes. It seems the defect in these *unc108* mutants is not in the initial generation of immature DCVs, but in the cooperative function of *unc108* with its effector, RIC-19, in retaining all the appropriate cargo during DCV maturation for normal physiological function (Edwards et al., 2009; Sumakovic et al., 2009). Rab6 has been shown to promote fission of Rab6-positive transport vesicles at the Golgi complex (Miserey-Lenkei et al., 2010). Myosin II and F-actin which are associated with Rab6-positive vesicles are essential for this process. Depletion of myosin II or depolymerization of F-actin led to inhibition of fission and appearance of Rab6-positive long tubules connected to the Golgi complex, concomitant with a decrease in Rab6-containing vesicles. Thus, myosin II and F-actin are effectors of Rab6-GTPase in vesicle fission at the Golgi. It has been proposed that contraction of short actin filaments by myosin II may increase membrane tension locally, which in turn could facilitate fission by the vesicle being pulled along microtubule tracks by the motor kinesin I. This study suggests an important role of Rab6 in cooperation with cytoskeletal proteins in vesicle fission. Rab3D working cooperatively with myosin Va has been implicated in DCV maturation by facilitating constitutive-like secretion and removal of, e.g. missorted furin, from the immature DCV (Kogel and Gerdes, 2010).

Dynamin was first discovered as a GTPase involved in clathrin-mediated endocytosis at the PM (Hinshaw, 2000), but more recently, evidence is emerging that it also plays a role in membrane fission and vesicle release at

the TGN. Dynamin has five functional domains: an N-terminal GTPase domain, a middle domain, a PH domain with variable hydrophobic loops, the GTPase effector domain (GED) and the C-terminal proline/arginine domain (PRD). The GTPase domain binds and hydrolyzes GTP (Chappie et al., 2010), while the middle domain and GED interact with each other to mediate self-assembly (Ramachandran et al., 2007) (http://onlinelibrary.wiley.com/doi/10.1111/j.1600-0854.2011.01250.x/full-b25). The PRD binds to SH3 domain-containing partners and is involved in targeting dynamin to clathrin-coated pits (CCPs) at the PM (Okamoto et al., 1997; Hinshaw, 2000). Subsequent to vesicle budding, in the presence of GTP, dynamin organizes into self-limited assemblies that continuously cycle at the membrane and drive vesicle release (Hinshaw, 2000; Pucadyil and Schmid, 2008). Current evidence suggests that shallow insertion of the hydrophobic loop in the PH domain of dynamin into the lipid bilayer leads to membrane deformation and fission (Ramachandran et al., 2009). There is also evidence suggesting that activity-dependent dephosphorylation of dynamin 1 is involved in fusion pore collapse during DCV exocytosis in chromaffin cells (Chan et al., 2010). What is known about the role of dynamin is mainly in endocytosis from the use of in vitro model membranes, liposomes or Hela cells. However, dynamin has been shown to be essential for transport of p75, a neurotrophin receptor, from the Golgi complex to the PM in neurons (Kreitzer et al., 2000), indicating that it is also involved in constitutive secretory vesicle formation at the TGN (Jones et al., 1998). While there have been no studies on the role of dynamin in DCV release at the TGN, it is likely that it also participates in membrane fission and release of DCVs subsequent to budding at the TGN, in (neuro)endocrine cells.

Clathrin coating is involved in CV and DCV budding at the TGN (Tooze and Tooze, 1986; Teuchert et al., 1999). Evidence that budding DCVs have a clathrin coat has been clearly demonstrated by electron microscopy of (neuro)endocrine cells (Orci et al., 1985; Tooze and Tooze, 1986). AP-1 and GGA are major APs that mediate clathrin coating on budding CVs, either independently or cooperatively (Bonifacino, 2004). Both AP-1 and GGA are found on immature DCVs, suggesting that they likely mediate clathrin coating of budding DCVs (Dittie et al., 1996; Kakhlon et al., 2006). An AP-1 adaptor complex bound to clathrin-coated immature vesicles in PC12 neuroendocrine cells has been reported and this binding is regulated by Arf1 (Dittie et al., 1996). Recently, it has been demonstrated in *Drosophila* epithelial cells of the larval salivary gland that AP-1 and clathrin co-localize at the TGN and in the mucin-containing immature secretory granules. Loss of either AP-1 or

clathrin inhibits formation of these secretory granules at the TGN, further indicating an essential role of AP-1 and clathrin in DCV biogenesis (Burgess et al., 2011). A number of other cytosolic proteins which have AP-1 or GGA-binding motifs may also assist in clathrin coating. These include phosphofurin acidic cluster sorting protein-1 (PACS-1) which facilitates binding of AP-1 to the acidic residues of the cytoplasmic tail of the integral membrane protein furin (Crump et al., 2001), and epsin-R that binds to PI4P, clathrin, and AP-1 (Mills et al., 2003). Subsequent to formation of the immature granule, the clathrin coat is removed by budding of constitutive-like clathrin-coated vesicles from the maturing DCVs (Tooze and Tooze, 1986).

SNARE proteins VAMP2, VAMP4, syntaxin 6, synaptotagmin I and IV are also recruited to immature DCV membranes and are subsequently removed, except for VAMP2 and synaptotagmin I, as the DCV matures to render them responsive to secretagogues (Eaton et al., 2000). The role of these SNARE proteins in DCV biogenesis is unclear. However, in some cells, such as PC12 cells, homotypic fusion of immature DCVs occurs and syntaxin 6 is required for the fusion (Tooze et al., 1991; Urbe et al., 1998).

Cytoskeletal proteins such as F-actin are found covering the cytoplasmic face of the Golgi complex and have been proposed to play a role in vesicular exit from the TGN (Godi et al., 1998; Fucini et al., 2000; Dubois et al., 2005; De Matteis and Luini, 2008; Miserey-Lenkei et al., 2010). Recently, we have found high levels of F-actin and the cytosolic actin-binding protein, γ-adducin, localized at the peri-Golgi area in AtT-20 endocrine cells. γ-Adducin has been shown to interact with the cytoplasmic tail of a transmembrane form of the prohormone-processing enzyme, CPE (Lou et al., 2010), and in POMC/ACTH-containing DCVs in AtT-20 pituitary cells. Transfection into AtT-20 cells of a dominant-negative construct containing a C-terminal domain of γ-adducin disrupted actin filaments and the interaction of γ-adducin with the CPE tail, resulting in inhibition of POMC/ACTH exit and accumulation of POMC in the Golgi complex. POMC/ACTH-containing DCVs were greatly diminished along and at the tips of the cell processes of these transfected cells, compared to control cells (Lou et al., 2012; Richter et al., 2008). We propose that γ-adducin may serve as an intermediary molecule to attach the budding immature DCV to actin filaments at the TGN to facilitate fission/release, analogous to caveolae being attached to actin filaments via actin-binding proteins during non-clathrin-dependent endocytosis at the PM (Richter et al., 2008). Furthermore, as described above, myosin II/F-actin has been shown to play a role in membrane fission to release Rab2-positive vesicles at the Golgi membrane.

Thus evidence is accumulating that cytoskeletal proteins are important in vesicle biogenesis at the Golgi complex.

5.3. Regulation of DCV Biogenesis

The biogenesis of DCVs can be regulated at the genetic, posttranscriptional and posttranslational levels (Fig. 2.3). Regulation of DCV biogenesis at the genetic level would be expected to be most important during development when progenitor cells differentiate to become specialized secretory cells that secrete hormones or neuropeptides, or non-DCV-containing cells. Another example where genes involved in regulating DCV biogenesis is of significance, is during endocrine, exocrine and neuronal cell regeneration from progenitors after injury in adults. In contrast, regulation at the posttranscriptional and posttranslational levels is likely used by differentiated secretory cells to replenish DCVs that have undergone exocytosis and released their cargo upon stimulation.

5.3.1. Genetic Regulation of DCV Biogenesis

The induction of DCV biogenesis at the transcriptome level is poorly understood. The receptor element 1-silencing transcription factor (REST)/the neuron-restrictive silencing factor (NRSF) has been proposed to be a negative regulator of DCV biogenesis. REST/NRSF binds a 23-bp DNA sequence element to cause suppression of the expression of various neuronal genes, including those necessary for secretion, as well as DCV proteins (Chong et al., 1995; Schoenherr and Anderson, 1995; Bruce et al., 2004). REST/NRSF expression is repressed in neuronal cells, but after epileptic and ischemic insults, REST/NRSF expression is derepressed, causing cell death (Palm et al., 1998; Calderone et al., 2003). WT PC12 cells do not express REST/NRSF, but in mutant cell lines that express REST/NRSF, such as PC12-REST and PC12-HZ4, expression of REST/NRSF is derepressed leading to inhibition of expression of various genes required for regulated secretion (Bruce et al., 2006). When a dominant-negative REST/NRSF construct was expressed in A35C cells, a REST/NRSF-expressing PC12 variant cell line without regulated secretory granules, messenger RNA (mRNA)-encoding proteins that are necessary for regulated secretion were elevated. However, DCV and SV proteins, such as CgA and synaptophysin, were not reexpressed at the protein level, and no DCV formation was observed (Pance et al., 2006), suggesting that REST/NRSF does not regulate expression of all genes necessary for DCV biogenesis in these cells.

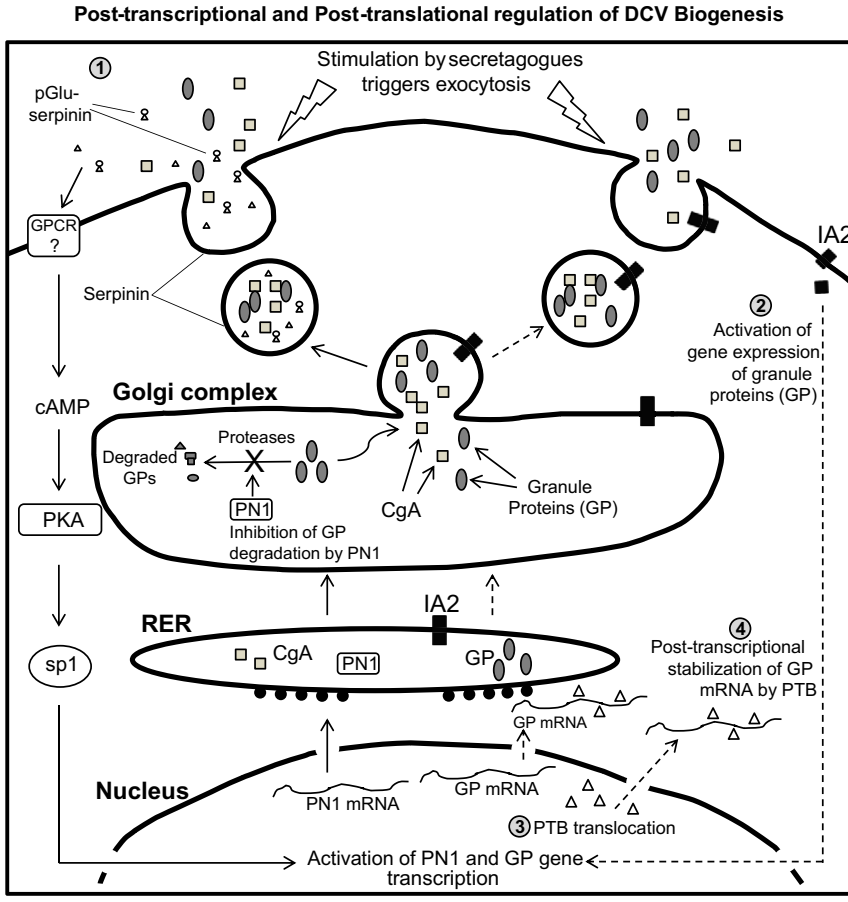

Figure 2.3 ***Multilevel mechanisms regulate DCV biogenesis.*** Formation of dense-core secretory granules in neuroendocrine and endocrine cells is regulated at multiple levels to maintain a steady-state pool of DCVs and to replenish stored pools after secretion. These mechanisms promote the level of granule proteins to enhance DCV biogenesis. Transcriptional (2) and posttranscriptional events (3, 4) regulating DCV biogenesis are illustrated by dotted arrows. The events involved in serpinin-induced posttranslational regulation of DCV biogenesis (1) are illustrated by solid arrows. In this posttranslational pathway, secreted serpinin and pGlu serpinin (CgA-derived peptides) bind to a putative G protein-coupled receptors (GPCR) which then leads to activation of a cAMP–PKA pathway, translocation of transcription factor sp1 into the nucleus and upregulation of PN-1 expression. This in turn inhibits granule protein (GP) degradation in the Golgi complex, increasing GP levels and enhancement of DCV biogenesis. For a color version of this figure, the reader is referred to the online version of this book.

Some astrocytes express high levels of REST/NRSF and do not contain DCV markers such as granins or neuropeptides (Prada et al., 2011). However, when the dominant-negative construct of REST/NRSF was expressed in these astrocytes, DCV formation was induced. Moreover, the DCVs were filled with NPY and SgII and released their contents in a Ca^{2+}-dependent manner. Astrocytes also form secretion-competent clear vesicles that contain glutamate (Haydon and Carmignoto, 2006; Parpura and Zorec, 2010) but formation of these vesicles seems to be independent of REST. This study also showed that not all proteins related to DCVs and secretion were increased after stable transfection of REST dominant-negative construct into astrocytes: SNAP25 was increased significantly, the other t-SNARE, Stx1a was only increased slightly in these astrocytes. SgII was elevated significantly, although all three proteins were well below that found in PC12 WT cells. In PC12–27 cells, a mutant cell line that is secretion incompetent, the SNAP25, Stx1a and SgII proteins were increased when stably transfected with the REST dominant-negative construct. In these cells, the rise of Stx1a was 12-fold, and that of SgII was only 2-fold (D'Alessandro et al., 2008). These differences indicate that control of gene expression by REST is cell specific.

Studies thus far would suggest that the negative regulation (suppression of expression) of genes involved in DCV biogenesis and secretion by the REST/NRSF transcription factor may be of significance during differentiation of cells to yield specific phenotypes. For example, for non-DCV-containing astrocytes during development, REST expression is high, and for astrocytes and neurons that contain DCVs, REST expression is low. REST may also play a role in maintaining the differentiated phenotype. However, in adults, after injury, differentiation of progenitors occurs to regenerate endocrine/exocrine and neuronal cells. Under these circumstances, another signal that downregulates REST expression in these progenitors may be present to facilitate differentiation into DCV-producing secretory cells. However, at present, not much is known about the level of REST in progenitors of endocrine/exocrine or neuronal cells, and more studies are necessary to investigate such a possibility.

5.3.2. Transcriptional Regulation of DCV Biogenesis

Another level of control of DCV biogenesis is at the transcriptional level. In insulin-secreting pancreatic β-cells, insulinoma-asssociated protein 2 (ICA512/IA-2), a transmembrane protein localized on DCV membranes, was shown to regulate DCV biogenesis (Harashima et al., 2005; Kim et al., 2006). When pancreatic β-cells are stimulated by glucose to release insulin,

the membrane of the exocytosed DCV is incorporated into the PM after the fusion process, thereby localizing ICA512/IA-2 to the cell membrane. The cytoplasmic tail of ICA512/IA-2 is then cleaved off and translocated into the nucleus where it induces expression of insulin (Fig. 2.3) (Trajkovski et al., 2004). Furthermore, when ICA512/IA-2 was overexpressed in MIN-6, an insulin-secreting β-cell line, insulin content and quantity of DCVs were increased (Harashima et al., 2005).

In another example, induction of exocytosis in sympathoadrenal chromaffin cells and PC12 pheochromocytoma cells resulted in upregulation of transcription of various DCV-related genes such as CgA, tyrosine hydroxylase, phenylethanolamine-*N*-methyltransferase, dopamine β-hydroxylase, and proenkephalin (Eiden et al., 1984; Rausch et al., 1988; Kilbourne et al., 1992; Hiremagalur et al., 1993; Tang et al., 1996; Mahapatra et al., 2003, 2005). Upregulation of expression of these genes and hence their proteins would be expected to drive DCV biogenesis. Indeed, it has been demonstrated that overexpression of granins and prohormones in fibroblasts can induce DCV biogenesis (Kim et al., 2001; Huh et al., 2003; Beuret et al., 2004; Stettler et al., 2009). However, the mechanism by which these genes are upregulated after an exocytosis event is unknown. It could be mediated by a peptide signal released from the vesicle, which then activates a signal transduction pathway leading to gene transcription (see Section 2.5.3.4 and Figure 2.3).

5.3.3. Posttranscriptional Regulation of DCV Biogenesis

Regulation of DCV biogenesis at the posttranscriptional level has been demonstrated in pancreatic insulin-producing cells (Knoch et al., 2004). In this mechanism, mRNA stability is increased leading to elevation of DCV protein biosynthesis and promotion of DCV formation (Fig. 2.3). Polypyrimidine tract-binding protein (PTB) is an RNA-binding molecule that regulates mRNA splicing, polyadenylation, 3′ end formation, internal ribosomal entry site-mediated translation, localization, and stability (Auweter and Allain, 2008). mRNAs of DCV proteins, such as insulin, insulinoma-associated protein 2 (ICA512/IA-2), CgA, and proprotein convertases, PC1/3 and PC2, all possess a PTB-binding site in their 3′-UTR (Knoch et al., 2004). Upon stimulation of secretion of insulin from pancreatic β-cells with glucose, PTBs were translocated from the nucleus to the cytoplasm where they bound the 3′-UTR of mRNAs encoding DCV proteins to stabilize them. As a consequence, translation and DCV protein levels were increased, leading to enhanced insulin secretory granule biogenesis (Knoch et al., 2004).

5.3.4. Posttranslational Regulation of DCV Biogenesis

Studies have indicated that CgA plays a very important role as a granulogenic protein in DCV biogenesis (see Section 2.3.2.2.). More recently, it was found that a C-terminal 26 amino acid fragment of CgA, named serpinin, which is secreted in an activity-dependent manner from AtT-20 cells, is an autocrine signal that regulates DCV biogenesis at the posttranslational level (Koshimizu et al., 2010). Investigations into the mechanism by which serpinin regulates DCV biogenesis revealed that it induced transcription of an mRNA encoding a protease inhibitor, protease nexin-1 (PN-1) that was found to be resident in the Golgi apparatus, although it was first identified as an extracellular protease inhibitor (Kim and Loh, 2006). DCV proteins appear to be constantly synthesized and degraded in the Golgi complex at steady state in PC12 and 6T3 cells. Enhanced degradation of DCV proteins occurred in CgA-deficient PC12 and 6T3 cells, and was prevented by reexpression of CgA. Furthermore, expression of PN-1 in 6T3 cells, in the absence of CgA, rescued DCV protein degradation and induced granule biogenesis. Serpinin and its pyroglutaminated form (pGlu-serpinin), which was more potent, enhanced PN-1 transcription through a receptor-mediated cyclic adenosine monophosphate (cAMP)–PKA signal transduction pathway (Koshimizu et al., 2011a, 2011b). Exogenous serpinin treatment of AtT-20 cells resulted in an increase in cAMP levels and PKA activity and caused translocation of the transcription factor, sp1, from the cytoplasm to the nucleus. Sp1 activated PN-1 transcription in the nucleus (Koshimizu et al., 2010, 2011b). This led to protection of DCV proteins from degradation, an increase in DCV proteins in the Golgi apparatus and promotion of secretory vesicle biogenesis. Treatment of PC12 cells with serpinin also increased PN-1 mRNA expression in PC12 cells, indicating that this mechanism is not unique to AtT-20 cells (Koshimizu et al., 2010). It was proposed that upon stimulation of DCV exocytosis and release of serpinins from (neuro)endocrine cells, expression of PN-1 would be increased, thereby stabilizing and increasing DCV protein levels in the Golgi complex and DCV biogenesis. This post-translational mechanism represents an efficient way to increase levels of all the proteins needed for DCV biogenesis after stimulated secretion (Fig. 2.3).

6. SV BIOGENESIS

SVs are formed from the PM by recycling of SV-specific proteins and lipids that are delivered to the PM via synaptic vesicle precursors (SVPs) or

SPTVs. The steps and protein and lipid components involved in SV biogenesis are reviewed below and summarized in Table 2.3. Three different modes of SV recycling from the PM have been proposed: kiss-and-run, complete fusion followed by clathrin-mediated endocytosis, or by bulk endocytosis (Ceccarelli et al., 1973; Heuser and Reese, 1973; Royle and Lagnado, 2003; Matthews, 2004). Kiss-and-run is thought to refill a readily releasable pool of SVs during mild stimulation while bulk endocytosis is for generation of SV reservoir after intense stimulation. In kiss-and-run mode, SV releases its content upon stimulation by forming a transient pore with the PM and then recycles by disconnecting itself from the PM (Ceccarelli et al., 1973; Fesce and Meldolesi, 1999; Harata et al., 2006). During kiss-and-run, SV membrane proteins and lipids are not intermixed with those in the PM. The kiss-and-run process has been observed during recycling of SVs in neurons and neuroendocrine cells as well as peptidergic vesicles in neuroendocrine cells (Klyachko and Jackson, 2002; Gandhi and Stevens, 2003; Zhang et al., 2007). In complete fusion followed by clathrin-mediated endocytosis, SVs collapse into the PM and release all their content. Subsequently, SV membrane proteins such as neurotransmitter transporters, are endocytosed via clathrin coating from the PM or early endosomes to form

Table 2.3 Protein and lipid components involved in SV/SLMV biogenesis

Step	Type	Proteins	Lipids	Discussed in section
Sorting at TGN	Sorting receptors, sorting adaptors	synaptophysin (VAMP2), ZnT3 (ClC-3), AP-2, AP-3, AP-4	Cholesterol, lipid rafts★	2.6.1, 2.6.2.2.2, 2.6.2.2.1
Vesicle endocytosis at PM	Clathrin based	Clathrin, AP-2, Stoned B, stonin 2, AP180, epsin, Dab2, PIPKIγ, PI4KIIα	PIP_2	2.6.2.2.1/ 2.6.2.1.2, 2.6.2.1.2
Vesicle sorting at EE	Non-clathrin based	AP-3, BLOC-1, Arf1, Casein kinase 1α-like kinase		2.6.2.2.1
Membrane curvature		Endophilin, amphiphysin	Cholesterol, lipid raft	2.6.2.2.1
Vesicle fission		Dynamin	PIP_2	2.6.2.2.1

EE= early endosome.
★Lipid rafts consist of platforms of lipids rich in cholesterol and sphingolipids.

empty SVs. In bulk (slow) endocytosis, endosomal intermediates are formed directly from the PM during intensive stimulation that causes excessive SV exocytosis (Richards et al., 2003; Royle and Lagnado, 2003; Evans and Cousin, 2007). Bulk endocytosis uptakes a relatively large PM area to balance sudden expansion of membrane during excessive exocytosis triggered by intense stimulation (Granseth et al., 2006; Wu and Wu, 2007). In the following sections, we will further discuss how proteins and lipids are sorted to SVs for biogenesis.

6.1. Sorting and Assembly of SV Proteins and Lipids

SVs contain a distinct set of proteins and lipids different from those in other membranous compartments, implying that there is selective sorting of SV-specific proteins and lipids away from non-SV components. The sorting appears to occur during formation of SVs from donor membranes such as the PM or early endosomes, or during formation of SVPs from the TGN. However, how SV-specific proteins and lipids are sorted to the SV is unclear. The selective sorting of SV proteins and lipids may start from specific protein–protein or protein–lipid interaction. SV-specific transmembrane proteins may recruit SV-specific proteins, interact with lipids across bilayer membranes, and bind to the cytoplasmic proteins involved in SV formation and localization. For example, synaptophysin mediates sorting of the SV-resident protein, synaptobrevin (VAMP2), but not syntaxin-1 and VAMP1, to synaptic vesicle precursors for transport to the presynaptic terminals in hippocampal neurons (Pennuto et al., 2003). The synaptophysin-based sorting of VAMP2 to the SVP is mediated by an interaction of synaptophysin with VAMP2 in the lumen of the TGN (Edelmann et al., 1995; Washbourne et al., 1995; Bonanomi et al., 2007). Then, the cytoplasmic domain of synaptophysin mediates targeting of synaptophysin/VAMP2-containing vesicles to the axonal terminal. Even in non-neuronal cells, synaptophysin reroutes VAMP2, but not synaptotagmin and syntaxin-1, from the PM to recycling endosomes (Bonanomi et al., 2007), suggesting that synaptophysin specifically mediates sorting of VAMP2 during its trafficking between membrane compartments. In addition, the interaction of synaptophysin with PM-localized cholesterol contributes to biogenesis of SLMVs, the SV counterpart in endocrine cells (Thiele et al., 2000), partly by mediating co-sorting of synaptophysin and VAMP2 (Galli et al., 1996; Mitter et al., 2003). Photoactivatable cholesterol specifically labels synaptophysin in the PM of MDCK (Madin Darby Canine Kidney) and PC12

cells upon UV irradiation while photoactivatable choline and inositol do not. Conversely, depletion of cholesterol reduces not only the levels of synaptophysin in SLMVs in PC12 cells but also the steady-state pool of SLMVs, suggesting that cholesterol is required for sorting of synaptophysin to SLMVs and for biogenesis of SLMVs. Hence, cholesterol-rich microdomains such as a lipid rafts appear to play a role as a platform for selective assembly of SV-specific membrane proteins and lipids.

The selective sorting or assembly of proteins and lipids during SV biogenesis may not necessarily lead to formation of SVs of uniform composition. Some microheterogeneity with respect to the types of proteins and lipids exists among SVs even within a single presynaptic bouton. The small differences in the composition of membrane proteins and lipids in donor membranes also contribute to generate the microheterogeneity in SVs. As a result, individual SVs with slightly different compositions are formed and have different functional characteristics, perhaps yielding different exocytosis rates (Valtorta et al., 2001; Newell-Litwa et al., 2007; Voglmaier and Edwards, 2007). However, even with different compositions, the majority of SVs in presynaptic boutons are usually of uniform size. Thus, one would expect that there should be some maturation process that would facilitate the generation of SVs of similar sizes. At present, little is known about the maturation process for SVs, however, high levels of continuous recycling of SVs during synapse maturation is proposed to contribute to vesicle size uniformity (Young and Poo, 1983; Matteoli et al., 1992; Antonov et al., 1999; Diefenbach et al., 1999). Another possibility is that SVs may be formed from uniformly sized microdomains on the donor membrane. In the following section, our discussion will focus on each protein and lipid sorted to SVs.

6.2. Molecules Involved in SV Biogenesis

6.2.1. Lipid Constituents

6.2.1.1. Bulky Lipids

Both SVs and synaptosomal PMs are rich in polyunsaturated fatty acids, such as docosahexanoic acid (Breckenridge et al., 1972). Gangliosides are usually found in synaptosomal membranes and bulky lipids such as cholesterol and sphingolipids are enriched in SVs. Depletion of cholesterol from the presynaptic membrane by treatment with methyl-β-cyclodextrin (MCD) reduces SV exocytosis at frog motor nerve endings, maybe by inhibiting the binding of proteins involved in docking and priming to the presynaptic

membrane (Petrov et al., 2010). Likewise, depletion of cholesterol from SVs by treatment of the nerve with MCD during prolonged stimulation decreases SV endocytosis, resulting in reduction in SV biogenesis at the motor nerve endings. Thus, cholesterol is required for both exocytosis and endocytosis of SVs. Additionally, cholesterol is involved in clustering of SNARE proteins (Chamberlain et al., 2001; Lang et al., 2001), SV proteins (Jia et al., 2006), and phosphatidylinositol 4,5-bisphosphate (PIP_2) (Pike and Miller, 1998) on the PM for facilitating SV biogenesis. Finally, the bulkiness of cholesterol appears to contribute to generation of higher curvature in the membrane (Gondre-Lewis et al., 2006; Kim et al. 2006), which facilitates formation of small sizes of SVs (Deutsch and Kelly, 1981; Martin, 2000; Thiele et al., 2000; Gondre-Lewis et al., 2006; Jia et al., 2006; Kim et al., 2006; Wasser et al., 2007). Since both cholesterol and sphingomyelin are major components of lipid rafts (Barenholz, 2004), lipid raft-like patches in the PM or in early endosomes are likely where most SV biogenesis occurs.

6.2.1.2. Phosphoinositides

Among several types of phosphoinositides, PIP_2 is the major lipid component of the vesicles and PM in SV exocytosis and endocytosis, i.e. SV biogenesis. PIP_2 in the PM provides a platform for assembly of clathrin coats during endocytosis (Cremona and De Camilli, 2001) and mediates actin nucleation via its interaction with actin-interacting proteins (Sechi and Wehland, 2000). AP-2 and the accessory clathrin adaptor protein, AP180, bind to the PM via their interaction with PIP_2 and initiate clathrin coating. Inhibition of the interaction of AP-2 with PIP_2 blocks recruitment of AP-2/clathrin to the PM, decreasing SV biogenesis (Jost et al., 1998). In the next steps, dynamin, a GTPase necessary for membrane fission and vesicular release, forms a polymeric stalk around the neck of endocytosed SVs and, upon GTP hydrolysis, mediates the pinching off of endocytosed SV from the PM (Cremona and De Camilli, 2001). The binding of dynamin to the neck of invaginated vesicle is also mediated by PIP_2 (Vallis et al., 1999).

The brain appears to have two major phosphatidylinositol 4-kinases (PI4K) that generate PIP_2: PI4K type II α (PI4KIIα) (Guo et al., 2003) and phosphatidylinositol phosphate kinase I-γ (PIPKIγ) (Wenk et al., 2001; Nakano-Kobayashi et al., 2007). PI4KIIα is associated with SVs at synapses and also found in other membranous compartments, such as the PM and Golgi complex in neurons (Guo et al., 2003). PIPKIγ, another PI4K concentrated in the brain, mediates formation of the clathrin coat and F-actin meshwork around SVs (Wenk et al., 2001). Synaptojanin-1 (synj-1),

a phosphatase of PIP_2, competes with PIPKIγ for binding to the PM and SVs to control the levels of PIP_2 during SV recycling (Wenk et al., 2001). Overexpression of the AP-2-interacting C-terminal domain of PIPKIγ inhibits SV endocytosis, suggesting that the interaction of PIPKIγ with AP-2 is important for clathrin coat formation and SV endocytosis. PIPKIγ KO (*PIPKIγ*$^{-/-}$) mice show various defects in neuronal activity and die shortly after birth (Di Paolo et al., 2004). *PIPKIγ*$^{-/-}$ mice have severe synaptic defects, such as decreased miniature current frequency and increased synaptic depression. Neurons from these knockout mice show defective SV endocytosis, fewer readily releasable SVs coated with clathrins and enlarged endosomes. It suggests that PIPKIγ is important for production of PIP_2 for clathrin-dependent biogenesis of SVs.

Stimulation of hippocampal neurons by membrane depolarization activates PIPKIγ that, in turn, increases the levels of PIP_2 at the presynaptic membrane, thus enhancing SV endocytosis (Nakano-Kobayashi et al., 2007). Similarly, NO generated by *N*-methyl-D-aspartate receptor activation increases SV endocytosis in a cyclic guanidine monophosphate (cGMP)-dependent manner, possibly by increasing the levels of PIP_2 in the presynaptic membrane of hippocampal neurons (Micheva et al., 2001, 2003) while decreasing PIP_2 in the cell body and dendrites (Micheva et al., 2001). The rate of SV endocytosis is increased in proportion to the levels of cGMP in stimulated neurons, while removing available PIP_2 suppresses the cGMP-induced SV endocytosis (Micheva et al., 2003). Thus, PIP_2 appears to be increased at the presynaptic terminals in an activity-dependent manner, thus enhancing SV endocytosis to prevent excessive expansion of the PM by SV exocytosis.

There are several phosphatases that decrease the levels of PIP_2 or other phosphoinositol phosphates in the vesicles and PM. Synj-1 is the major phosphatase important for SV recycling. Synj-1 is expressed predominantly in the nervous system and mediates conversion of PIP_2 and phosphatidylinositol-3,4,5-trisphosphate ($PI(3,4,5)P_3$) to PI4P and $PI(3,4)P_2$ (McPherson et al., 1994; Haffner et al., 1997). The C-terminal SH3 domain of synj-1 interacts with various proteins involved in SV biogenesis and actin organization at the presynaptic terminals. Mice lacking synj-1 (*Synj1*$^{-/-}$ mice) die early after birth and show greater synaptic depression and accumulation of PIP_2 and clathrin-coated SVs at nerve terminals (Cremona et al., 1999; Luthi et al., 2001). In neurons, deletion of synj-1 causes slower SV endocytosis in response to both short and repetitive stimuli while having little effect on the exocytosis rate (Mani et al., 2007). Another study using *Synj1*$^{-/-}$ mice

shows that deletion of synj-1 decreases the pool of SVs available for the next round of exocytosis while increasing the pool of clathrin-coated SVs (Kim et al., 2002). It suggests that accumulated PIP_2 interferes with transition from clathrin-coated SVs to readily releasable SVs maybe by inhibiting removal of the clathrin coat. Furthermore, knockdown of synj-1 ($Synj1^{-/-}$) in zebra fish causes abnormal synaptic transmission at the cone photoreceptor synapses (Van Epps et al., 2004). The cone photoreceptor terminals of $Synj1^{-/-}$ zebra fish show accumulation of irregular actin polymers, uneven distribution of ~50% fewer readily releasable SVs, and enlarged endosomes. This finding suggests that abnormal accumulation of PIP_2 at the photoreceptor terminals increases random actin polymerization and inhibits SV biogenesis. Taken together, the function of synj-1 in lowering the levels of PIP_2 seems to be important not only for regulation of actin polymerization at presynaptic terminal but also for clathrin uncoating to generate the readily releasable pool of SVs.

6.2.2. Protein Constituents

6.2.2.1. Cytoplasmic Proteins in SV Biogenesis

Two AP complexes, AP-2 and AP-3, mediate biogenesis of SLMVs and SVs in endocrine cells and neurons; however, they use different mechanisms and donor membranes (Desnos et al., 1995; Faundez et al., 1998; Blumstein et al., 2001). AP-2 aids clathrin-mediated endocytosis of SVs from the PM while AP-3 mediates clathrin-independent SV biogenesis mostly from early endosomes. AP-3 mediates early recycling and maturation of SVs during synapse maturation in developing nerves (Zakharenko et al., 1999) whereas AP-2 facilitates SV recycling from the PM in mature presynaptic boutons.

AP-2 was well reviewed by Traub (2003). Briefly, the AP-2 heterotetramer consists of two large (α and β2), one medium (μ2), and one small (δ2) subunit. AP-2 binds to the PM via the interaction of its α subunit with PIP_2. Both α and β2 subunits interact with clathrin heavy chain, thus initiating formation of the clathrin lattice around the budding vesicle. The μ2 subunit of AP-2 functions as a cargo selector while the δ2 subunit is primarily structural, and recognizes YXXØ signals in the cytoplasmic domain of transmembrane protein cargo, thus facilitating endocytosis of the cargo. Various scaffolding and actin-interacting proteins such as AP180, epsin, and Dab2 collaborate with AP-2 for clathrin recruitment and assembly.

In *C. elegans*, unc-11, a homolog of mammalian AP180, is expressed specifically at nerve terminals (Nonet et al., 1999). Loss of unc-11 by nonsense mutations causes defective synaptic transmission at cholinergic,

glutamatergic, and GABA-ergic synapses. The unc-11 mutations specifically inhibit the targeting of synaptobrevin (VAMP2), but not other presynaptic proteins (e.g. synaptogyrin, synaptotagmin, Rab-3), to SVs. Moreover, the sizes of SVs at the presynaptic terminals in unc-11 mutants are larger than those in WT. These results suggest that AP180 may mediate the targeting of synaptobrevin to the presynaptic terminals and may control the size of SVs for proper synaptic transmission at cholinergic, glutamatergic, and GABA-ergic synapses in *C. elegans*.

In *Drosophila*, the loss-of-function mutation of Stoned B, an AP-2-interacting protein, causes formation of irregular shapes of vesicles and depletion of functional SVs at the larval motor nerve terminals (Stimson et al., 2001). The Stoned B mutation increases the retention of cysteine string protein and synaptotagmin at the presynaptic membrane and slows the rate of endocytosis of SV proteins from the membrane. Stonin 2, a human ortholog of *Drosophila* Stoned B, is enriched at the presynaptic terminal and is involved in clathrin-mediated endocytosis in rat cortical neurons (Walther et al., 2001). Stonin 2 directly interacts with and co-localizes with AP-2 and synaptotagmin 1 at the presynaptic terminal to facilitate AP-2-dependent internalization of synaptotagmin 1 to SLMVs and SVs in PC12 cells and cortical neurons, respectively (Diril et al., 2006).

AP-3 consists of δ, β3A/β3B, μ3A/μ3B, and σ3A/σ3B, mutations of which are linked to defects in biogenesis of endosomes, lysosomes, and lysosome-related organelles (Faundez et al., 1998; Dell'Angelica, 2009). AP-3 is expressed throughout the brain with especially high levels in the striatum and hippocampus, and is primarily localized to presynaptic axonal terminals (Newell-Litwa et al., 2010). AP-3 mediates SV biogenesis in an Arf1-dependent manner (Faundez et al., 1998). AP-3 was shown to mediate biogenesis of SLMV from early endosomes in a clathrin-independent manner in PC12 cells (Shi et al., 1998) while a later study showed that subunits β3A and β3B of AP-3 can interact with clathrin heavy chain (Peden et al., 2002). The recruitment of AP-3 to early endosomes for SLMV biogenesis is regulated by casein kinase 1α-like kinase that phosphorylates the β3A/β3B subunit of AP-3 (Faundez and Kelly, 2000). Inhibition of the kinase blocked recruitment of AP-3 to early endosomes and thus SLMV formation in PC12 cells. Mice deficient in AP-3 showed altered SV biogenesis in brain (Blumstein et al., 2001; Newell-Litwa et al., 2010). Mice lacking the AP-3 δ subunit showed enlarged SVs in the dentate gyrus but had minimal effects on presynaptic compartments in CA3 pyramidal neurons in the hippocampus and in the striatum (Newell-Litwa et al., 2010).

Thus, AP-3 seems to mediate SV or SLMV biogenesis from early endosomes in an Arf1-dependent and clathrin-independent manner, but its influence on these processes may be different depending on the brain region.

The protein complex, "biogenesis of lysosome-related organelles complex 1 (BLOC-1)", appears to collaborate with AP-3 to mediate SV biogenesis from early endosomes. BLOC-1 is an octamer made up of BLOS1–3, cappuccino, dysbindin, muted, pallidin, and snapin subunits (Di Pietro and Dell'Angelica, 2005). Both BLOC-1 and AP-3 are required for biogenesis of lysosomes and lysosome-related organelles. Mice with loss-of-function mutations of BLOC-1 or AP-3 develop Hermansky–Pudlak syndrome due to defective sorting of membrane proteins from endosomes to lysosomes and lysosome-related organelles (Li et al., 2004; Di Pietro and Dell'Angelica, 2005). AP-3 directly interacts with BLOC-1 via the interaction of its μ subunit with dysbindin, a subunit of BLOC-1 (Taneichi-Kuroda et al., 2009). Vesicular co-localization of AP-3 with dysbindin was observed in neurons of the dentate gyrus and throughout CA1 to CA3 subfields, and at presynaptic terminals and axonal growth cones of cultured hippocampal neurons. These findings suggest that AP-3 and BLOC-1 may collaborate to mediate SV biogenesis in the hippocampus. A recent study (Newell-Litwa et al., 2010) shows that mice lacking either pallidin (*Pldnpa/pa*) or muted (*Mutedmu/mu*) display similar neurological defects to those lacking AP-3, and BLOC-1 and AP-3 formed a complex in fractions enriched in brain nerve terminals (synaptosomes). However, BLOC-1 and AP-3 appeared to work sometimes in an incoherent manner (Newell-Litwa et al., 2010). Loss of function of the BLOC-1 alleles, Pldn(pa/pa) and Muted(mu/mu), had no effect while loss of AP-3 enhanced SV biogenesis in the dentate gyrus. Loss of AP-3 inhibited SV biogenesis in the striatum while loss of BLOC-1 had no effect. In addition, lack of dysbindin, the BLOC-1 subunit, in PC12 cells and hippocampal neurons showed various defects in neurosecretion (Chen et al., 2008). Overall, these mice showed depletion of readily releasable SVs, slower and fewer exocytic events, and larger sizes of SVs, while no change in the levels of proteins involved in the SV exocytic machinery was evident.

Some cytoplasmic proteins are involved in mechanical processes of SV biogenesis, e.g. invagination and fission of SVs. Endophilin-1, a protein enriched in brain presynaptic nerve terminals, participates in multiple stages in clathrin-coated endocytosis, from early membrane invagination to SV uncoating (Reutens and Begley, 2002). Using its C-terminal SH3 domain, endophilin binds to the proline-rich domains of synaptojanin and dynamin.

The N-terminus of endophilin binds directly to lipids on the membrane and mediates generation of membrane curvature by using its lysophosphatidic acid acyl transferase activity (Reutens and Begley, 2002). Synj-1, described in Section 2.6.2.1.2, regulates recruitment of dynamin, amphiphysin, and other proteins onto budding vesicles by changing PIP_2 levels in the PM (Mani et al., 2007). Amphiphysin is a major dynamin-interacting protein that is recruited to the neck of the endocytosing vesicles (Wu et al., 2009). Amphiphysin enhances membrane curvature as well as the GTPase activity of dynamin, thus facilitating invagination and fission of SVs from the PM. Dynamin is a GTPase required for biogenesis of SVs or SLMVs from the PM and early endosomes (Scaife and Margolis, 1997), and consists of four different domains—an N-terminal GTPase domain, a PH domain, a GED, and a C-terminal proline/arginine-rich domain. Dynamin is recruited to CCPs via the interaction of its proline/arginine-rich domain with the SH3 domain of amphiphysin (Hill et al., 2001). It mediates constriction of the neck between budding vesicles and the PM by forming spiral rings around the neck, resulting in vesicle fission.

In addition to the above mechanical proteins, the following proteins also affect SV biogenesis. α GDP dissociation inhibitor (αGDI) that regulates the GTPase activity and membrane targeting of Rabs is also important for SV biogenesis. Loss of function of αGDI impairs several steps in SV biogenesis and recycling in the hippocampus by causing abnormal accumulation of different Rabs throughout the intermediate compartments (Bianchi et al., 2009). Mice deleted of αGDI show defects in glutamate secretion from hippocampal synaptosomes, and short-term plasticity and memory. The small GTPase, Arf6, a mediator of endocytosis, affects SLMV biogenesis in PC12 cells (Powelka and Buckley, 2001). Expression of the constitutively active mutant of Arf6 increased the number of SLMVs while that of the dominant-negative Arf6 mutant decreased it.

6.2.2.2. Membrane Proteins in SV Biogenesis

The SV membrane contains several transmembrane proteins, such as synaptophysin, synaptobrevin, SV protein 2 (SV2), etc., and several types of neurotransmitter and ion transporters. There are four different classes of neurotransmitter transporters: VAChTs (ACh), vesicular glutamate transporter (VGLUT: glutamate), vesicular GAT (GABA/glycine), and VMATs (monoamines). The first three transporters are targeted to SVs while VMATs are found mostly in small peptidergic vesicles that are morphologically and dimensionally different from SVs. The specificity of targeting of SV

membrane proteins appears to be determined by specific motifs in the cytoplasmic domain of the proteins. For example, the di-leucine and Met-Leu (degenerate di-leucine) sequences are used for sorting of SV membrane proteins to SLMVs in an AP-3/Arf1-dependent manner (Blagoveshchenskaya et al., 1999). AP-3 is known to bind to the di-leucine motif directly (Odorizzi et al., 1998). In PC12 cells, point mutations of the di-leucine or Met-Leu motifs in the cytoplasmic domain of synaptotagmin-1 significantly reduce sorting of the proteins to SLMVs. Given that synaptotagmin-1 can interact with AP-2 via an interaction of its C2B domain with the μ2 subunit of AP-2 (Grass et al., 2004), sorting of synaptotagmin to SLMVs or SVs appears to be mediated by AP-2. We also cannot rule out the possibility that AP-3 (Odorizzi et al., 1998) may affect SV sorting of synaptotagmin-1 via its interaction with the di-leucine motif of synaptotagmin-1. VAMP2 appears to contain an SV-specific motif in its cytoplasmic domain (Hao et al., 1997). Deletion of VAMP2 amino acid residues 31–38 inhibited sorting of VAMP2 to SLMVs without affecting its endocytosis. By contrast, deletion of the VAMP2 amino acid residues 41–50 inhibited both SLMV targeting and endocytosis of VAMP2 whereas a point mutation (N49A) of VAMP2 increased the extent of targeting of VAMP2 to SLMVs by 200-fold. Thus, VAMP2 (synaptobrevin) appears to contain an SV-specific motif that is functionally involved in endocytosis.

The cytoplasmic domain of the zinc transporter, Zn transporter (ZnT) 3, has an AP-3-interacting domain that helps sorting of ZnT3 to SLMVs in PC12 cells (Salazar et al., 2004b). Inhibition of AP-3 blocked the sorting of only ZnT3 but not synaptophysin, but inhibition of AP-2 decreases the sorting of only synaptophysin but not ZnT3 to SLMVs. ZnT3 and synaptophysin are indeed found in different SLMV populations, accordingly, the SV sorting signals of synaptophysin and ZnT3 may be differentiated to bind specifically to AP-2 or AP-3, respectively, resulting in sorting to different SLMV pools. ZnT3 also helps sorting of a chloride channel, ClC-3, to SLMVs in an AP-3-dependent manner (Salazar et al., 2004a). The co-sorting of ZnT3 and ClC-3 to SLMVs and, likely, SVs is required to establish proper ionic composition inside of SLMVs/SVs. The nerve terminals of mossy fiber hippocampal neurons in mice deficient in AP-3 show significantly lower levels of ZnT3 and ClC-3. Thus, some neurological defects found in mouse models deficient in AP-3 may be caused by failure of co-sorting of ClC-3 and ZnT3 to SVs in the brain.

However, it appears that the cytoplasmic domains of SV membrane proteins do not share motifs specialized for sorting to SV (Prado and Prado,

2002; Voglmaier and Edwards, 2007). The cytoplasmic tail of synaptophysin has tyrosine-based repeats, which are similar to the internalization and lysosomal targeting sequences of lysosome-associated membrane proteins (Marks et al., 1996). VGLUT1 binds to endophilins via its proline-rich cytoplasmic domain and co-localizes with endophilins at the synaptic terminals of differentiated rat neocortical neurons in primary culture (De Gois et al., 2006; Vinatier et al., 2006). The interaction of VGLUT1 with endophilin is, however, not required for VGLUT1 endocytosis under moderate stimulation but is required under intensive stimulation. As such, deletion of the endophilin-binding domain of VGLUT1 inhibited endocytosis of VGLUT1 only during prolonged stimulation at high frequency.

Mutation or deletion of SV membrane proteins interferes with biogenesis and recycling of SVs in addition to loss of their innate functions. For example, mutations of VAMP2 or synaptotagmin-1 affected formation of SVs in glutamatergic nerve systems (Deak et al., 2004; Fremeau et al., 2004; Poskanzer et al., 2006; Wallen-Mackenzie et al., 2006). Loss of VAMP2 causes a delay in the refilling of readily releasable pools of SVs after stimulation without any drastic effect on the total number of presynaptic SVs (Deak et al., 2004). Synaptotagmin-1 was shown to regulate both the rate of formation and the size of SVs during endocytosis by using its calcium-sensing C2B domain (Poskanzer et al., 2006). The study showed that mutations of the polylysine motif in the synaptotagmin-1 C2B domain alter vesicle size while mutations in Ca^{2+}-binding aspartate residues affect only the rate of SV exocytosis. Moreover, loss of VGLUTs reduced the reserve SV pool and caused formation of larger, more elongated vesicles and tubulovesicular structures in neurons, which results in a decrease in fast excitatory synaptic activity (Fremeau et al., 2004; Wallen-Mackenzie et al., 2006). This decrease could not be reproduced by inhibition of vesicle filling or release (Augustin et al., 1999; Parsons et al., 1999; Verhage et al., 2000). Therefore, loss of VGLUTs appears to physically interfere with biogenesis of glutamatergic SVs.

7. GOLGI-TO-PM VESICLE TRAFFICKING

Post-Golgi transport of vesicles from the cell body where they are synthesized to the PM is microtubule dependent (Goldstein and Yang, 2000; Rudolf et al., 2001; Neco et al., 2003; Smith et al., 2003; Guzik and Goldstein, 2004). A recent review gives a good perspective of the functional significance of the orientation and posttranslational modification of

microtubules and actins, and microtubule motors in vesicular transport (van den Berg and Hoogenraad, 2012). Briefly, the plus ends of microtubules are oriented toward the axonal terminals and the minus ends toward the cell body. In distal dendrites, the plus ends of microtubules are oriented toward the dendritic terminals, but in proximal dendrites, the plus and minus ends of microtubules are intermingled. Conversely, F-actins are enriched in the axonal terminal boutons and dendritic spines and are not abundant along the axons and dendrites.

The microtubule motors include microtubule plus end-directed motors, the kinesins, which are the major conveyers for anterograde transport toward axonal and dendrite terminals, and cytoplasmic dynein, a minus end-directed motor, responsible for retrograde transport to the cell body or dendrite (Goldstein and Yang, 2000; Yano et al., 2001). Cytoplasmic dynein interacts with the multiprotein complex, dynactin, for proper function (Gill et al., 1991; Schroer and Sheetz, 1991). Dynactin is involved in both retrograde and anterograde transport on microtubules (Deacon et al., 2003; Dell, 2003). At the actin-rich matrix beneath the PM, myosin Va mediates transport of vesicles on actin filaments (Bridgman, 1999; Varadi et al., 2005). In the following section, and Fig. 2.4, we will provide an overview of post-Golgi transport of DCVs and SVs.

7.1. DCV Transport

7.1.1. Neuropeptide/Hormone-Containing Vesicles

The molecular mechanisms by which DCVs are transported along microtubules in peptidergic neurons and endocrine cells are poorly understood, but more insights are gradually emerging. In paraventricular nucleus neurons of the hypothalamus, DCVs containing thyroid-releasing hormone are transported by unidentified microtubule motor(s) (Alexander et al., 2005). In *C. elegans*, DCVs containing phogrin (IDA-1) and PC2 (Egl-3) are transported along both axons and dendrites by kinesin superfamily protein (KIF) 1A [uncoordinated (Unc)-104] (Zahn et al., 2004). Loss of Unc-104 prevents localization of phogrin and PC2 to nerve terminals. In two endocrine cell lines, AtT-20 and PC12, immature DCVs containing CgB are transported from the TGN to the proximity of the PM in a microtubule- and actin-dependent manner and mature at the actin-rich cortex beneath the PM (Rudolf et al., 2001). In anterior pituitary cells, both anterograde and retrograde transports of POMC/ACTH are mediated by a microtubule motor complex comprised of dynactin, cytoplasmic dynein, kinesin-2, and

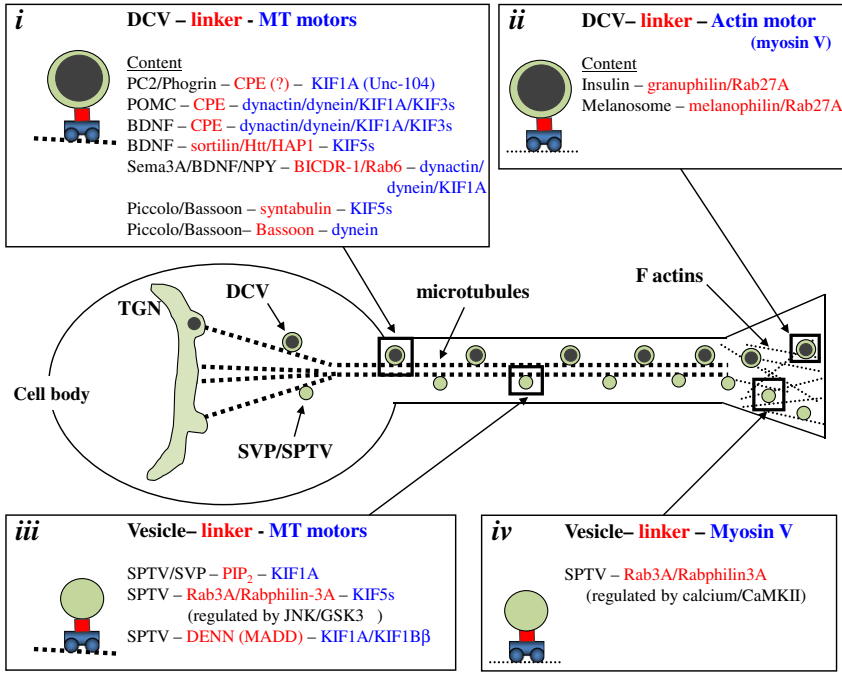

Figure 2.4 ***Proteins involved in post-Golgi transport of DCVs and SVs.*** Panel (i): Secretory and membrane proteins in DCVs are shown in black. CPE functions as a receptor (in red) for microtubule motors (in blue) on DCVs containing PC2/phogrin, POMC, and BDNF. Sortilin/Htt/HAP1 links BDNF vesicles to microtubule (MT) motors. BICDR-1 and Rab6 recruit motor proteins onto DCVs containing Sema3A/BDNF/NPY. PTV contains Piccolo, Bassoon, SNAP25, N-cadherin, Rim1, CgB, Rab3A, Munc18, syntaxin-1, and syntabulin (see Section 2.7.2.1). The interaction of syntabulin with kinesin-1 (KIF5s) mediates anterograde transport of PTV, and that of Bassoon with cytoplasmic dynein for retrograde transport. Panel (ii): Myosin Va mediates transport of DCVs along F-actins beneath the PM. Granuphilin and melanophilin connect myosin Va to DCVs via their interaction with Rab27A on DCV. Panel (iii): SVP and SPTV are transported by microtubule motors to maturing and matured synapses, respectively. SVPs and SPTVs contain SV proteins including synaptophysin, SV2, VAMP2, PIP_2 and/or DENN/MADD (see text). These proteins mediate recruitment of kinesin-3 (KIF1A) onto SVP and SPTV. Rab3A and rabphilin-3A recruit kinesin-1 onto SPTVs, which is under control of JNK and GSK3β. Panel (iv): Rab3A and rabphilin-3A connect myosin V to SPTV, which is regulated by calcium levels and CaMKII. For interpretation of the references to color in this figure legend, the reader is referred to the online version of this book.

KIF1A (Park et al., 2008). The motor complex is linked to DCVs via their interaction with the cytoplasmic tail of CPE on DCVs. Competitive inhibition of overexpressed CPE cytoplasmic tail decreases both the velocity and real-time advancement of movements of POMC/ACTH vesicles.

Overexpression of CPE also decreases the localization of ACTH vesicles and regulated secretion of ACTH itself at terminals of anterior pituitary cells. In hippocampal neurons, the speed, but not the advancement of DCVs along neurites (both axons and dendrites), can be enhanced by PKA activation but not by depolarization with high K^+ (Washburn et al., 2002). A recent study reported an interesting observation: The polarity of DCV movement toward either axon or dendrite is determined not by the type of neuropeptide in DCVs but by the type of neuron (Ramamoorthy et al., 2011). In hippocampal GABA-ergic neurons of transgenic mice expressing NPY-green fluorescent protein (GFP), DCVs containing NPY-GFP were transported to both axons and dendrites whereas in hypothalamic GABA-ergic neurons, NPY-GFP-containing DCVs are found only in the axons. Hypothalamic primary neuron cultures transfected with NPY-Venus or immunostained for endogenous Agouti-related peptide showed specific targeting of these DCVs to axons only, unlike in hippocampal neurons.

In early neuronal differentiation, DCV transport required for neurite outgrowth in neurons is temporally regulated by Bicaudal-D-related protein 1 (BICDR-1) that interacts simultaneously with Rab6 on DCVs and with the microtubule-based motor complex that consists of dynactin, cytoplasmic dynein, and kinesin-3 (Schlager et al., 2010). In zebra fish, during early nerve development, the expression of high levels of BICDR-1 holds Rab6-containing DCVs at the pericentrosomal area causing inhibition of anterograde secretory transport and neuritogenesis. In fully developed nerve fibers, the expression of BICDR-1 is decreased, thus releasing Rab6-DCV for anterograde transport to future sites of neurite outgrowth.

In addition to microtubule motors, the F-actin motor, myosin Va, also affects movement of DCVs along axons and dendrites in cultured hippocampal neurons (Bittins et al., 2009). The dominant-negative form of myosin Va reduces retrograde, but not anterograde, transport of DCVs, suggesting that myosin Va facilitates only retrograde DCV transport. Rab27A connects myosin Va to DCVs for F-actin-based transport in pancreatic β-cells (Brozzi et al., 2011). Granuphilin-a, granuphilin-b, and rabphilin-3A are also a part of the Rab27A–myosin Va complex. Inhibition of binding of the granuphilins to Rab27A blocks myosin Va-mediated DCV transport. The role of rabphilin-3A in DCV transport remains unclear. In melanocytes, Rab27A connects myosin Va to melanophilin on melanosomes for transport of melanosomes to the PM (Izumi et al., 2003; Seabra and Coudrier, 2004). An effector protein, Rab3 GTP/GDP exchange protein, mediates correct targeting of Rab27A to melanosomes (Tarafder et al., 2011). Subsequent to

granuphilin/myosin Va-mediated DCV transport to the periphery, myosin Va and its interacting protein, exophilin8 (also known as myosin VIIA and Rab interacting protein/Slac2-c), mediate clustering and immobilization of insulin vesicles on F-actin in pancreatic β-cells (Mizuno et al., 2011).

7.1.2. Neurotrophin Vesicles

The dendritic secretion of neurotrophins [e.g. NGF, neurotrophin-3 (NT-3), neurotrophin-4 (NT-4), and BDNF] is important for neuritogenesis and synaptogenesis (Lessmann et al., 2003; Ernsberger, 2009). NGF, NT-3, and NT-4 are sorted to the CSP (Goldstein and Yang, 2000) while BDNF is transported through the RSP (Lou et al., 2005). Microtubule-based transport system is thought to mediate anterograde transport of neurotrophins to the dendritic terminals for secretion.

The microtubule-based transport of BDNF is better understood than other neurotrophins. The study of Huntingtin (Htt), a protein whose polyglutamine (polyQ) form is implicated in the pathogenesis of Huntington's disease, elucidates some aspects of BDNF transport. Htt forms a complex with Htt-associated protein-1 (HAP1) and dynactin (Gauthier et al., 2004; Kwinter et al., 2009). These studies showed that the Htt/HAP1/dynactin complex mediates anterograde transport of BDNF in hippocampal neurons. WT Htt enhanced and polyQ Htt inhibited BDNF vesicle movement along neurites. Deletion of HAP1 by siRNA eliminated Htt-mediated BDNF vesicle movement. Moreover, polyQ Htt sequestered HAP1–dynactin from binding to microtubules, effectively inhibiting BDNF vesicle movement, and this appears to contribute to the pathology observed in Huntington's disease. Indeed, in the brains of Huntington's disease patients, the interaction of polyQ Htt with HAP1 and dynactin is significantly increased, which likely results in reduction of BDNF transport and secretion. A recent study shows that sortilin stabilizes the htt–HAP1–dynactin complex on BDNF vesicles, which is proposed to prevent degradation of pro-BDNF and enhance processing of pro-BDNF (Yang et al., 2011). In addition, insulin growth factor-1 (IGF-1)/Akt-induced phosphorylation of Htt appears to determine the directionality of BDNF vesicle traffic (Colin et al., 2008; Zala et al., 2008). Phosphorylation of Htt at serine 421 facilitates recruitment of kinesin-1 to the Htt/HAP1/dynactin complex and enhances anterograde BDNF transport and secretion (Colin et al., 2008). By contrast, dephosphorylation of Htt causes detachment of kinesin-1 from Htt/HAP1/dynactin complex. This detachment makes cytoplasmic dynein-mediated retrograde transport toward the cell body more dominant over anterograde

transport of BDNF to the dendritic terminals (Colin et al., 2008). Interestingly, Ser421 phosphorylation also suppresses inhibitory effects of polyQ Htt and causes polyQ Htt to work as an enhancer of BDNF transport (Zala et al., 2008). Likewise, activation of IGF-1/Akt signaling pathway can restore BDNF vesicle transport and release in neurons containing only polyQ Htt.

The lumenal domain of CPE mediates sorting of the precursor of BDNF, pro-BDNF, to the RSP at the TGN (Lou et al., 2005) while the cytoplasmic tail of CPE on BDNF vesicles recruits a microtubule-based motor complex comprising of kinesin-2, kinesin-3, cytoplasmic dynein, and dynactin to transport the BDNF vesicle along dendrites of hippocampal neurons (Park et al., 2008). Unc-104 mediates anterograde transport of secretory vesicles containing CPE (egl-21) and ACh to the neuromuscular junction in *C. elegans* (Jacob and Kaplan, 2003). Loss of Unc-104 led to failure in localization of CPE/ACh-containing DCVs to the neuromuscular junction, resulting in reduced ACh secretion. KIF1A was also found to mediate BDNF transport in cultured hippocampal neurons (Lo et al., 2011) and in this study BDNF was sorted to CgA-containing DCVs. In addition, inhibition of histone deacetylase (HDAC) by trichostatin A is reported to indirectly enhance vesicular transport of BDNF (Dompierre et al., 2007). The inhibition of HDAC6 increases acetylation of α-tubulins of microtubules, which enhances binding of microtubule motors to microtubules and facilitates microtubule-based transport of BDNF. In the brains of Huntington's disease patients, the acetylation of α-tubulins is decreased, which would decrease binding of microtubule motors to microtubules, thus inhibiting BDNF transport. This study provides some premise for use of HDAC inhibitors as therapeutic agents for treatment of Huntington's disease.

7.2. Synaptic Vesicles

7.2.1. Piccolo–Bassoon Transport Vesicle

Structural proteins (e.g. Piccolo, Bassoon, Rim, and the liprins-α) that constitute the active zone (Schoch and Gundelfinger, 2006) at the presynaptic terminals appear to be carried to nascent presynaptic sites by Piccolo-Bassoon transport vesicles (PTVs) (Fenster et al., 2000; Zhai et al., 2001; Shapira et al., 2003; Fejtova and Gundelfinger, 2006; Regus-Leidig et al., 2009). PTVs purified from developing E18 rat brain have characteristics of DCVs with a diameter of ~80 nm and contain Bassoon, syntaxin-1, SNAP25, N-cadherin, Rim1, and CgB but not VAMP2, synaptophysin,

synaptotagmin, and GAT1 (Zhai et al., 2001). Another study shows that PTVs also contain Munc18 and Rab3A and that two or three PTVs are required for constitution of a new active zone (Shapira et al., 2003). PTVs aggregate with small clear vesicles during axonal transport to the active zone (Tao-Cheng, 2007). This aggregate has a diameter of 0.13–0.22 mm, and consists of one or two PTVs and five or six clear vesicles that express SV2, synaptotagmin, synapsin-1, and VAMP2 (synaptobrevin). A recent study showed that calneuron, a family protein of neuronal calcium sensor-1, regulates formation of PTVs from the TGN by sequestering PI4Kβ which mediates the synthesis of phospholipids required for formation of PTVs at the TGN (Mikhaylova et al., 2009). High calcium levels release PI4Kβ from the inhibitory binding of calneuron, thus facilitating PI4Kβ-mediated formation of PTVs at the TGN.

Bassoon, itself, plays a role in assembly of PTVs at the TGN. Both Bassoon and Piccolo are associated with the TGN in rat hippocampal neurons and PTVs cannot be formed if association of Bassoon with the TGN is blocked by low temperature, or if a Bassoon mutation inhibits binding of Bassoon to the TGN (Dresbach et al., 2006). In transgenic mice containing mutated Bassoon, fewer PTVs are formed and the protein levels of Piccolo and other active zone proteins are decreased in retinal photoreceptor ribbon synapses (Regus-Leidig et al., 2009). Despite the importance of Bassoon and Piccolo in the assembly of the active zone, mice deficient in both Bassoon and Piccolo do not show any detectable electrophysiological defects (Mukherjee et al., 2010). Loss of both Bassoon and Piccolo also does not affect glutamatergic and GABA-ergic synaptic transmission in hippocampal slices and neurons, but clustering of SVs at the active zone is decreased. These findings suggest that Bassoon and Piccolo play a redundant role and are not necessary for neurotransmission.

Kinesin-1 is a major microtubule motor that transports PTVs from the cell body to the distal axonal terminals. Transport of SVPs, however, is mostly mediated by kinesin-3 (KIF1A) (Goldstein et al., 2008). Syntabulin links kinesin-1 to syntaxin-1 on PTVs in axons of developing neurons (Cai et al., 2007). Knockout of syntabulin causes accumulation of PTVs in the cell body and reduction of SVs at the axonal terminals, resulting in a reduction in the amplitude of postsynaptic currents. Conversely, Bassoon of PTVs directly interacts with light chains (DLC1 and DLC2) of cytoplasmic dynein for bidirectional movement of PTVs along the axon (Fejtova et al., 2009). Inhibition of this Bassoon–dynein interaction by using a competitive dominant-negative peptide of Bassoon interferes with correct

targeting of PTVs to nascent presynapses and the neuron consequently forms immature presynapses. This study also showed that myosin Va associated with PTVs along with cytoplasmic dynein.

7.2.2. Synaptic Vesicle Precursor/Synaptic Protein Transport Vesicle

In developing axons, SVPs also called SPTVs (Park et al., 2011) deliver SV proteins, such as synaptophysin, to the PM constitutively (De Camilli and Jahn, 1990; Kelly, 1991). In adult axons, SPTVs are still the major vesicles that deliver SV proteins to the PM. Rab3A and its effector, rabphilin-3A, were shown to mediate axonal transport of SPTV (Li et al., 1995; Li, 1996). Rab3A appears to mediate transport of synaptophysin- and synapsin-1-containing SPTVs in both cell bodies and axonal terminals of motor neurons (Li et al., 1995). Rabphilin-3A appears to be involved in SPTV transport at the proximal segments of axons but not at the distal segments in spinal nerves (Li, 1996). DENN (MADD), an Rab3 GTP–GDP exchange factor, interacts directly with the stalk of KIF1A or KIF1Bβ and regulates the function of KIF1s in SVP transport in hippocampal neurons (Niwa et al., 2008). Overexpression of the dominant-negative domain or knockdown of DENN inhibits transport of Rab3-containing SPTVs to the terminals. KIF1-bound DENN (MADD) binds via its N-terminal domain to SPTV-associated Rab3-GTP more strongly than free Rab3-GDP, and forms a KIF1–DENN–Rab3–GTP complex on SPTVs which enhances axonal SVP transport. This suggests that GDP/GTP exchange on Rab3 may regulate KIF1A-mediated SVP transport. PIP_2 is also involved in SV transport as noted in the previous section. PIP_2 is required for binding of Unc-104 (KIF1A: kinesin-3) to SVs for axonal SV transport along microtubules of *C. elegans* (Klopfenstein et al., 2002; Klopfenstein and Vale, 2004; Kumar et al., 2010). The PH domain of Unc-104 directly binds to PIP_2 on the SV membrane. Loss of binding of Unc-104 to PIP_2 not only blocks axonal SV transport but also causes ubiquitin-mediated degradation of Unc-104 (Kumar et al., 2010).

Fast axonal transport of VAChT-containing SPTVs has been observed in motor, sensory, and autonomic axons of the peripheral nervous system (Li et al., 1998). Using the method "stop-flow/nerve crush," the authors demonstrated that small SPTVs containing VAChT, SV2, and synaptophysin are present in motor axons and in autonomic postganglionic neurons. In cholinergic SN56 cells, VAChT appears to first travel to the PM in the soma, recycled to early/recycling endosomes, and finally departs to the axon for function at the terminal (Santos et al., 2001). A VAChT mutant inept for

endocytosis is retained in the PM of the cell body and is never routed to early/recycling endosomes and SPTVs in the neurites. Synaptophysin is carried in SPTVs to neurite terminals of sympathetic neurons but was shown to use an alternative route to the terminals through small DCVs that contain VMATs and dopamine β-hydroxylase (Bauerfeind et al., 1995). However, small DCVs do not appear to be the major vehicle for delivery of synaptophysin to the nerve terminals of sympathetic neurons. During exit from the TGN for axonal transport, synaptophysin guides sorting of VAMP2, but not VAMP1 and syntaxin-1, to SPTVs in hippocampal neurons (Pennuto et al., 2003). The cytoplasmic domains of VAMP2 and synaptophysin form heterodimers, which promote the sorting of VAMP2 to SPTVs. Since VAChT exists with synaptophysin in the same vesicles in cholinergic neurons and chromaffin cells (Varoqui and Erickson, 1998; Park et al., 2011), it is likely that synaptophysin and VAChT travel together in the same SPTV during axonal transport.

Axonal transport of SPTVs is decreased during the pathogenic processes of neurodegenerative diseases, such as Alzheimer's disease (AD). c-Jun N-terminal kinase (JNK), a signaling protein activated by neuroinflammation, appears to play a major role in inhibition of axonal SPTV transport (Stagi et al., 2005, 2006). It is postulated that tumour necrosis factor (TNF) activates JNK and increases the levels of phospho-JNK along the neurites of hippocampal neurons (Stagi et al., 2006). Activated JNK, in turn, phosphorylates and dissociates KIF5B, a heavy chain of kinesin-1, from microtubules, resulting in inhibition of kinesin-1-mediated axonal transport of SPTV containing synaptophysin and mitochondria in cultured primary neurons. Pretreatment of hippocampal neurons with a JNK inhibitor, SP600125, restores binding of KIF5B to microtubules and consequently axonal transport of SPTVs. NO that is generated excessively by activated microglia also activates JNK and inhibits axonal transport of synaptophysin-containing vesicles in primary neurons (Stagi et al., 2005). In addition, loss of presenilin-1 (PS1), which is implicated in AD neuropathology, interferes with kinesin-1-mediated axonal transport of synaptophysin-containing SPTVs (Pigino et al., 2003). Loss of PS1 in mice ($PS1^{-/-}$) generates excessive amounts of active GSK3β that over-phosphorylates the kinesin light chain of kinesin-1, resulting in continuous dissociation of kinesin-1 from SPTVs and inhibition of axonal SPTV transport.

In addition to microtubule-based motors, myosin Va also plays a role as transporter of SPTVs and SVs through the actin cortex at the axonal terminal. At nerve endings, under resting conditions, myosin Va associates

with SVs via the direct interaction of its tail domain with SV-localized synaptobrevin-2 and synaptophysin, and this interaction immobilizes SVs (Prekeris and Terrian, 1997). However, upon Ca^{2+} influx due to membrane depolarization, myosin Va dissociates from SVs, allowing free movement of SVs toward the PM for tethering and docking. The GTP-bound form of Rab3A directly binds both the tail domain of myosin Va and rabphilin-3A. This was shown in extracts of giant squid axoplasm as well as mouse frontal cortex (Wollert et al., 2011). Rab-GDI inhibits myosin Va-mediated SV movement while calcium/calmodulin-dependent kinase II (CaMKII) enhances the binding of Rab3A to myosin Va, and myosin Va-mediated SV movement. Thus, Rab3A may regulate myosin Va-mediated transport of SV and likely SVP/SPTV as well through the actin cortex at the active zone in a GTP/CaMKII-dependent manner.

8. VESICLE TETHERING AND DOCKING FOR EXOCYTOSIS

Since priming and exocytosis of DCV and SV are well reviewed elsewhere (Sorensen, 2005; Stojilkovic, 2005; Rizo et al., 2006; Malsam et al., 2008; Parpura and Mohideen, 2008; Martin, 2012), we will not discuss SNARE-mediated priming and exocytosis. Instead, we will review recent findings of molecular mechanisms involved in docking and tethering of DCVs and SVs.

8.1. Exocytosis of DCVs

During DCV exocytosis, the optimum levels of PIP_2 should be maintained for proper exocytosis (Milosevic et al., 2005). Overexpression of the phosphatase synj-1 in chromaffin cells decreases the levels of PIP_2 in the PM, reduces the readily releasable pool of DCVs, and inhibits regulated secretion of catecholamines, whereas increased PIP_2 enhances the priming of DCVs and DCV exocytosis.

Many of the resident proteins of the actin matrix involved in vesicular trafficking and localized on membranes of vesicles are also important for the docking and exocytosis process. In DCVs, Rab3A and Rab27 mediate tethering and docking of DCVs at the PM of neurons and endocrine cells (Darchen et al., 1995; Fukuda, 2008; Tsuboi, 2009). Rab27A, however, plays a dominant role in DCV exocytosis and is required, for example for glucose-induced insulin secretion in pancreatic β-cells (Aizawa and Komatsu, 2005; Kasai et al., 2005). The decreased

docking of insulin DCVs in Rab27A KO mice was attributed to a reduction in the interaction between granuphilin on DCVs and syntaxin-1a on the PM.

Granuphilins are specifically expressed in pancreatic β-cells, chromaffin cells, and the pituitary gland, but not in pancreatic α cells and brain, and appears to negatively regulate exocytosis (Wang et al., 1999; Fukuda et al., 2002; Yi et al., 2002; Fukuda, 2003; Kasai et al., 2008). Granuphilin-a may be specific for DCV and not SLMV exocytosis because it was found only in DCVs in pancreatic β-cells (Wang et al., 1999). Knockout of granuphilin increases both spontaneous and stimulated fusion of DCVs to the PM, which decreases the number of DCVs proximal to the PM. The inhibitory effect of granuphilin is also lost when binding of granuphilin to Rab27A or syntaxin-1 is blocked. These data and others (Brzezinska et al., 2008; Kariya et al., 2010) strongly suggest that loss of Rab27s or their effectors decreases the docking/fusion of DCVs to the PM.

In PC12 cells, rabphilin and Noc2, like granuphilin, are also recruited to DCVs via their interactions with Rab27A (Fukuda et al., 2004) and play a role in DCV docking. In one study, overexpression of rabphilin inhibits high KCl-dependent NPY secretion, but another shows that overexpression of WT rabphilin significantly increases DCV docking without affecting the exocytosis kinetics (Tsuboi, 2009). Hence, it is unclear whether rabphilin is a negative or a positive regulator of DCV exocytosis. In PC12 cells, Noc2 negatively regulates Rab3A-mediated DCV exocytosis (Tsuboi, 2009). Moreover, overexpression of Noc2, but not Noc2 mutants unable to bind Rab3a, inhibited Ca^{2+}-triggered exocytosis (Haynes et al., 2001). In addition to Rab3A and Rab27, there are other Rabs, such as Rab11B and Rab18, that contribute to the mechanisms of regulated DCV exocytosis (for more on these Rabs, see Khvotchev et al., 2003; Vazquez-Martinez et al., 2007).

CAPS, the Ca^{2+}-dependent activator protein for secretion, was shown in *C. elegans* to specifically dock DCVs (Hammarlund et al., 2008). It contains a dynactin-1 binding domain and a DCV binding domain which promote formation of the open form of syntaxin, important for tethering, docking and priming of DCVs (Hammarlund et al., 2008; Lin et al., 2010). In granule neurons of developing cerebellum, CAPS2 was found to be associated with DCVs containing NT-3, BDNF, and CgB (Sadakata et al., 2004), and shown to play a role in increasing activity-dependent secretion of these neurotrophins in granule neurons and PC12 cells (Wassenberg and Martin, 2002). This suggests that CAPS mediates the activity-dependent

secretion of neurotrophins for survival and differentiation of cerebellar granule neurons.

8.2. Exocytosis of SVs

Like DCVs, various Rab proteins are involved in tethering and docking of SVs to the presynaptic terminals. SV recycling and exocytosis is associated with 11 different Rab proteins, but Rab3a and Rab27b have emerged as the two major Rabs involved in Ca^{2+}-induced neurotransmitter release in the nervous system (Takai et al., 1996; Geppert et al., 1997; Mahoney et al., 2006; Pavlos and Jahn, 2011). Both Rab3A and Rab27B tightly associate with SVs under resting conditions (Yu et al., 2008; Pavlos and Jahn, 2011). This association is differentially regulated during Ca^{2+}-induced exocytosis: Rab3A is dissociated from SVs while Rab27B maintains its tight association (Pavlos et al., 2010). After Ca^{2+} influx, Rab-GDI easily retrieves dissociated Rab3A but not Rab27B from SVs, suggesting that only Rab3A is regulated by the GTP/GDP switch during Ca^{2+}-induced SV exocytosis.

In Rab3A KO mice, SV docking and exocytosis is impaired but not blocked. The readily releasable SV pool was intact but the rate of Ca^{2+}-induced fast phase SV fusion was enhanced (Geppert et al., 1997). After depolarization, however, Rab3A KOs had fewer docked SVs and delayed replenishment of these docked SVs compared to normal terminals. This is consistent with the phenotype of Rab3a KOs which display defective short- and long-term synaptic plasticity in the hippocampus, altered circadian motor activity with more active exploratory behavior (D'Adamo et al., 2004).

The major role of CAPS is in DCV docking/priming to the PM but they also function SV exocytosis. CAPS-1 and CAPS-2 are required for fast phasic transmitter release by enhancing vesicle priming (Jockusch et al., 2007). CAPS-deficient neurons have very few SVs competent for fusion, resulting in defects in fast phasic synaptic release, suggesting that CAPS is required for the makeup of readily releasable pools of SVs.

9. NEURONAL DISORDERS INVOLVING SV DEFECTS

9.1. Schizophrenia

Neuropeptide and neurotransmitter dysregulation is widely implicated in pathogenesis of schizophrenia (SCZ). Postmortem brains from schizophrenic patients had decreased levels of somatostatin, NPY, vasointestinal

peptide (VIP) and cholecystokinin (CCK) (Gabriel et al., 1996). These results were confirmed for somatostatin, NPY and CCK by microarray analysis studies, which also showed a significant reduction in transcripts of neuropeptides associated with GABA-ergic neurons and GABA metabolism (Hashimoto et al., 2008). These data indicate a role for impaired GABA synthesis and signaling, as well as neuropeptides, in patients with SCZ.

When the SV marker synaptophysin is studied, results are in conflict with the idea of a global presynaptic reduction of proteins, although VAMP was proposed to be downregulated in SCZ brains (Halim et al., 2003). DCV markers CgA, CgB and SgII, however, were shown to be altered in regionally specific ways in SCZ brain (Willis et al., 2011). Deficiency of these proteins known to be involved in DCV biogenesis (see Section 2.5.2) might cause impairment of packaging of neuropeptides and BDNF, resulting in feedback inhibition and downregulation of synthesis of these molecules. As previously discussed, BDNF is a key regulator of synaptic plasticity and therefore, cognitive function, and thus is proposed in the pathogenesis of SCZ. BDNF mRNA and protein are downregulated in the dorsolateral prefrontal cortex of schizophrenic patients, indicating that intrinsic and target neurons of the cortex may receive dampened trophic support provided by BDNF (Weickert et al., 2003; Wong et al., 2010). It remains to be investigated whether transcriptional control of BDNF genes and stabilization of its mRNA and protein at the TGN can influence packaging and availability at the synapse.

9.2. Alzheimer's Disease

From a histopathological point of view, vesicular deficiencies have been a hallmark in AD and it is precisely the lack of synaptic function that manifests in affected individuals (Shankar and Walsh, 2009; Waites and Garner, 2011; Willis et al., 2011). Cellular deficiencies include defective sorting and processing of amyloid precursor protein (APP) at the TGN and in endosomes, as well as defects in formation of vesicles (Small and Gandy, 2006). Lipid rafts are involved in aggregation of proteins at the TGN, in vesicles and at the PM. Via their aggregative function, these lipid rafts mediate the segration of γ-secretase and its substrate, the C-terminal fragment (CTF) of APP, for cleavage in the TGN. Depletion of cholesterol, a major constituent of lipid rafts, disrupts the proximity of association of γ-secretase and CTF, and induces non-plaque-forming alpha-secretase activity (Vetrivel and Thinakaran, 2010). Granins such as CgA, CgB, and SgII are extensively co-localized with amyloid-β plaques in the hippocampus

of mouse models of Alzheimer's and in human postmortem AD brain, suggesting a potential role of these granins in AD pathology. (Willis et al., 2011). CgA activates microglial stress cascades of neurotoxicity, an effect amplified when combined with Aβ (Twig et al., 2005; Hooper and Pocock, 2007; Hooper et al., 2009), and may be an avenue of intervention in AD patients to reduce neurotoxicity. A precise role for the granins in vesicular defects of Alzheimer's is yet to be revealed, however, recent analysis of granin peptides (CgA, CgB, SgII), compared to APP and Aβ in the cerebrospinal fluid supports the proposition that APP and Aβ may be primarily secreted through a RSP, with enzymes separate from BACE1 within the constitutive pathway (Mattsson et al., 2010). If validated, this idea will be consistent with the findings that APP was found at neuronal terminals in vesicular organelles not associated with SV markers (Ikin et al., 1996), and soluble APP undergoes stimulated release in neuroendocrine bovine chromaffin cells in response to cholinergic stimulation (Efthimiopoulos et al., 1996).

APP is processed by several proteases: α-secretase to generate non-amyloidogenic peptides, β-secretase to generate amyloidogenic competent peptides, followed by γ-secretase to cleave α-CTF or βCTF, respectively (Vetrivel and Thinakaran, 2006; Marks and Berg, 2010). It is the production and secretion of Aβ within the amyloidogenic pathway to form amyloid plaques that is detrimental to neuronal function in disease states. The next paragraphs will primarily address recent work addressing molecules functioning at the TGN.

In terms of vesicular budding and sorting at the Golgi, there are many Alzheimer protein-specific defects reported. A marked reduction in PLD activity resulting in dysregulated membrane phospholipid metabolism is a key feature in AD (Kanfer et al., 1986), and downregulation of PLD1 is associated with increased beta-amyloid plaque formation. Presenelin 1 (PS1), mutations in which causes inherited familial AD, is one of four members of the γ-secretase complex that regulates processing of β-amyloid precursor protein (βAPP) to generate Aβ42, the plaque-forming protein in AD (Citron et al., 1997; Chau et al., 2012). By binding to PLD1, PS1 recruits it to the Golgi/TGN as a negative regulator of Aβ formation. Overexpression of PLD1 decreased the association of γ-secretase components whereas down-regulation of PLD1 by short hairpin RNA (shRNA) increased the association of γ-secretase components as well as its proteolytic activity (Cai et al., 2006). Furthermore, PLD1 itself can increase vesicular budding and trafficking of βAPP, even while it reduced Aβ generation by inhibiting cleavage of βAPP at the TGN (Cai et al., 2006).

AP-4 is the non-clathrin-associated AP that mediates sorting of transmembrane cargo in post-Golgi compartments. Bonifacino's group recently showed that the μ4 subunit of AP-4 interacts with APP, and mediates trafficking of APP to endosomes (Burgos et al., 2010). Disruption of this interaction reduces localization to the endosome and increases γ-secretase activity to produce the pathogenic Aβ peptide (Burgos et al., 2010). Therefore, AP-4 can be said to be protective against Aβ production by targeting APP away from γ-secretase activity.

Estrogen's neuroprotective effects and effectiveness as reducing β-amyloid plaques in AD are attributed to its stimulation of vesicular budding of APP-containing vesicles from the TGN and into post-Golgi compartments. 17β-estradiol recruits the small GTPase, Rab11, to the TGN from the cytosol and can induce the budding of APP-containing vesicles from the TGN by influencing the redistribution of PI composition at the TGN membrane, and into post-Golgi vesicles. As discussed previously, changes to the lipid concentration in the TGN can induce increased membrane curvature so as to promote budding from the TGN (Huttner, 2000; Gondre-Lewis, 2006). Thus, PI was detected in post-Golgi compartments after treatment with estrogen.

The mechanisms discussed above provide a foundation to develop therapeutic strategies that manipulate lipid raft components, budding and processing at the Golgi and endosomes to ameliorate the severity of the disorder by decreasing Aβ formation. One strategy is to increase APP-containing vesicles. If less APP is available in the TGN to be later converted to Aβ, then the secretion of Aβ and plaque formation will be reduced, which could be neuroprotective.

9.3. Fragile X Syndrome

Fragile X is an x-linked inherited intellectual disorder caused by the silencing of the *fmr1* gene due to the hypermethylation of its promoter (Oberle et al., 1991). The resulting absence of fmr1 protein reduces synaptic plasticity by reducing long term potentiation of synaptic inputs, a condition that could be restored with exogenous BDNF although basal BDNF and pro-BDNF levels are comparable to control (Lauterborn et al., 2007). Based on later studies addressing regulated secretory release of neuropeptide and neuromodulators, it seems that the cargo packaged in DCVs, possibly including BDNF, does not undergo effective regulated secretion. The GTPase Rab3A involved in vesicle docking and fusion is significantly

downregulated in cerebral cortex of *fmr1*$^{-/-}$ mice, which exhibited severely impaired—no peaks by MALDI-MS—neuropeptide release even in the absence of significant differences in DCV density (Annangudi et al., 2010). GABA-ergic receptor- and somatostatin-expressing neurons and the size of pancreatic islets were significantly decreased in the *fmr1* KO mice which had higher plasma glucose levels, indicative of a more widespread effect on neuroendocrine function (El Idrissi et al., 2012). These effects on endocrine function, especially the stress response, are exacerbated in patients with both autism spectrum disorder and Fragile X (Roberts et al., 2009). Although a mental retardation disorder, Fragile X patients experience neuroendocrine symptoms such as abnormal stress responses, sleep abnormalities, and growth patterns, but the number of studies addressing the biology of these aberrations are few (Hessl et al., 2004; El Idrissi et al., 2012). Further studies in the biogenesis, morphology and secretory mechanisms in *fmr1* KO may reveal a significant role for the gene product in SV and DCV biology, although currently there is no evidence for such a role for fmr1.

9.4. Smith–Lemli–Opitz Syndrome

Smith–Lemli–Opitz syndrome is an autosomal recessive genetic disorder caused by a mutation in the gene encoding 7-dehydrocholesterol reductase (7-DHC), an enzyme necessary for the conversion of 7-dehydrocholesterol to cholesterol during cholesterol biosynthesis (Porter, 2008). SLOS is one of many congenital malformations, inclusive of lathosterolosis (Brunetti-Pierri et al., 2002), where patients make insufficient enzymes to catalyze the synthesis of the final product, cholesterol. Cholesterol plays an important role as precursor to steroids and secreted hormones in the adrenal medulla and gonads. When the proper synthesis of these hormones is impaired, packaging of cargo into secretory vesicles is reduced, and this in turn attenuatess the formation of DCVs. In addition, cholesterol is an important and integral component of membranes. In SLOS mice, there is a defect in the ability of DCVs to form properly (Gondré-Lewis et al., 2006). We have shown using artificial membranes that cholesterol is required for the rigidity of the membrane, a feature that cannot be duplicated by 7-DHC or lathosterol substitution. Consistently, a prominent feature of cholesterol-deficient mice was the decreased number or total absence of secretory vesicles in the anterior pituitary, adrenal gland, and endocrine and exocrine pancreas. DCVs in mice lacking cholesterol had abnormally large, immature, unpinched vesicles with no dense core. Cells from these mice were

functionally impaired in their ability to respond to stimulation via the RSP; however, this function could be restored when cholesterol was supplied to the cells (Gondre-Lewis et al., 2006).

10. CONCLUSIONS AND FUTURE DIRECTIONS

The works reviewed here highlight the dynamics of SV and DCV biology which allow the brain and professional secretory cells of the endocrine and exocrine systems to respond to physiological stimuli. Regardless of the tissue type, there is a shared mechanism for SV or DCV targeting to the PM. The content of DCVs are sorted to and concentrated in specific regions of the TGN by the actions of granins, CPE, and V-ATPases. DCV budding from the TGN is dependent on cholesterol, lipid rafts, PAs and/or DAG, as well as a number of kinases, GTPases and APs. Maturation of DCVs is facilitated by acidification by V-ATPase and condensation of contents by actions of water channels, the AQPs. DCVs are trafficked along microtubule motors, dynactin/dynein and the KIF proteins, and on actin motor, myosin V. When at the periphery, they release their hormone, neuropeptide, or enzyme cargo and the DCV membrane components are recycled.

Recently, we have identified a post-Golgi sorting compartment which carries membrane and soluble proteins associated with both DCVs and SPTVs, implying a shared machinery to deliver synaptic and peripheral proteins (Park et al., 2011). Studies in the future will provide more clarity on the sorting mechanisms of SPTV components at the TGN as compared to DCV components, and the extent of convergence and divergence of the pathways.

For the multitude of neuropeptides and hormones with biological action packaged within DCVs, understanding of the mechanisms of genetic, transcriptional, posttranscriptional, translational, and posttranslational regulation of DCV biogenesis is steadily emerging. Our group and others have begun to elucidate some steps where potential key regulators such as REST/NRSF at the genetic level in neurons and astrocytes and IA-2 at the transcriptional level in insulin-secreting β-cells are involved. Many DCV proteins have been identified as possessing a PTB-binding site, and thus PTB may function as a master posttranscriptional stabilizer of DCV-associated mRNA to increase translation. The CgA-derived peptide, serpinin, through PN-1 gene regulation, can increase availability of DCV proteins at the TGN

by controlling protein degradation and hence promote DCV biogenesis. Although these findings are important in understanding some mechanism of DCV biogenesis, there is great paucity of information regarding genetic control of this process.

SVs follow a somewhat similar path as DCVs in that a mature, filled SV has components from SVPs or SPTVs which were formed at the TGN and trafficked to the periphery with the aid of KIF1A and other KIF family of motors. The lipid PIP_2, along with cholesterol and lipid rafts, is critical for SVs to bud and pinch from the PM. These recycled SVs then interact with SPTVs and acquire components important for responding to subsequent stimuli. Unlike for DCVs, an abundance of research regarding the mechanisms involved in SV biogenesis has been conducted. Still, issues involving *how* SV-specific proteins and lipids are sorted to SVs need further research. There is also very little known about SVPs and transport vesicles, and the nature of their interaction with SVs.

We have discussed a few neurological disorders with impairment of vesicular budding and/or trafficking. In some cases, the defective, mutated proteins and lipids responsible for the various pathologies give insight into the complexity of the interplay required for vesicular biogenesis, trafficking, docking, and release. These disorders may expand the repertoire of molecules believed to be involved in vesicular function and, together with this expanding field in the future, may provide avenues of intervention for amelioration of disease.

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