Multivesicular Bodies in Neurons: Distribution, Protein Content, and Trafficking Functions

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Summary

Multivesicular bodies (MVBs) are intracellular endosomal organelles characterized by multiple internal vesicles that are enclosed within a single outer membrane. MVBs were initially regarded as purely prelysosomal structures along the degradative endosomal pathway of internalized proteins. MVBs are now known to be involved in numerous endocytic and trafficking functions, including protein sorting, recycling, transport, storage, and release. This review of neuronal MVBs summarizes their research history, morphology, distribution, accumulation of cargo and constitutive proteins, transport, and theories of functions of MVBs in neurons and glia. Due to their complex morphologies, neurons have expanded trafficking and signaling needs, beyond those of “geometrically simpler” cells, but it is not known whether neuronal MVBs perform additional transport and signaling functions. This review examines the concept of compartment-specific MVB functions in endosomal protein trafficking and signaling within synapses, axons, dendrites and cell bodies. We critically evaluate reports of the accumulation of neuronal MVBs based on evidence of stress-induced MVB formation. Furthermore, we discuss potential functions of neuronal and glial MVBs in development, in dystrophic neuritic syndromes, injury, disease, and aging. MVBs may play a role in Alzheimer’s, Huntington’s, and Niemann-Pick diseases, some types of frontotemporal dementia, prion and virus trafficking, as well as in adaptive responses of neurons to trauma and toxin or drug exposure. Functions of MVBs in neurons have been much neglected, and major gaps in knowledge currently exist. Developing truly MVB-specific markers would help to elucidate the roles of neuronal MVBs in intra- and intercellular signaling of normal and diseased neurons.

Keywords
Endosome; Trafficking; Axonal Transport; Sorting; Release; Endocytosis; Degradation

1. Definition of MVBs and scope of this review

Based on their original discovery and historical tradition, MVBs are defined – as their name implies – by morphological criteria at the ultrastructural level. MVBs appear in ultrathin sections as spherical organelles characterized by a single outer (“limiting”) membrane that
encloses a variable number of small spherical or ellipsoidal vesicles within a matrix. The entire MVB typically has a diameter of 250–1,000 nm. Most MVBs have a round or slightly oval shape (Fig. 1A-E), although tubular forms or extensions also exist. MVBs contain at least two and up to dozens of smaller vesicles within the membrane-enclosed lumen (an average of 24 internal vesicles/MVB. Cooney et al., 2002). The small vesicles as well as the matrix, the lumenal space between the internal vesicles, are typically clear, but they can be of varying electron densities (Roizin et al., 1967). The internal vesicles can have heterogeneous sizes with a diameter of 50–100 nm, although they frequently are uniform in size, about 60–70 nm in diameter. The outer (limiting) membrane is of a similar thickness as the membrane of the internal vesicles and the plasma membrane, with a thickness of about 5.2 nm (Yamamoto, 1963; but see Roizin et al., 1967). MVBs can be surrounded by and be connected to tubular compartments (Cooney et al., 2002). Associations with Golgi stacks, endoplasmic reticulum and secretory vesicles have also been described (Palay, 1960; Peters et al., 1991; Roizin et al., 1967). MVBs move within cellular compartments along microtubules (Gruenberg and Stenmark, 2004; Saito et al., 1997).

MVBs have been described in neurons of numerous animal species and classes, including vertebrates and invertebrates (Roizin et al., 1967). In neurons, MVBs are most commonly located in cell bodies and dendrites. MVBs are occasionally observed in normal axons or axon terminals in vivo (Castel et al., 1992; Delcroix et al., 2003; Kapeller and Mayor, 1969). When they occur in PNS axons, they are more frequent in the axoplasm at nodes of Ranvier than at internodal segments (Altick et al., 2009; Berthold and Rydmark, 1995). With appropriate ultrastructural preservation, the distinct features of MVBs are relatively easy to recognize. Occasionally, MVBs can be mistaken for dense endosomes (when their matrix and vesicles are relatively dense), for phagocytotic vacuoles (Roizin et al., 1967), residual bodies (Roizin et al., 1967; Schmied and Holtzman, 1987), dense lamellar bodies (Smith, 1980), multilamellar, cup-shaped or other endosomes and cisternae (Birks et al., 1972; Holtzman et al., 1973; Weldon, 1975), elongated, membrane-limited profiles (Claude et al., 1982b), or for large vesicles (if there are less than two vesicles within the lumen). Adequate ultrastructural morphology is necessary to distinguish these different types of organelles, and serial thin sections may be required for definitive conclusions (LaVail et al., 1980). Several distinct morphological types of MVBs have been quantified in axons (Altick et al., 2009; LaVail et al., 1980), but not in other neuronal compartments.

Cellular, perikaryal MVBs can stain positive for acid phosphatases and hydrolases (Gatzinsky et al., 1988; Holtzman and Novikoff, 1965; Holtzman et al., 1973; Paula-Barbosa et al., 1978; Roizin et al., 1967; Schmied and Holtzman, 1987). MVBs are thought to function primarily as late or prelysosomal endosomes with sorting functions for internalized transmembrane receptors and their ligands (Katzmann et al., 2002; Mukherjee et al., 1997; Myromslien et al., 2006; Reggiori and Pelham, 2001). Some investigators define MVBs to comprise only one particular subset of late endosomes (Mukherjee et al., 1997) or consider them to be a shuttle/carrier between early and late endosomes in the degradation pathway towards lysosomes (Hurley, 2008; Piper and Katzmann, 2007; Piper and Luzio, 2001; Russell et al., 2006). Other investigators use a broader definition of MVBs and believe that all endosomes along the degradation pathway contain multivesicular elements (Gruenberg and Stenmark, 2004), or recognize two distinct types of MVBs, one for the degradative pathway and one for the recycling/exocytic pathway (Mathivanan et al., 2010). At a functional level, some studies have distinguished the endosomal carrier vesicle, defined as the transport intermediate between early and late endosomes, from MVBs or multivesicular endosomes (Aniento and Gruenberg, 1995). Obviously, investigators apply the term “multivesicular body” to a more or less restricted set of functionally or structurally defined endosomes. On one extreme, use of non-specific MVB markers may cast the net too wide and lead to an unwarranted broad inclusion of endosomes, while a very narrow
functional interpretation may deny MVB status where structural evidence and historical
definition would suggest otherwise, thus leading to ambiguity in the field. In this review, we
consider primarily work that includes ultrastructural evidence to support conclusions derived
in conjunction with biochemistry, physiology, and imaging at the light microscopical level.

Excellent recent reviews are available about the molecular composition of MVBs in non-neuronal cells (Gruenberg, 2001; Gruenberg and Stenmark, 2004; Katzmann et al., 2002;
Murk et al., 2002; Piper and Katzmann, 2007; Piper and Luzio, 2001; Raiborg et al., 2003;
Sorkin and von Zastrow, 2002). The goal of our review is to compile and organize
information available about the morphology, distribution, and potential functions of MVBs
specifically in neurons and glia. Neuronal MVBs appear to be highly heterogeneous with
distinct functional subtypes both in the somatodendritic compartment (Saito et al., 1997) as
well as the axon (Altick et al., 2009). Some molecules such as early endosome antigen 1
(EEA1) and lysobisphosphatidic acid (LBPA) have been suggested to have specific
associations with MVBs, but these markers turned out to be stage-dependent for temporally-
defined classes of endosomes, i.e. early and late endosomes, respectively (White et al.,
2006), rather than for MVBs. Accordingly, these markers are considered ambiguous for
MVB identification, because they label subsets of endosomes at different stages of
maturation (Maxfield and McGraw, 2004). Compiling information about neuronal MVBs
requires considerable effort, because information about these MVBs is currently scattered
among various specialty journals. The lack of a comprehensive review of this topic has led
to numerous omissions and misconceptions. For example, the association of MVBs with
postsynaptic sites has been discovered and re-discovered independently on multiple
occasions in the past half century (Cooney et al., 2002; Pappas and Purpura, 1961; Paula-
Barbosa et al., 1978; Rind et al., 2005; Schnapp, 1997; von Bartheld et al., 1996). Some
conflicting views about the distribution and functions of neuronal MVBs may be based on a
lack of awareness of relevant studies. This review is designed to provide a synopsis of
previous work, to facilitate access to pertinent original reports, to reveal gaps in current
knowledge, and to discuss controversies and recent progress.

2. History of major concepts

The MVB organelle was discovered, first pictured, and briefly described in neurons by Palay
and Palade (1955) in their paper entitled The Fine Structure of Neurons: “There appears a
particular vesicular formation limited by a single membrane and filled with small, circular
profiles which may represent membrane invaginations.” This discovery was erroneously
attributed in recent reviews to a different article that was authored by Palade in 1955 (see
Katzmann et al., 2002; Piper and Katzmann, 2007; Vajjhala et al., 2007; Winter and Hauser,
2006). Soon after Palay and Palade’s 1955 paper, Roberto Sotelo and his colleagues
introduced the name “multivesicular body” for these organelles (Estable et al., 1957; Sotelo
and Porter, 1959). Although MVBs were first identified in neurons, the majority of
subsequent work on MVBs has focused on non-neuronal cells, while neuronal MVBs have
been largely neglected. A chronology of major insights regarding MVB functions,
bio genesis, protein transport, and their molecular characterization, with emphasis on
neurons, is listed in Table 1.

In the earliest studies on neuronal MVBs, these organelles appeared to be associated
primarily with Golgi stacks and were thought to be involved in neurosecretion rather than
endocytosis (Palay, 1960). This view changed with the observation that tracers such as
ferritin, horseradish peroxidase (HRP), and thorium dioxide accumulated in MVBs (Birks
et al., 1972; Holtzman and Peterson, 1969; Rosenbluth and Wissig, 1964; Weldon, 1975), and
a primary role in the endocytic-degradative pathway was recognized (Holtzman, 1971;
Kristensson and Olsson, 1971). Consistent with this notion was the finding of acid

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phosphatase and hydrolase activity in at least some MVBs (Gatzinsky and Berthold, 1990; Gatzinsky et al., 1988; Holtzman, 1971; Holtzman and Novikoff, 1965; Overly et al., 1995; Paula-Barbosa et al., 1978; Roizin et al., 1967; Saito, 1983).

In the 1970s and 1980s, it was realized that MVBs accumulate many internalized macromolecules, apparently en route to lysosomal degradation. In neurons, internalized molecules that accumulate in MVBs include endogenous proteins such as nerve growth factor (NGF) (Bernd and Greene, 1983; Claude et al., 1982a; Schwab and Thoenen, 1976), but also exogenous proteins, among them tetanus toxin (Parton et al., 1987; Schwab and Thoenen, 1978) and various neuronal tracers, notably HRP (Berthold and Mellstrom, 1982; Buchner et al., 1987; Bunge, 1977; Bunt and Haschke, 1978; Chan et al., 1980; Chu-Wang and Oppenheim, 1980; Feher, 1984; Kadota et al., 1994; LaVail and LaVail, 1974), and lectins (Broadwell and Balin, 1985; Buchner et al., 1987; Steindler and Cooper, 1986). The HRP studies in particular suggested a role for MVBs in the retrograde axonal transport of macromolecules (Feher, 1984; Grafstein and Forman, 1980), a topic that will be discussed in more detail below (sections 3.5 and 7.2).

MVBs have attracted particular interest because internalized signaling molecules and their receptors accumulate in this organelle. For most of the internalized trophic factors examined, MVBs consistently scored with the highest labeling densities among the subcellular organelles, comparable only with dense endosomes and lysosomes (Butowt and von Bartheld, 2001; Claude et al., 1982a; Claude et al., 1982b; Rind et al., 2005; Schwab and Thoenen, 1977; Schwab et al., 1979; Schwab and Thoenen, 1976; Stieber et al., 1984; von Bartheld et al., 1996).

Several investigators noted an association of MVBs with postsynaptic sites (Fig. 1C-D) (Chicurel and Harris, 1992; Cooney et al., 2002; Pappas and Purpura, 1961; Paula-Barbosa et al., 1978; Rind et al., 2005; Schnapp, 1997; von Bartheld et al., 1996). This association is of interest because it raises the intriguing possibility of a function for MVBs in retrieving, storing and supplying transmembrane receptors for various ligands and transmitters into postsynaptic membranes (see section 4.2). Storage of receptors and their recruitment for membrane insertion has major importance for mechanisms of synaptic plasticity (Cooney et al., 2002; Grampp et al., 2008; Kennedy and Ehlers, 2006), including trafficking of G-protein coupled receptors (Bloch et al., 2003; Eden et al., 2009; Grimes and Miettinen, 2003; Sorkin and von Zastrow, 2002; Woodman, 2009).

Knowledge about the molecular composition and functions of MVBs has advanced primarily in non-neuronal cells, particularly in yeast mutants. However, an important difference between yeast and mammalian cells is that in yeast, proteins targeted to the membranes of the internal vesicles of MVBs are destined for degradation only and are not sorted to recycle back to the limiting MVB membrane or to the cellular membrane (Gruenberg and Stenmark, 2004). Despite a high degree of conservation in the sorting machinery between yeast and mammalian cells (Russell et al., 2006), in order to unravel the full spectrum of sorting mechanisms and signals relevant for multi-cellular organisms and human disease, MVB research cannot solely rely on yeast models. Studies on the trafficking of transferrin, low density lipoprotein (LDL) and epidermal growth factor (EGF) receptors in vertebrate cells proved to be particularly insightful and helped to generate the classic model of the fate of internalized ligand/receptor complexes: The ligand binds to its receptor on the surface membrane; the ligand/receptor complex is internalized; the ligand dissociates from the receptor in the acidic environment of early endosomes; in the endosome the receptor is sorted to either be recycled (considered to be the default and more efficient pathway) or retained to be targeted to the internal vesicles of the late endosome. The soluble ligand remains in the lumen of the endosome and proceeds in the late endosome towards...
degradation in the lysosome (Goldstein et al., 1985; Haigler et al., 1979; Maxfield and McGraw, 2004; McKanna et al., 1979; Mukherjee et al., 1997). This simple model has been refined to account for differences between different types of ligands and receptors among signaling molecules (Maxfield and McGraw, 2004; Mayor and Pagano, 2007; Mukherjee et al., 1997; Russell et al., 2006; Sorkin and von Zastrow, 2002).

Recycling may be a particularly important function in neuronal compartments that can be as far away as one meter or more from the cell body. Among the major advances in MVB research was the realization that MVBs are sites for sorting of proteins between recycling and degradation pathways – and that passage through the MVB is not necessarily a one-way street. There is now considerable evidence for additional sorting functions in MVBs (Eden et al., 2010; Felder et al., 1990; Katzmann et al., 2001; Uchil and Mothes, 2005; Vajjhala et al., 2007; Valdez, et al., 2007; Vanlantingh and Ceresa, 2009). Unifying insights into the function of this organelle are emerging as the molecular composition of MVBs is being defined (Babst, 2005; Gruenberg, 2001; Gruenberg and Stenmark, 2004; Hurley, 2008; Katzmann et al., 2002; Maxfield and McGraw, 2004; Piper and Luzio, 2001; Wollert and Hurley, 2010). A molecularly based account of neuronal MVB function is also important for understanding membrane trafficking in normal as well as diseased or dystrophic conditions, with relevance for neurodegenerative diseases (sections 11.2–11.3).

One of the recent controversies in the field of neuronal MVBs has focused on the question whether MVBs are the main retrograde carrier of trophic signals from axon terminals to the cell body. The endosomal signaling hypothesis postulates that endosomes provide platforms for signaling, after and possibly during axonal transport (Campenot and MacInnis, 2004; Grimes and Miettinen, 2003; Howe and Mobley, 2004; Oppenheim and von Bartheld, 2008; Wu et al., 2009; Zweifel et al., 2005). The identity of these endosomes has been a matter of disagreement: are MVBs involved in retrograde axonal transport of trophic signals and are they possibly the main axonal carrier for target-derived trophic factors (Bronfman et al., 2007; Sandow et al., 2000; Weible and Hendry, 2004)? While there is no doubt that neurotrophic factors accumulate in MVBs in cell bodies, until recently it was uncertain whether the neurotrophic factors reside in MVBs already during axonal transport or whether MVBs are generated from trophic factor-carrying axonal endosomes only after reaching the cell body. According to two recent studies, Cui et al. (2007) and Altick et al. (2009), MVBs in axons are not the retrograde carrier of several major neurotrophic factors, thus indicating that the accumulation of these trophic factors in MVBs within the cell body and dendrites constitutes an event that occurs subsequent to the retrograde axonal transport of the neurotrophic factor in a non-MVB organelle.

Another recent uncertainty concerns potential release mechanisms of MVBs: Do neuronal MVBs release their content via vesicular intermediates with “back-fusion” events (Kleijmeer et al., 2001; Murk et al., 2002; Rind et al., 2005; Uchil and Mothes, 2005), or does the limiting membrane fuse with the plasma membrane and release internal vesicles as exosomes (Faure et al., 2006; Lachenal et al., 2011; Putz et al., 2008; Smalheiser, 2007; Weldon, 1975), or can neurons employ both mechanisms? Modes of release have potentially important disease-relevant implications, for example the release of amyloid-beta 42 and prions from neuronal MVBs in the pathophysiology of Alzheimer’s disease and Prion disease, respectively. These aspects will be discussed with more details in later sections of this review (sections 8.1–8.2; 11.3.2, 11.5).
3. Distribution of MVBs in neuronal compartments

This section describes where MVBs are typically located in neurons and concludes that MVBs are differentially distributed between neuronal compartments.

Neuronal MVBs reside predominantly in cell bodies (Holtzman et al., 1973; Peters et al., 1991; Waxman et al., 1995) (Fig. 1A, B) and dendrites (Chicurel and Harris, 1992; Cooney et al., 2002; Pappas and Purpura, 1961; Rind et al., 2005; Roizin et al., 1967) (Fig. 1C-D). MVBs can be present in PNS and CNS axons and terminals, but, as will be discussed in detail below, they are about 50 times more abundant in the soma or dendrites than in axons. Furthermore, the formation of MVBs in axons may occur largely in response to a wide range of non-physiological (“dystrophic”) stimuli (Altick et al., 2009). This section will first review in detail current knowledge about MVBs in cell bodies, dendrites, and PNS and CNS axons.

3.1. Neuronal cell bodies—The fractional area of MVBs (= the relative volume occupied by MVBs, compared to the total space of the compartment analyzed) in vertebrate neuronal somata is similar in vivo and in vitro and ranges from 0.2% to 1.8% (for references, see Table 2). MVB volume is dynamically regulated and can change with external influences such as pharmacological agents, electrical stimulation and neuronal activity. After treatment with the lysosomal inhibitor, methyamine (10 mM) (Claude et al., 1982b), the frequency of MVBs was normal, but the MVBs were larger and appeared more often “empty.” The volume of MVBs increases in neurosecretory cells when neurohypophysial secretion is stimulated by water deprivation (Theodosis, 1982), when neurons lack prosaposin, a glycoprotein with neurotrophic activities (Chu et al., 2005), or when the membrane trafficking chaperone pincher or the neurotrophin receptor TrkB are overexpressed (Valdez et al., 2005). On the other hand, some other pharmacological agents, notably the lysosomal inhibitor chloroquine, result in a decrease of the fractional area of MVBs (reduced numbers and/or smaller-sized MVBs) (Claude et al., 1982b). The usual location of MVBs has been described as the “cellular cytoplasm within or close to the vicinity of Golgi complex” (Holtzman et al., 1973; Peters et al., 1991; Roizin et al., 1967). MVBs can also be associated with smooth endoplasmic reticulum (Peters et al., 1991; Roizin et al., 1967), vesicles, and tubules (Cooney et al., 2002; Parton et al., 1992). MVBs may also connect with electron-dense membranes or clathrin coats – visible as “plaques” at the ultrastructural level (Peters et al., 1991).

An association or transition of MVBs to secondary lysosomes has been postulated (Gruenberg and Stenmark, 2004; Kaasinen et al., 2008; Morales et al., 1999; Mukherjee et al., 1997; Rink et al., 2005; Roizin et al., 1967; Saxena et al., 2005; Stoorvogel et al., 1991), although this dynamic flux is difficult to verify by morphological techniques (Peters et al., 1991; Roizin et al., 1967) and even more so in the absence of MVB-specific markers. Nevertheless, MVBs in neuronal cell bodies most likely interface between early and late or prelysosomal endosomes, as demonstrated in non-neuronal cells (Hurley, 2008; Katzmann et al., 2002; Mukherjee et al., 1997). This may not be the only function of MVBs, as different subtypes of neuronal MVBs are known to exist (Altick et al., 2009; McCaffrey et al., 2009; Saito et al., 1997).

3.2. Dendrites—MVBs reside in proximal and distal dendrites (Pappas and Purpura, 1961; Rind et al., 2005; Roizin et al., 1967). MVBs have been reported to associate preferentially or specifically with postsynaptic densities on dendrites, including spines and spinules (Chicurel and Harris, 1992; Cooney et al., 2002; Kadota and Kadota, 2002; Pappas and Purpura, 1961; Paula-Barbosa et al., 1978; Rind et al., 2005; Schnapp, 1997; von Bartheld et al., 1996). Four studies have quantified MVBs in dendrites, but only one of them determined
the fractional area (FA) of MVBs (Rind et al., 2005). This study found the dendritic FA of MVBs to be in a similar range as in the neuronal soma (0.2–1.8%, Table 2). The other three studies quantified MVBs in shafts below spines or in spineheads of hippocampal (Chicurel and Harris, 1992; Cooney et al., 2002) and amygdalar dendrites (Ostroff et al., 2010). In the later two studies MVB frequency per dendrite length was 0.1–0.4 MVB per 10 μm or 5–15 MVBs per 10 μm, respectively. Interestingly, MVB frequency increased in dendritic shafts below spines after tetani or mild focal seizures (Kadota and Kadota, 2002; Kraev et al., 2009) and after fear conditioning (Ostroff et al., 2010). Dendritic MVBs can be associated with tubules (Cooney et al., 2002; Parton et al., 1992) and smooth endoplasmic reticulum (Chicurel and Harris, 1992), and MVB-tubule complexes at spine origins have been suggested to be storage, sorting, and recycling centers for membrane-associated proteins, while isolated MVBs (MVBs not associated with tubules) may preferentially carry sorted material to the soma (Cooney et al., 2002). In addition, association of MVBs with electron dense structures (densities or clathrin coats) has been reported, occasionally in neurite growth cones (Birks et al., 1972; Peters et al., 1991; Povlishock, 1976).

MVB formation and/or their preferred association with synapses appear to be influenced by trophic factors obtained from target areas (Fig. 2A-D) (Rind et al., 2005; White et al., 2006). Numerous signaling events depend on endosomal trafficking, as recently reviewed (Sorkin and von Zastrow, 2002, 2009; Wu et al., 2009). It has been shown that dendritic MVBs are transported by a novel kinesin that is restricted to the dendritic compartment (Saito et al., 1997), which is suggestive for a dendrite-specific function of these MVBs. MVBs in dendrites may be organelles en route to the cell body, or they may move from cell body to dendrites, or they may shuttle in both directions (see sections 7.1–7.2, below). It is likely that MVBs carrying cargo into dendrites have different functions than those carrying cargo confined within the soma, since the dendrite-specific kinesin did not co-label with many of the late endosomal or prelysosomal markers (Saito et al., 1997) that can be found in non-neuronal MVBs and are often used for identification of MVBs.

3.3. PNS axons—Seemingly controversial sets of data have been reported describing the distribution of MVBs in PNS axons and terminals. MVBs are rare in most parts of normal PNS axons in vivo (Berthold et al., 1993; Berthold and Rydmark, 1995; Gatzinsky et al., 1988; Hollenbeck, 1993). A recent quantification showed that MVBs are about 50-fold less frequent in PNS axons than in their soma or dendrites (Altick et al., 2009). MVBs have been shown in the presynaptic terminals of axons in sympathetic ganglia after prolonged (1-5 min) electrical stimulation (Kadota and Kadota, 1982). MVBs are consistently found in the distal region of the internodal segment at the node of Ranvier, often in specialized membrane sacs that belong to the “axon-Schwann-cell-network,” a structure that is thought to be a hot spot of membrane and protein exchange and other interactions between axons and their myelinating glia (Berthold et al., 1993; Berthold and Rydmark, 1995; Gatzinsky, 1996; Gatzinsky et al., 1988). The MVBs found at these internodal sites are not localized within the mainstream axoplasm, but rather appear segregated within secluded compartments. Interestingly, this distribution of MVBs is restricted to the PNS domain of the axon; within the CNS pathway of the same myelinated axons, the (rare) MVBs are distributed within the axoplasm without nodal concentration (Gatzinsky et al., 1988). Thus, the accumulation of MVBs at the node of Ranvier in the PNS does not seem to be the consequence of a simple rheologic phenomenon, since similar constrictions and reductions of the effective axonal diameter occur in the CNS portion of the same axons, yet no such accumulation of MVBs occurs there. It has been suggested that these MVBs function to sequester and remove toxic macromolecules that have direct access to PNS terminals and are transported retrogradely, but are degraded before they reach the CNS soma that is protected by the blood-brain-barrier (Gatzinsky, 1996).
3.4. PNS terminals and sensory receptor cells—There are several reports of MVBs in PNS terminals or sensory receptors in vivo, including neuromuscular junctions, mechanoreceptors and free nerve endings, hair cells, photoreceptor cells, and autonomic ganglia. We will comment on each of these categories.

MVBs are rarely seen in normal neuromuscular junctions (Csillik and Knyihar-Csillik, 1980; Lloyd et al., 2002), but the numbers of MVBs are increased in genetically altered, dystrophic, and aging neuromuscular junctions (Boaro et al., 1998; Diaz-Cintra et al., 2004; Fergestad et al., 1999; Jia et al., 1993).

Mechanoreceptors and free nerve endings are, by anatomical criteria, the dendritic (receiving) ends of the bipolar sensory ganglion cells, and thus not directly comparable with “true” efferent axon terminals. However, MVBs in mechanoreceptors are relatively frequent and characteristically located at the base of finger-like protrusions where the sensory cytoplasm of mechanoreceptors and free nerve endings contact the surrounding connective tissue (Andres, 1966; Andres and von During, 1973; Byers, 1984, 1985; Byers and Yeh, 1984). The function of MVBs at this location is unknown, but the fact that the fingerlike protrusions in lamellated receptors are exposed to mechanical deformations and are thought to convert such deformations into axon potentials is consistent with the notion (advanced below) that stress can give rise to MVBs.

MVBs have also been noted among the afferent and efferent innervation of outer hair cells within the mammalian inner ear (Omata and Schatzle, 1980) and in cardiac ganglion terminals (Pauziene and Pauza, 2003). Presently, the function of MVBs at these locations is unknown and largely unexamined. MVBs in terminals may contribute to slow turnover of membrane components of the synaptic terminal (Wittich et al., 1994). Ultrastructural studies consistently report MVBs in shedding photoreceptor cells (Calderon et al., 2002; Calman and Chamberlain, 1982; Williams and Blest, 1980; Schmied and Holtzman, 1987). MVB numbers can fluctuate in parallel with circadian shedding and synthesis of the photoreceptor membrane (Williams and Blest, 1980; Calderon et al., 2002; Chamberlain and Barlow, 1984; LaVail, 1976). MVBs are present in the inner segment of photoreceptor cells and seem to have a role in the endocytic pathway, based on work showing MVBs harboring the tracers HRP and gold-labeled interphotoreceptor-retinoid-binding protein, apparently en route to degradation (Hollyfield and Rayborn, 1987; Hollyfield et al., 1985). Moreover, MVBs have been noted in photoreceptor cells of diseased retinas, although their significance has not been explored (Matthes and LaVail, 1989; Montiani-Ferreira et al., 2005). Similar to retinal photoreceptors, light-sensitive pinealocytes contain MVBs, which vary in number with age (Lewczuk et al., 2004; Vigh and Vigh-Teichmann, 1993). The role of MVBs in these cell types remains to be determined.

Besides a physiological function of MVBs in PNS terminals, it needs to be considered that MVBs may be generated artificially, by dystrophic conditions. In some studies (Chu-Wang and Oppenheim, 1980; Omata and Schatzle, 1980; Pauziene and Pauza, 2003), the tissues were not immediately fixed. Delays of up to 8 hours (and lack of oxygen during this delay) could have rendered the terminals ischemic or dystrophic. As noted above, axons in dystrophic conditions have an increased number of MVBs, presumably in response to such insults (Altick et al., 2009; Schroer et al., 1992), in addition to an increase in the number of other organelles, such as autophagosomes and mitochondria (Dixon, 1967; Einheber et al., 2006; Korthals et al., 1978; Marty and Peschanski, 1994; Matthews and Raisman, 1972; Wang et al., 2006). In support of this notion, MVBs can rapidly form de novo (Chu-Wang and Oppenheim, 1980), apparently within minutes (Birks et al., 1972; Piper and Luzio, 2001; Weldon, 1975), and the number of MVBs can increase significantly within hours of a mechanical insult or drug treatment (Altick et al., 2009; Bresnahan, 1978; Claude et al.,...
1982b). The occurrence of MVBs in PNS terminals is in contrast to the rarity, if not absence, of MVBs along the axon shaft. As a possible explanation for the apparent paucity of MVBs in axon terminals, it has been suggested that MVBs may be rapidly removed from the terminals by retrograde axonal transport to the soma (Holtzman et al., 1973). If this was true, however, then a larger number of MVBs should be visible en route or in transit in the axons shaft – which is not the case (Altick et al., 2009).

3.5. Accumulation of MVBs at axonal ligatures—Accumulation of MVBs at the distal side of axonal ligatures has been reported in multiple studies, and upon superficial consideration, this may be taken as reasonable and convincing evidence that MVBs are the carriers of many, if not all retrogradely transported macromolecules along axons (Bronfman et al., 2007; Kaasinen et al., 2008; LaVail and LaVail, 1974; Parton et al., 1992; Sandow et al., 2000; Smith, 1980; Tsukita and Ishikawa, 1980; Valdez et al., 2005; Weible and Hendry, 2004). This notion, however, needs to be reconciled with the apparently contradictory finding that MVBs are very rare along 99% of normal PNS axons (Altick et al., 2009; Berthold et al., 1993) and along CNS axons (Janetzko et al., 1989). A possible explanation is that MVBs may be rapidly generated from smaller vesicles in response to dystrophic conditions. This was the prevailing notion in the 1960s (Blumcke and Niedorf, 1966; Holtzman and Novikoff, 1965; Kapeller and Mayor, 1969; Smith, 1980; Zelena et al., 1968). Such conditions may include the slowing of axonal transport at sites of mechanical constriction or cooling (Hirokawa et al., 1990; Tsukita and Ishikawa, 1980). Thus, the accumulation of MVBs at ligatures or freeze-blocks in axons could be the result of local slowing of small transport vesicles (Grimes et al., 1997) or other membranous material. Such vesicles en route may not form MVBs under physiological conditions within the axon, but may do so rapidly in response to a wide range of dystrophic conditions. As we will argue below (sections 3.6–7, 11.2), this explanation is entirely consistent with older and more recent experimental evidence.

3.6. CNS axons—MVBs have only rarely been documented in normal CNS axons under physiological conditions (Janetzko et al., 1989; Roizin et al., 1967; Vitalis et al., 2008). Some investigators concluded that MVBs may not exist there at all (Castel et al., 1992; Hirano and Llena, 1995; Pappas and Purpura, 1961). Based on the apparent lack of MVBs in CNS axons, Pappas and Purpura (1961) suggested that MVBs may aid in the ultrastructural distinction between dendritic and axonal processes. When “dystrophic” conditions are generated, by injection of horseradish peroxidase (HRP) or lectins or by other lesions, MVBs have been reported within CNS axons and CNS axon terminals (Bunt and Haschke, 1978; LaVail and LaVail, 1974; LaVail et al., 1980). At the concentrations applied, both HRP and lectins are considered to be toxic for neurons (Bunt and Haschke, 1978), and their internalization, along with the formation of new MVBs, may constitute part of the neuron’s response to a “dystrophic” condition, a response that seeks to remove and thereby eliminate the internalized toxic material by retrograde axonal transport and eventual degradation in lysosomes (Gatzinsky, 1996; Grafstein and Forman, 1980).

3.7. Neurites and growth cones in vitro—Neurites grown in vitro from embryonic or neonatal sympathetic ganglia contain relatively sparse MVBs, some of which are spherical with diameters averaging 750 nm (Bunge, 1977), while others are elongated tubular with diameters of 60–130 nm and up to 2 μm in length (Birks et al., 1972; Weldon, 1975). In these studies, two types of neurite varicosities were described with one of them containing relatively large numbers of MVBs (Birks et al., 1972). Neurite growth cones contain both spherical and tubular MVBs with no characteristic location within the growth cone (Birks et al., 1972; Povlishock, 1976). Neurites and neurite growth cones also contain cup-shaped bodies (‘C’ bodies) or ‘sacs’ (vesicle-filled structures that often have a curving avesicular
portion or “tail”); they are believed to be early (precursor) forms of MVBs (Birks et al., 1972; Bunge, 1977; Weldon, 1975; Wessells et al., 1974 – see section 5). Cup-shaped bodies contain a few vesicles and an occasional myelin figure.

MVBs seem to be more frequently encountered in PNS axon terminals and neurites in vitro than in vivo. The in-vitro conditions may be considered a form of dystrophic state for neurons, and in many in-vitro studies, neurons were furthermore exposed to additional toxic markers or potential inducers of MVBs, such as HRP and thorium dioxide (Birks et al., 1972; Holtzman et al., 1973; Parton et al., 1992; Teichberg and Holtzman, 1973). Thus, results from in-vitro studies may not reflect the normal physiological status of MVBs in axons or axon terminals in vivo.

4. Accumulation of molecules in neuronal MVBs

This section distinguishes and lists two types of molecules: those constitutive for MVBs (housekeeping), and the cargo molecules that are processed

The molecules within MVBs can be classified into two broad categories: constitutive molecules and cargo molecules. Constitutive molecules are not necessarily unique to MVBs, but are essential to make up either the organelle structure or serve house-keeping functions of MVBs, for example vesicle budding, providing an appropriate internal milieu (pH), recognition of signal sequences, ubiquitination and sorting functions, and transport along microtubules. Different families of molecules, for example ESCRT proteins, pincher, kinesins, phosphatases and kinases, accomplish these types of functions (Falguieres et al., 2008; Piper and Katzmann, 2007). Cargo molecules, on the other hand, are the membrane-bound receptors, ligands, internalized proteins and macromolecules that are sorted, processed and transported by MVBs. Different subtypes of MVBs may be composed of different constitutive molecules, and may also accumulate and transport different types or combinations of cargo molecules (McCaffrey et al., 2009; Saito et al., 1997; White et al., 2006). The cargo molecules can be further divided into those that are endogenous to the neurons (e.g., receptors for neurotransmitters or trophic factors, Fig. 2A-D) and those that are exogenous (e.g., HRP tracers or toxins). A large variety of molecules has been shown to accumulate in neuronal MVBs. Accumulation of identified molecules is typically evidenced by radiolabel, immunolabel, or enzyme histochemistry. Major categories and examples of such molecules in neuronal MVBs are listed in Table 3.

4.1. Constitutive molecules—Constitutive proteins associated with MVBs have been identified almost exclusively in non-neuronal cells. Numerous in-vitro studies have characterized molecules necessary for MVB morphogenesis, maintenance, protein sorting, and MVB transport along microtubules. The described features of MVBs most likely also apply to neuronal MVBs. MVBs and the molecules associated with MVB formation and function have been the topic of several excellent recent reviews (Gruenberg and Stenmark, 2004; Hurley, 2008; Katzmann et al., 2002; Maxfield and McGraw, 2004; Piper and Katzmann, 2007; Russell et al., 2006; Williams and Urbe, 2007; Woodman and Futter, 2008). MVBs originate from endosomes that have undergone invagination of the limiting membrane. Four protein complexes, ESCRT 0-III, composed of several subunits each, function to “escort” cargo to endosomes, recruit necessary molecules, such as phosphatidylinositol and respective kinases, sequester lipids and cargo for sorting, and promote invagination and cleavage to form internal vesicles.

Ubiquitination is a major signal for sorting of membrane proteins into MVBs (Babst, 2005; Davies et al., 2009; Hicke and Dunn, 2003; Katzmann et al., 2001; Raiborg et al., 2003; Russell et al., 2006; Woodman, 2009). Ubiquitination of cargo proteins occurs at the plasma membrane or the membrane of early endosomes, which promotes delivery of cargo into the...
endosomal-lysosomal system (Davies et al., 2009; Haglund et al., 2003; Longva et al., 2002; Ren et al., 2008; Sigismund et al., 2005; Thelen et al., 2008). De-ubiquitination is necessary for appropriate sorting of cargo by the MVB (Agromayor and Martin-Serrano, 2006; Babst, 2005; Kyuuma et al., 2007; Nikko and Andre, 2007; Richter et al., 2007; Row et al., 2006). The subunits of the ESCRT 0, I and II complexes have ubiquitin binding motifs and are believed to sort cargo via ubiquitin binding (Hurley, 2008; Luzio et al., 2009; Russell et al., 2006; Wollert and Hurley, 2010). While ubiquitination is not the only route into the endosomal-lysosomal system and thus into MVBs (Calistri et al., 2009; Piper and Katzmann, 2007; Reggiori and Pelham, 2001), this route is clearly significant, because mutations of the proteins involved in the ubiquitination/de-ubiquitination activity can lead to neurodegenerative diseases (Lee et al., 2007; Matsuda and Tanaka, 2010; Rubinsztein, 2006; Rusten and Simonsen, 2008; Tamai et al., 2008).

Rab GTPases are another class of proteins that are associated with the endocytic pathway. It has been established that Rabs 4, 5, 7, 9, and 11 function to delineate membrane domains of endosomes which will either invaginate to form internal vesicles, bud off as small vesicles to recycle to the plasma membrane, or become part of lysosomes for degradation (Vonderheit and Helenius, 2005; Zerial and McBride, 2001). Thus Rabs are present at each stage within the endosomal-lysosomal system, including MVBs.

In addition to ESCRT complexes and Rab GTPases, other individual molecules have been identified as being associated with MVBs and/or endosomes. These include the LAMP family, often used to mark the lysosomal pathway (Futter et al., 1996), mannose 6-phosphate receptor (van Deurs et al., 1993), STAM and AMSH (Agromayor and Martin-Serrano, 2006; Kyuuma et al., 2007), LBPA (Kobayashi et al., 1999; Matsuo et al., 2004), and UBPY (Row et al., 2006), and several phosphatidylinositol kinases (Heilmeyer et al., 2003; Odorizzi et al., 1998). These proteins have roles in ubiquitination and de-ubiquitination, sorting, maturation, transport, lysosomal fusion, and exosome release. In spite of their seemingly specific functions, these proteins are found in numerous endosomal and cellular compartments, besides MVBs, thus confounding the molecular definition of MVBs (Maxfield and McGraw, 2004; Sorkin and von Zastrow, 2002, 2006; van Meel and Klumperman, 2008). Interestingly, of all the signaling proteins associated with endosomal compartments as listed by Sorkin and von Zastrow (2002), only one was localized to the endosomal/MVB pathway by electron microscopy, all others were localized by indirect methods, such as immunofluorescence, immunoprecipitation, and subcellular fractionalization. It is a difficult task to assign specific markers to all of the stages of organelle morphology from endosome to lysosome (van Meel and Klumperman, 2008). Thus, MVBs, and possibly MVB subtypes (McCaffrey et al., 2009; White et al., 2006) still await full molecular characterization.

For the most part, neuronal MVBs presumably utilize the same molecules and processes for biogenesis and function as other cell types, but some aspects may be neuron-specific. Studies of neurons have identified several proteins that associate with MVBs, such as neuron-specific kinesin, which moves MVBs anterogradely (− to + direction) along microtubules (Saito et al., 1997); neuron-specific dynein isoform, which moves signaling endosomes retrogradely (+ to − direction) along microtubules (Ha et al., 2008); pincher, which is involved in macroendocytosis of Trks and NogoΔ20 (Joset et al., 2010; Valdez et al., 2005); and Ndfip 1, which is required for exosome secretion (Putz et al., 2008). On the basis of these recent findings, it seems probable that although MVBs are composed of and utilize the same proteins present in other cell types, neuron-specific molecules exist that associate with MVBs in neurons.
4.2. Endogenous cargo molecules—Endogenous cargo molecules within neuronal MVBs may be integral membrane proteins such as channels, transporters and receptors, or they may be soluble molecules. The soluble molecules typically are internalized by receptor-mediated mechanisms. Different categories of endogenous molecules have been identified and localized in neuronal MVBs, including growth factors/trophic factors (Fig. 2A-D) (Butowt and von Bartheld, 2001, 2009; Claude et al., 1982a; Claude et al., 1982b; Rind et al., 2005; Schwab and Thoenen, 1977; Schwab and Thoenen, 1976; von Bartheld et al., 1996) and their receptors (Pioro et al., 1991; Valdez et al., 2005). Another important category is comprised by receptors for neurotransmitters, ion channels, and G-protein-coupled receptors (Bloch et al., 2003; Grimes and Miettinen, 2003; Sorkin and von Zastrow, 2002, 2009; Vitalis et al., 2008), and their ligands, such as the neuropeptides neurotensin, calcitonin gene-related peptide (CGRP), substance P and small cations, for example calcium (Table 3). Calcium has been localized to the internal vesicles of neuronal MVBs (Buchs et al., 1994; Csillak and Knyihar-Csillak, 1980; Fifkova et al., 1983; Zareba-Kowalska and Gajkowska, 1990). This indicates that MVBs sequester and store calcium. Calcium may participate in MVB housekeeping functions, such as formation of internal vesicles and sorting (Luzio et al., 2007). Neurosecretory material has been localized to MVBs in some experimental paradigms. The number of MVBs in neurosecretory cells increases in parallel with an increase in neurosecretory molecule synthesis, suggesting a role in either processing the neurosecretory molecules or degrading excess material from the previous synthesis and release cycle (Bord and Bell, 1977; Morris and Dyball, 1974; Morris and Steel, 1975, 1977). Another example for endogenous cargo is the LR11 (lipoprotein) receptor which binds apolipoprotein A that is involved in cholesterol metabolism. Cholesterol appears to be required for the diversion of growth factor receptors from MVBs to the Golgi network instead of targeting to lysosomes (Miwako et al., 2001; Offe et al., 2006). This may point to integration of signaling mediated by cholesterol and growth factor receptors at the level of the MVB (Eden et al., 2010; Miwako et al., 2001), a concept that may be relevant for the pathophysiology of Niemann-Pick disease (Kobayashi et al., 1999) as discussed in more detail in section 11.3.4.

Additional membrane and binding proteins that have been localized to neuronal MVBs include interphotoreceptor-retinoid-binding protein (IRBP), amyloid precursor protein (APP), and prion proteins. While IRBPs apparently are destined for degradation (Hollyfield et al., 1985), prion proteins (PrP<sup>C</sup>) and APP are targeted to MVBs, from where they may be released as exosomes (Rajendran et al., 2006; Takahashi et al., 2002; Vella et al., 2008). Whether the pathogenic scrapie prion form (PrP<sup>Sc</sup>) follows a similar pathway is unclear (see section 11.5). Cannabinoid receptors accumulate in MVBs in neuronal cell bodies as well as in axonal MVBs (Morozov and Freund, 2003; Vitalis et al., 2008). Transporters for dopamine and monoamine have also been localized to MVBs (Table 3), and the monoamine transporter-2 has been localized to the limiting membrane of MVBs (Nirenberg et al., 1996).

Information about the precise localization of cargo proteins within MVB compartments is available for only a few types of endogenous cargo and one constitutive-type MVB molecule. Such information is important, because growth factors and other signaling molecules may retain signaling capacity when they are targeted to the limiting membrane of MVBs, but not when they are sequestered within MVB’s internal vesicles (Mukherjee et al., 1997; Sorkin and von Zastrow, 2002, 2009). In cortical neurons, amyloid-beta 42 accumulates in the limiting membrane of MVBs (Takahashi et al., 2002). In hypoglossal motoneurons, the internalized neurotrophic factors BDNF and GDNF are localized primarily to the internal vesicles of MVBs when those MVBs are located in the neuronal cell body (possibly destined for degradation), while they are preferentially localized at the limiting membranes when the MVBs are located in dendrites (possibly destined for signaling and/or recycling, see Fig. 3A-C) (Rind et al., 2005). Consistent with the notion that tyrosine kinase
activity regulates trafficking into a degradation pathway (Felder et al., 1990), in cultured sympathetic neuronal cell bodies overexpressing the neurotrophin receptor TrkB, most TrkB was immuno-localized to the internal vesicles of MVBs (88%), with only 12% in the limiting membrane (Valdez et al., 2005). In similar experiments, the constitutive MVB molecule and membrane trafficking chaperone, pincher, on the other hand, was localized primarily to the limiting membrane (77%) and only 23% to internal vesicles (Valdez et al., 2005). As with all over-expression studies, the caveat is that normal pathways may have been overloaded with the additional protein, thus prompting an abnormal load into a non-physiological ‘overflow’ (Birks et al., 1972). Nevertheless, these studies suggest that sorting of cargo within MVBs is not only stage- but also location-dependent (dendrite vs. soma).

4.3. Exogenous cargo molecules—Exogenous molecules are typically internalized by non-receptor mediated mechanisms: pinocytotic (bulk) uptake/transport. This category includes tracers such as ferritin, thorium dioxide, HRP and lectins (Berthold et al., 1993; Birks et al., 1972; Broadwell and Balin, 1985; Bunge, 1977; Chu-Wang and Oppenheim, 1980; Feher, 1984; Grafstein and Forman, 1980; Haigler et al., 1979; Holtzman and Peterson, 1969; LaVail and LaVail, 1974; Richardson et al., 1997; Rosenbluth and Wissig, 1964; and Weldon, 1975), toxins (Parton et al., 1987; Rind et al., 2005; Schwab and Thoenen, 1977; Schwab and Thoenen, 1976), aminoglycoside antibiotics (de Groot et al., 1990), and viruses (Ashok and Hegde, 2006; Morita and Sundquist, 2004; Uchil and Mothes, 2005). One major purpose for internalization of exogenous cargo into axonal MVBs may be to “detoxify” and protect the CNS (Gatzinsky, 1996). Although exogenous cargo such as tracers (gold, lectin, HRP) is typically found in axonal MVBs, several studies have suggested that a substantial fraction of the MVBs in axons may have formed in response to the endocytosed toxin rather than being recruited to transport the external cargo from the periphery to the CNS (Altick et al., 2009; Birks et al., 1972).

5. Genesis of MVBs
This section provides a brief summary of the steps involved in MVB biogenesis, especially ESCRT functions, as known from studies in non-neuronal cells

The biogenesis of neuronal MVBs was studied in considerable morphological detail in the 1960s and 1970s. These early studies applied electron dense markers to cultured neurons and examined how they were taken up and processed by growth cones and neurites. Markers included ferritin, horseradish peroxidase, and thorium dioxide (Birks et al., 1972; Bunge, 1977; Holtzman and Petersen, 1969; Richardson et al., 1997; Rosenbluth and Wissig, 1964; Weldon, 1975; Wessells et al., 1974). These studies suggested the following sequence of MVB dynamics: MVBs are formed from pinocytotic vesicles and vacuoles through intermediate structures that are called cup-shaped bodies or sacs (‘C’-bodies) (Theodosis, 1982; Wessells et al., 1974), sometimes also called pre-multivesicular bodies (Birks et al., 1972; Weldon, 1975). Coated vesicles and vacuoles of 50–500 nm in diameter are derived from macropinocytosis (Hirsch et al., 1968). Apparently through fluid loss, the vacuoles collapse rapidly (within 3–15 minutes, Weldon, 1975) into cup-shaped bodies. The cup-shaped bodies form internal vesicles to become MVBs. Plasmalemma appears to provide the source for the MVB’s limiting membrane (Birks et al., 1972; Bunge, 1977). Based on quantification of organelle populations after tracking uptake and processing of exogenous ferritin, the MVB population turns over within 2 hours (Birks et al., 1972). It needs to be noted that the neurite culture paradigm examines regenerating neurites rather than neurites that advance their very first growth cone, as normally in vivo (Bunge, 1977). This model of MVB biogenesis in cultured neurites was not further explored at the molecular level; therefore, it is not known how pre-MVBs (cup-shaped bodies) relate to current models of
MVB biogenesis (primarily studied in yeast and other non-neuronal model systems) (Gruenberg and Stenmark, 2004; Maxfield and McGaw, 2004; Piper and Katzmann, 2007).

The defining event in MVB biogenesis is the formation of internal vesicles within an endosome (Fig. 4). Internal vesicle formation requires several steps: targeting of endocytosed proteins to specific locations in the membrane of the endosome; sequestering, via tubular extensions, of cargo destined for recycling from other cargo that is targeted for degradation; recruitment of specific Rabs (Vonderheit and Helenius, 2005; Zerial and McBride, 2001); and activity of AAA-type ATPases and phosphatidylinositol-3-phosphate. Both molecules are necessary for invagination of endosomal limiting membrane to form internal vesicles (Babst et al., 1998; Fernandez-Borja et al., 1999). The formation of internal vesicles in an endosome, essentially the genesis of an MVB, is accomplished within 4–30 minutes after endocytosis of markers at the plasma membrane (Mayor et al., 1993; Piper and Luzio, 2001).

Recent reviews provide a detailed account of the machinery that orchestrates MVB formation, including the role and composition of ESCRT complexes, phosphatidylinositols, and Rabs (Babst, 2005; Falguieres et al., 2008; Hurley, 2008; Vonderheit and Helenius, 2005; Williams and Urbe, 2007). Our knowledge about the mechanisms of biogenesis of MVBs is based on evidence from non-neuronal cell types including reticulocytes, endothelial cells, immortalized cell lines, and cells of the immune system (Gruenberg and Stenmark, 2004; Piper and Katzmann, 2007; Russell et al., 2006; Woodman and Futter, 2008). These mechanisms, described below, have not been confirmed in neurons. However, all the molecular and morphological components identified and described in other cells are present in neurons, thus supporting the notion that MVB biogenesis is conserved in different types of cells.

Several lines of work have identified factors that are pertinent for internal vesicle formation: asymmetry in the lipid bilayer; pH gradient between the lumen of the endosome and the cytosol; molecular interactions via ESCRT-III proteins; and specialized lipid composition, such as LBPA, the molecular structure of which can bend membranes (Lawrence et al., 2010; Matsuo et al., 2004; Piper and Luzio, 2001; Theos et al., 2006). Wollert and Hurley (2010) showed distinct roles of each of the ESCRT complexes during MVB formation in a cell-free system. According to their model, ESCRT-0 clusters the cargo, i.e. is involved in sorting, ESCRTs I and II form the membrane neck; then ESCRT-III is recruited to the neck. Three ESCRT-III subunits are necessary for complete scission of the internal vesicle from the MVB limiting membrane (Wollert and Hurley, 2010). In intact cells, it is thought that ESCRT complexes become sequentially associated with endosomes, and cargo is treadmilled along the endosomal membrane until final incorporation into an internal vesicle (Babst, 2005; Vajjhala et al., 2007). A different scenario proposes that ESCRT complexes are arranged concentrically around cargo on the endosomal membrane, thereby effectively sequestering cargo from the cytosol during vesicle formation (Nickerson et al., 2007). The recent work by Wollert and Hurley suggests a third scenario in which cargo is confined by ESCRT-0, then ESCRT-I and II bend the membrane and form the buds at the site of the ESCRT-0-cargo complex. Thereafter, ESCRT-III cleaves the buds to form the internal vesicle containing the cargo, excluding all ESCRT subunits. Several molecules, such as ALIX, Tsg101, and annexins that participate in MVB biogenesis and function are known to interact with calcium-binding proteins. Annexins in particular participate in several different steps of MVB formation and function (Futter and White, 2007).

Regardless of the precise assembly of ESCRTs on an endosome, when ESCRT proteins are lacking or dysfunctional, MVB formation and function are impaired (Babst, 2005; Hurley, 2008; Piper and Katzmann, 2007; Rusten and Simonsen, 2008; Tamai et al., 2008).
study, using yeast, HEp2, and HeLa cells, found that recognizable MVBs can still form in the absence of individual key subunits of all four ESCRTs (Stuffers et al., 2009). However, trafficking of the EGF receptor is impaired and the morphology of the MVBs is severely altered, possibly compromising proper function of the endosomal-lysosomal system. Cell-free systems designed to recapitulate MVB formation indicate that formation of internal vesicles, the determining feature of MVBs, is dependent upon Hrs and STAM, components of ESCRT-0; and Vps4, an ATP-binding protein (Sun et al., 2010). The plasticity of MVB formation reflects the complexity of the process, and while there seems to be variability in MVB morphogenesis, the relationship of this variability in form to proficient function in physiologically relevant systems is not yet fully understood (Traub, 2010).

During and subsequent to internal vesicle formation and maturation, MVBs vary in size, shape, number of vesicles, electron density of lumen and internal vesicles, subcellular location and protein content (Altick et al., 2009; Clague and Urbe, 2008; Kobayashi et al., 2002; van Meel and Klumperman, 2008; White et al., 2006). It has been observed that with time, MVBs enlarge, increase the number of internal vesicles, become more electron-dense, and localize increasingly within the peri-nuclear region of cells. This series of changes has been called “maturation” (Ohashi et al., 2000; Stoorvogel et al., 1991), which sometimes has been taken to imply that the MVB no longer contains recycled proteins and is not competent to receive additional endocytosed cargo (Woodman and Futter, 2008). Maturation is completed some time after cargo endocytosis, estimated at 8–10 minutes in vitro after endocytosis (Mayor et al., 1993). Rab conversion, the replacement of Rab5 with Rab7, is a sign of progression from an “endosomal MVB” to a mature MVB (Deinhardt et al., 2006; Ohashi et al., 2000; Rink et al., 2005; Stoorvogel et al., 1991). This progression is also accompanied by a change in pH. The recycling endosomes have been reported to have a pH of 6.4–6.5, sorting endosomes have been measured at pH 5.9–6.0, and the pH of mature MVBs has been reported to be 5.0–6.0 (Maxfield and McGraw, 2004; Woodman and Futter, 2008). The acidic pH facilitates the dissociation of receptor/ligand pairs as well as initiates protein degradation, which is completed in the lysosome. It is possible that as the luminal matrix of a maturing endosome becomes progressively more acidic, the internal vesicles may form to maintain a protected physiological environment. Until recently, it was thought that any protein sequestered into the lumen of an internal vesicle was destined for degradation (Fader and Colombo, 2009). Some recent findings in neurons and glia suggest that internal vesicles of MVBs may also be released as exosomes (Bulloj et al., 2010; Fevrier and Raposo, 2004; Lachenal et al., 2011; Putz et al., 2008; van Niel et al., 2006) (see section 6.2). In conclusion, genesis of MVBs and particularly the characteristic formation of the MVB’s internal vesicles are orchestrated through complex actions of constitutive molecules, not unlike those that form synaptic vesicles and regulate their fusion for transmitter release (Smith et al., 2008). In fact, it may be speculated that the similar size of synaptic vesicles and MVBs’ internal vesicles point to an optimal ratio of membrane circumference and luminal volume that is common between the processes of exo- and endocytosis.

6. Recycling and release of internal vesicles

This section examines how three pathways may contribute to the processing of cargo within neuronal MVBs

The proteins contained within internal vesicles of MVBs can have one of three fates: extracellular release of the proteins in intact internal vesicles by fusion of the limiting membrane of MVBs to the plasma membrane (exosome release), back-fusion to recycle proteins to the plasma membrane, or degradation in lysosomes (Fig. 4). Early in MVB research it was hypothesized that the internal vesicles may be either released into the cytoplasm, or may be destined for neurotranscretion (Palay, 1960). Subsequently, the role of
MVBs in the degradation pathway was discovered, but in the 1990s and early 2000s, recycling to the plasma membrane via the so-called “back-fusion” pathway to the plasma surface membrane moved to the forefront (Murk et al., 2002), only to be followed in most recent years by an emphasis on release as exosomes (for expulsion and/or intercellular communication) (Faure et al., 2006; Lachenal et al., 2011; Mathiavanan et al., 2010; Putz et al., 2008; Smalheiser, 2007; Vingtdeux et al., 2007). In this section, we will discuss both the back-fusion pathway and the exosome release pathway, and examine currently existing evidence for these two pathways of MVBs in neurons.

6.1 Back-fusion/recycling pathway—This pathway is in many ways a reversal of the biogenesis of MVBs (Denzer et al., 2000; Fevrier and Raposo, 2004; Murk et al., 2002; Uchil and Mothes, 2005). It requires a dynamic remodeling of MVBs (Fig. 5). The typical spherical MVBs that contain multiple internal vesicles morph into elongated tubules that are largely depleted of internal vesicles (Murk et al., 2002). According to current models, the limiting membrane expands by the insertion of membrane from the internal vesicles. Such back-fusion allows internally stored proteins to become incorporated into the limiting membrane, from where they can be transported to the surface plasma membrane (Kleijmeer et al., 2001). The final movement from the tubules to the plasma membrane is believed to occur through small transport vesicles. A membrane shuttle mechanism may explain the insertion of proteins from temporary storage in MVBs into the plasma membrane upon stimulation. This mechanism is well established for antigen – e.g., major histocompatibility complex (MHC) class II – presenting cells of the immune system (Murk et al., 2002). Whether other cells such as neurons can use MVBs as temporary protein and membrane storage compartments is not known but is an attractive hypothesis. A storage function of neuronal MVBs is particularly intriguing, given the frequent localization of MVBs at postsynaptic sites (Chicurel et al., 1992; Cooney et al., 2002; Ostroff et al., 2010; Pappas and Purpura, 1961; Paula-Barbosa et al., 1978; Rind et al., 2005; Schnapp, 1997; von Bartheld et al., 1996), the loading of postsynaptic MVBs with neuromodulatory agents (e.g. neurotrophic factors, Rind et al., 2005; von Bartheld et al., 1996), and the evidence that MVBs with tubular extensions exist in neurons and serve distinct regions of synaptic sites within hippocampal (Chicurel et al., 1992; Cooney et al., 2002) and amygdalar dendrites (Ostroff et al., 2010). Three-dimensional reconstruction of ultrathin serial sections provided evidence for tubular extensions of neuronal MVBs at postsynaptic sites (Cooney et al., 2002). Furthermore, the trophic factors BDNF and GDNF reside primarily in the limiting membrane of MVBs in dendrites (Rind et al., 2005, see also Fig. 3), consistent with a back-fusion recycling mechanism. This mechanism could be used to insert receptors into specific sites that are often distant from the cell body, such as axodendritic synapses (Bloch et al., 2003; Cooney et al., 2002). Thus, several lines of circumstantial evidence support back-fusion as a mechanism for protein recycling in neuronal MVBs.

6.2 Exosome release—The concept of exosome release as an alternative or addition to back-fusion has gained considerable attention in the past decade. This mechanism involves fusion of the MVBe outer limiting membrane with the plasma membrane and release of internal vesicles as exosomes (Denzer et al., 2000; Fevrier and Raposo, 2004; Harding et al., 1983; Murk et al., 2002). In this case, proteins associated with internal vesicles are not incorporated (at least not directly) into the plasma membrane, but rather become expelled into the extracellular space. After release, exosomal vesicles may come in contact with the same releasing cell or with other cells. These exosomal vesicles may continue to bind ligands on the outside, with the extracellular domain of receptors facing the outside and the cytoplasmic domain on the inside of the internal vesicle (the vesicular content represents cytoplasmic compartment, see Fig. 4). The continued binding of the ligand to the
transmembrane receptor depends on binding affinities and the milieu of the extracellular environment. Exosome release is an established mode of vesicle release and an important form of intercellular communication for hematopoietic cells of the immune system (Denzer et al., 2000; Fevrier and Raposo, 2004; Murk et al., 2002). It is currently not known whether this mode of release occurs for MVBs in neurons in vivo, but a number of recent papers have speculated on the potential significance of exosome release as a mechanism for removal of proteins from neurons as well as for communication between neurons and between neurons and other cells (Bulloj et al., 2010; Faure et al., 2006; Fevrier et al., 2004; Lachenal et al., 2011; Putz et al., 2008; Smalheiser, 2007).

Current evidence for exosome release relies on the isolation of organelles and detection of proteins (known to reside in exosomes) in media of cultured neurons. After isolation, exosomal fractions are often used for immunolabeling followed by electron microscopic analysis (Bulloj et al., 2010; Faure et al., 2006; Fevrier et al., 2004; Lachenal et al., 2011; Putz et al., 2008; Vingtdeux et al., 2007). A potential problem is that some of these exosomal markers are not localized exclusively to exosomes. For example, the heatshock protein Hsp70, is also known to accumulate in synaptosomes (Joshi et al., 2006), and flotillin-1 distributes equally to the pre- and postsynaptic side of synapses (Swanwick et al., 2010). Therefore, it is difficult to unequivocally demonstrate that such organelles are indeed exosomes derived from neuronal MVBs. The use of tetanus toxin C fragment (Lachenal et al., 2011) as an “MVB marker” may also be potentially confounding due to its preferred presynaptic localization (about 10-fold compared to MVBs, Rind et al., 2005). Nevertheless, the notion of exosome release is supported by the size of the vesicles, and the findings that they are immunopositive for proteins known to traffic through MVBs (Mathivanan et al., 2010). Snapshot images of putative exosomes during release (“caught in the act”) are rare (Weldon, 1975; Lachenal et al., 2011), and it is difficult to conclusively identify a partially fused MVB. The recent studies cited above provide evidence consistent with the notion that exosomes are released by neurons in vitro, however whether such release would also happen in vivo awaits further scrutiny. Direct evidence for release by neurons in vivo is preferable to in vitro protein correlates (Thery et al., 2002); a truly specific MVB marker would help to resolve such uncertainties. In conclusion, more and preferably direct evidence of neuronal exosome release is desirable – a challenge due to current technological limitations.

7. Neuron-specific transport functions of MVBs

This section reviews possible intraneuronal transport pathways of MVBs and the empirical evidence for which ones are actually utilized.

From a morphological standpoint, neurons are specialized cell types with unique geometrical cell shapes unlike any other cell type: they have extremely long processes (axons and dendrites) relative to the size of the cell body, and therefore have extended transport needs, as compared with cells that are geometrically more “simple” (Nixon, 2005). Transport of organelles, membrane proteins and signaling molecules in neurons therefore may require novel strategies or employ modified and expanded transport functions of mechanisms utilized by simple cells. In this context, it is intriguing that neurons, but no other cell type, express an MVB-specific kinesin (KIFC2) that exclusively distributes within the soma and dendrite, but not the axon, and that moves a distinct type of MVBs apparently unrelated to the endosome-to-lysosome pathway (Saito et al., 1997). Furthermore, MVBs in dendrites have been shown to contain molecules that were originally obtained by retrograde axonal transport (Rind et al., 2005). Taken together, this suggests that a novel type of neuron-specific MVBs contributes to transport needs of neurons at a wider and largely unexplored scale when compared to those in simple cells.
How are MVBs involved in trafficking needs of neurons? Theoretically, MVBs may participate in several distinct trafficking pathways as illustrated in Fig. 6. These pathways may be distinguished as (1) intrasomal (somato-somatic); (2) axo-somatic and axo-dendritic; (3) soma-to-dendrite and interdendritic; and (4) soma-to-axon. We will consider the empirical evidence, if available, for each of these categories of MVB trafficking.

(1) **Intrasomal and dendro-somal**—MVBs may form at the plasma membrane in cell bodies or dendrites and move membranes and internalized proteins from peripheral to central sites within the soma. This is the “classical” endosome-to-lysosome pathway as seen in non-neuronal cells (Katzmann et al., 2002; Maxfield and McGraw, 2004; Mukherjee et al., 1997; Sorkin and von Zastrow, 2002). This pathway is also compatible with the initial delivery of internalized material to the soma from more distant sources (axon or dendrite) via vesicles or endosomes (not necessarily via MVBs), and subsequent transfer to MVBs in the soma (Altick et al., 2009; Rind et al., 2005). There is abundant evidence for apparently “normal” (conventional) MVB composition (endocytotic markers and cargo) in neuronal cell bodies, as summarized in Table 3.

(2) **Axo-somatic**—This category encompasses all MVB-mediated retrograde axonal transport that originates from either nerve terminals or from the axon shaft, and delivers the axonal MVB to either the soma (axo-somatic), or beyond the soma to proximal or distal dendrites (axo-dendritic). To be considered for this category, the MVB itself must be transported within the axon, not any endosomal (non-MVB) precursors. Evidence for such a MVB transport pathway requires physical evidence that the MVB is transported in the axon, not just components that later accumulate in somal or dendritic MVBs. Such evidence for retrograde axonal transport of tracer-labeled MVBs in vitro was provided by time-lapse video followed by ultrastructural analysis of the transported organelle (Parton et al., 1992). Early studies had indicated that endogenous, receptor-binding proteins such as growth factors tend to reside during axonal transport in small vesicles or tubules rather than MVBs (reviewed in Schwab, 1977; Schwab et al., 1979; Schwab and Thoenen, 1978), while exogenous, bulk-transported proteins and compounds conjugated to tracer molecules reside in MVBs during axonal transport (Breuer et al., 1975; Hirokawa et al., 1990; LaVail and LaVail, 1974; Parton et al., 1992; Sandow et al., 2000; Tsukita and Ishikawa, 1980; Turner and Harris, 1974; Weible and Hendry, 2004). Recent work (Altick et al., 2009) confirms this earlier notion of a more restricted role of MVBs for retrograde axonal transport and points to a small vesicular transport endosome, consistent with studies characterizing NGF- and TrkA-containing endosomes (Cui et al., 2007; Delcroix et al., 2003; Grimes et al., 1997). Retrograde transport of cannabinoids in MVBs may be an exception to this general rule (Vitalis et al., 2008). Thus, although MVBs can move retrogradely along axons, only a select type of cargo appears to utilize them as retrograde carriers.

(3) **Soma-to-dendrite or interdendritic**—Endocytosed material (delivered from the soma plasma membrane and/or from other dendrites or axons) accumulates in MVBs, which transport the cargo to dendrites. This category also includes the special case of MVB transport between adjacent dendrites or between adjacent spines within the same dendrite. The most convincing (albeit indirect) evidence for such a transport function of MVBs is provided by the work of Cooney et al., 2002 who showed that collections of adjacent spines are supplied by MVBs – likely involved in recycling of signaling molecules and synaptic plasticity. Taken together with evidence of a neuron-specific type of MVBs in the somal dendrite (Saito et al., 1997) and recent studies showing that postsynaptic MVBs can contain trophic factors derived from the axonal target (Rind et al., 2005), these data are highly suggestive for a distinct MVB type specialized for somatic and dendritic transport and storage. This is consistent with the surprising lack of endocytotic markers in this MVB type.
(Saito et al., 1997), apparently differing from other MVB types in the soma. The “dendritic” MVB type appears to lack proteins that are typically found in MVBs in the soma of non-neuronal cells, including Rab5, dynamin, Lamp2, and Trk as well as proteins typically found in organelles of the endosome-to-lysosome pathway, such as transferrin receptor and Lamp1 (Saito et al., 1997). Thus, neurons appear to utilize a novel type of MVB for dendritic functions that differ considerably from the conventional somal MVB type.

(4) Soma-to-axon (anterograde axonal)—There is currently no evidence that MVBs are transported anterogradely within axons, based on HRP transport experiments or ligation and cooling experiments of peripheral nerves, since there was virtually no HRP labeling of MVBs indicative of anterograde MVB movement (LaVail and LaVail, 1974), and no accumulation of MVBs proximal to axonal ligatures or cooling sites (Hirokawa et al., 1990; Smith, 1980; Tsukita and Ishikawa, 1980).

8. Multiple functions of neuronal MVBs

This section argues that MVBs in neurons may have expanded, neuron-specific functions beyond those seen in non-neuronal cells

It is obvious from the above accounts that neuronal MVBs are involved in numerous fundamental cell biological processes, and therefore are likely to have multiple functions, based on cellular and subcellular context. The primary role of MVBs is traditionally placed in the endocytic pathway, where MVBs are heavily involved in sorting and adequate routing of endocytosed cargo. Since endosomal and membrane trafficking are closely intertwined with cellular signaling (Scita and Di Fiore, 2010; Sorkin and von Zastrow, 2002, 2009; Zwang and Yarden, 2009), it is not surprising that MVBs, and in particular neuronal MVBs, appear to have important functions in intracellular – and, possibly, in intercellular – signaling. Such functions appear to involve sorting/recycling; regulation of receptor surface expression, therefore implications for synaptic plasticity; intra- and intercellular communication, via intercompartmental transport and exosome release; neurosecretion, via degradation and/or processing of neurosecretory materials; signal termination; and clearance of toxins and unnecessary proteins, via degradation or export to the extracellular space or neighboring cells. In this section, we will summarize these multiple functions of neuronal MVBs and examine the empirical evidence.

Sorting/recycling—MVBs are part of the endocytic pathway. In the endo-lysosomal pathway receptors and ligands internalized from the plasma membrane are sorted and then targeted into particular pathways. After sorting in the MVB, cargo can be routed back to the plasma membrane, moved to internal vesicles for exosome release, or retained within the membrane of internal vesicles for eventual lysosomal degradation (Grant and Donaldson, 2009; Gruenberg and Stenmark, 2004; Piper and Katzmann, 2007; Raiborg et al., 2003; Russell et al., 2006). Thus, MVBs sort between “garbage” and proteins that can be recycled – thus saving energy for replacement and transport. Clutter is removed and housekeeping functions prevent accumulation of dementia-inducing protein aggregates, as summarized in a recent commentary in the Journal of Cell Biology, suitably entitled “clean-up or go crazy” (Williams, 2007). MVBs keep the cellular machinery functional, reduce inventory, and help with “just-in-time” delivery of cargo where it is needed. As shown in Table 3, numerous proteins have been localized in neuronal MVBs, but only few studies have examined sorting/recycling in neurons (Butowt and von Bartheld, 2009; Cooney et al., 2002; Grimes and Miettinen, 2003; Rind et al., 2005; Valdez et al., 2005). Nevertheless, this fundamental MVB function is believed to be applicable to neurons as it is to other cell types, as mentioned above.
Regulation of receptor surface expression—After signal termination, receptors and ligands are recycled to the plasma membrane, degraded, or possibly released via exosomes. Degradation conclusively terminates signaling and alters a cell’s molecular make-up as compared to recycling, which returns the receptors to the plasma membrane, re-setting the signaling competence of the cell. In cultured hippocampal cells, endocytosed receptors, e.g., TrkA vs. EGF receptor, follow different routes within MVBs, distinguished by the co-localization with specific Rab proteins (Valdez et al., 2007). Dendritic MVBs accumulate and recycle internalized markers from neighboring spines, thus serving as a local store for endocytosed material (Cooney et al., 2002). In neurons, protein degradation and recycling play important roles in synaptic potentiation, depression, and circuit maintenance.

Intra- and intercellular communication—Neuron morphology places extreme demands on an efficient transportation system between neuronal compartments. Communication between axon terminals and the cell body, between dendrites and the cell body, and between axons and dendrites is necessary for cell homeostasis as well as cellular plasticity related to circuitry development, maintenance and synaptic plasticity. MVBs are a major retrograde transport organelle, although many cargo types still remain to be defined. It is known that MVBs in dendrites contain proteins that were originally endocytosed in axon terminals (Rind et al., 2005; von Bartheld et al., 1996), suggesting that MVBs not only sort material, but also participate in transport of endocytosed material between neuronal compartments. Exosome release, an alternative to degradation and recycling, has potential significance for intercellular communication as well as a possible function for exporting unwanted proteins and toxins from the cell (Thery et al., 2002) and has been documented in cultured neurons (Bulloj et al., 2010; Faure et al., 2006; Fevrier et al., 2004; Lachenal et al., 2011; Putz et al., 2008; Vingtdeux et al., 2007), see section 6.2 above for full discussion.

Neurosecretion—In neurosecretory cells, the number of MVBs increases with neurosecretory activity and then decreases during the recovery period of secretion (Borg and Bell, 1977; LaVail, 1976; Morris and Dyball, 1974; Morris and Steel, 1975, 1977). Some MVBs appear to contain neurosecretory granules and/or aggregates (Morris and Dyball, 1974; Morris and Steel 1975, 1977), suggesting that MVBs process or sort secretory molecules originating in the Golgi and cytoplasm.

Signal termination—After endocytosis at the plasma membrane and cellular signaling, incorporation of signaling molecules into internal vesicles of an MVB is believed to terminate signaling (Mukherjee et al., 1997; Sorkin and von Zastrow, 2002, 2009). Signal termination could be accomplished in two different ways: 1) release of ligand from its receptor after the receptor has been endocytosed at the plasma membrane and has been incorporated into the outer membrane of the MVB. This process reverses the orientation of the receptor/ligand pair (Fig. 4), with the ligand now facing the luminal side of the MVB’s outer membrane and thus being exposed to a lower pH (5.9–6.0), which inhibits ligand-receptor interactions. 2) sequestration of the entire receptor/ligand complex into internal vesicles of MVBs. In this case, the ligand faces the luminal matrix of the MVB, and the cytoplasmic domain of the receptor extends into the lumen of the internal vesicle (Fig. 4), where signaling is blocked from the cytoplasm. Formation of MVBs in non-neuronal cells has been observed within minutes of ligand/receptor endocytosis (Maxfield and McGraw, 2004). The rapid formation is possible because endocytosis and MVB biogenesis are independent of gene transcription (Zwang and Yarden, 2009). Therefore this mechanism of signal termination is fast and efficient.

Clearance of toxins and unnecessary proteins—Some neurons of the peripheral nervous system extend processes out of the central nervous system, thus beyond the blood-
brain barrier. It has been suggested that MVBs in axons, specifically MVBs at the node of Ranvier, function as a back-up barrier system. Based on evidence from HRP transport and acid phosphatase studies, it appears that MVBs in peripheral axons accumulate toxins and other exogenous substances and route them out of the neuron and into neighboring Schwann cells (Berthold and Mellstrom, 1982; Gatzinsky, 1996; Gatzinsky et al., 1997).

MVBs in neurons have been shown to contain protein-aggregates that accumulate in Huntington’s, Parkinson’s and Alzheimer’s disease as well as frontotemporal dementia (Alegre-Abarrategui et al., 2009; Filimonenko et al., 2007; Nixon, 2005; Takahashi et al., 2002). In neurons, the dysfunction of either MVBs or autophagosomes can lead to neurodegeneration. Recent work has connected the autophagosome pathway with the endosomal-lysosomal pathway; specifically ESCRT subunits are necessary for both pathways. Mutations in ESCRT subunits result in accumulation of non-membrane bound protein aggregates (Filimonenko et al., 2007). Similarly, loss of ESCRT-III subunit Msnf7-2 causes autophagosome accumulation and neuronal cell loss (Lee et al., 2007). These observations, along with others from non-neuronal cells, have led to the hypothesis that the endosomal-lysosomal pathway and the autophagy pathway are interactive and interdependent most likely via their mutual use of ESCRT proteins (Fader and Colombo, 2009; Rusten and Simonsen, 2008). Thus, MVBs in neurons are integral to proper protein clearance and aggregation prevention, which is essential for neuron viability.

9. MVBs in glial cells

In this section, we review information about the role of MVBs in glial cells and their function in glia-neuron homeostasis

MVBs have been described in all major types of glial cells, including Schwann cells (Pannese et al., 1988a; Pannese et al., 1988b), oligodendrocytes (Ogawa et al., 2004; Trapp et al., 1989), astrocytes (Ng et al., 2009), microglia (Potolicchio et al., 2005), Müller glia (LaVail and Margolis, 1987), and Bergmann glia (Baude et al., 1994; Herndon, 1964; Lively and Brown, 2010). Tanyocytes, an ependymal/glial cell type, also contain MVBs that are often connected to tubular structures – possibly reflecting the extensive endocytotic and transcytotic trafficking in this cell type (Rodriguez et al., 2005). MVBs in glial cells contain proteolipid protein, a myelin membrane component (Trajkovic et al., 2006); myelin-associated glycoprotein (Trapp et al., 1989); cathepsin D, a lysosomal enzyme (Trapp et al., 1989); and SCI/Hevin, an extracellular matrix protein (Lively and Brown, 2010). Some of these proteins are not destined for degradation, but rather are being used for neuropil construction and maintenance. There is accumulating evidence that oligodendrocytes, astrocytes, and microglia release exosomes, and that these exosomes are internal luminal vesicles derived from glial MVBs (Faure et al., 2006; Hsu et al., 2010; Potolicchio et al., 2005; Tomboli et al., 2010; Trajkovic et al., 2008; Tytell, 2005).

Glial MVBs have been implicated in specific cellular functions related to neurons. It has been proposed that glial MVBs are involved in the transport of material from axons to Schwann cells (Fabricius et al., 1993; Gatzinsky, 1996; Gatzinsky et al., 1997; Pannese et al., 1988a). This idea is supported by the findings that the tracer HRP and fluorescent nanospheres injected into hind limb muscle localize to MVBs at the nodes of Ranvier and in the axon-Schwann cell network (Berthold and Mellstrom, 1982; Gatzinsky et al., 1997). Consistent with this hypothesis, we have observed that quantum dots, endocytosed at the axon terminal, can reach neighboring glial cells (A.L. Altick, L.M. Wiggins, C.S. von Bartheld, unpublished). Likewise, it has been shown that axonal transport along myelinated nerve fibers in the CNS, including transport of MVBs, is dependent upon oligodendrocytes (Edgar et al., 2004). Signals originating in the neuron result in myelin membrane exocytosis from glial cells (Trajkovic et al., 2006). In related work, it has been shown that LDLs use a
novel transport pathway to cross the blood-brain-barrier. The pathway involves MVBs in the epithelial cells of the blood vessels in the brain. Activated astrocytes regulate the uptake and transcytosis of LDL from the blood. In this case, the cargo, LDL, accumulates within the luminal matrix, but outside the internal vesicles of glial MVBs (Candela et al., 2008). Myelin-associated glycoprotein that accumulates in glial MVBs has been suggested as a candidate for transducing axonal influences onto oligodendrocytes (Trapp et al., 1989), but also, in the reverse direction, in potentially transferring trophic signals from oligodendrocytes via exosomes to axons (Kramer-Albers et al., 2007). Therefore, glial MVBs appear to assist in functional neuron-glial relationships and possibly inter-cellular trafficking.

Much information with regard to MVBs in glial cells has been gained from studies on brain diseases. MVBs are frequently mentioned in studies documenting changes in oligodendrocytes in animals with myelinating pathology (Lassmann et al., 1997; Nakadate et al., 2006), from models of Niemann-Pick disease (Suzuki et al., 2003), and in astrocytes and oligodendrocytes of the lateral geniculate nucleus in aged rats (de la Roza et al., 1985). MVBs are increased in number, enlarged, and have aberrant morphology in glia of prion-infected animals (Ersdal et al., 2009). MVBs in oligodendrocytes are associated with cholesterol trafficking, and disruptions in this trafficking can lead to neurodegeneration (Liu et al., 2010). Finally, following a kindling protocol simulating pre-epileptic activity, MVBs are enlarged in dendrites contacting activated astrocytes, and translocate to the neck of the dendritic spine (Kraev et al., 2009). MVB’s potential association with diverse pathologies, from demyelinating diseases to neurodegeneration, such as Alzheimer’s and Niemann-Pick diseases (see section 11.3.2 – 11.3.3 below), suggests that MVBs are critical for both glial and neuronal cell homeostasis.

10. MVBs: repertoire of techniques

This section compiles information about the range of techniques used to examine neuronal MVBs and comments on advantages and disadvantages

As mentioned previously, MVBs can currently be identified with certainty only by electron-microscopic analysis. A specific marker for MVBs would be tremendously useful for light-microscopic studies, but is yet lacking. Here, we summarize the types of techniques that have been and are currently used to examine MVBs, and particularly neuronal MVBs.

**Morphological techniques**—MVBs can be identified in thin sections, with standard processing for electron microscopic analysis. This approach can be expanded to serially reconstruct individual MVBs (Cooney et al., 2002; LaVail et al., 1980). MVBs in thin sections can also be analyzed by quantitative techniques, e.g., to determine fractional areas or volumes within defined compartments such as cell bodies, dendrites and axons (for references, see Table 2), to compare changes in MVBs in different groups, such as experimental treatment or age (Boaro et al., 1998; de la Roza et al., 1985; Johnson et al., 1975; Nakadate et al., 2006; Piori et al., 1991). Researchers have examined protein content of neuronal MVBs by using a variety of techniques (Table 3). Enzyme histochemistry (e.g. for acid phosphatase), immunocytochemistry with antibodies labeled by biotin-streptavidin-HRP, gold-conjugated antibodies, or quantum-dot conjugated proteins or antibodies are the most commonly used techniques to localize specific proteins to MVBs (Altick et al., 2009; Chu-Wang and Oppenheim, 1980; Holtzman, 1971; Neutra et al., 1985, Vu et al., 2005). Such techniques have the advantage of providing high-resolution spatial information, e.g., whether internal vesicles within MVBs contain proteins of interest, or the limiting membranes of MVBs, or both (Mobius et al., 2003). One can also ask whether MVBs accumulate certain (neuronal) tracers that can be visualized by various methods, e.g. HRP, lectins, utilizing natural electron-dense properties (e.g., quantum dots), or fluorescence that
can be photo-converted to electron dense properties (Fomina et al., 2003; Maranto, 1982; von Barthaeld et al., 1990). The content of MVBs can be further explored by introducing radioactive isotopes such as $^{125}$I into proteins and localizing them by autoradiography at the electron-microscopic level after coating thin sections with a monolayer of photographic emulsion to develop silver grains (von Barthaeld, 2001). This technique allows for quantification of labeling densities between organelles that lend themselves for easy statistical analysis, and group analysis even allows one to enhance practical resolution to distinguish labeling of internal vesicles of MVBs versus label that is localized to the limiting membranes (Rind et al., 2005). Techniques that visualize proteins within MVBs differ significantly in their resolution, sensitivity, and the degree of preservation of structural integrity.

**Physiological and biochemical techniques**—Movement of neuronal MVBs can be examined in vitro by fluorescent label at the light-microscopic level in time-lapse video microscopy, followed by morphological analysis of such identified organelles, including MVBs, at the ultrastructural level (Lively and Brown, 2010; Tsukita et al., 1980). Endosomes, including MVBs, have been examined by biochemical means, i.e., purification followed by Western blots or mass spectrometry (McCaffrey et al., 2009). Finally, structural components of MVBs can be examined by biochemical or molecular techniques to identify MVB components such as exosomes, although concerns about reliability and validation persist (Thery et al., 2002; Traub, 2010).

**Dynamic imaging techniques**—Organelles can be labeled with fluid-phase markers (ovalbumin-gold and/or HRP) and tracked in vitro by time-lapse video, followed by ultrastructural analysis to identify the transported organelle (Parton et al., 1992). Similarly, quantum dot-labeled NGF has been tracked in axons and the endosomes were subsequently identified by electron microscopy, taking advantage of the fact that quantum dots are both fluorescent and electron dense (Cui et al., 2007). Caveats are that the markers used to track the organelle movement may affect the trafficking or sorting (Neutra et al., 1985), and that with current techniques the organelle has to be identified retrospectively as an MVB by electron microscopy. Therefore, an authentic MVB marker would be exceptionally valuable for dynamic imaging studies of neuronal MVBs.

**Genetic/molecular techniques**—Constitutive MVB proteins can be mutated, deleted or altered by transfection or genetic manipulations, and the effects of such manipulations on MVB structure and function can be assessed. Most of these approaches have utilized simple model systems and in-vitro settings (e.g., yeast, tissue culture; Almeida et al., 2006; Cataldo et al., 2008; Liao et al., 2007), but naturally occurring mutations that are of relevance for MVB function can also be studied in human populations (e.g., humans with neurodegenerative disease, Skibinski et al., 2005; Urwin et al., 2010a, b).

In summary, although a variety of morphological techniques are available for analysis of neuronal MVBs, one major handicap is the lack of a sensitive and specific molecular marker that would allow researchers to unambiguously identify MVBs, and possibly distinct types of MVBs in fluorescence and confocal light microscopy. Such a tool would without doubt accelerate the pace of MVB research and allow further insights into this multifaceted organelle.
11. Neuronal MVBs in development, diseases and aging

This section examines how MVBs have been implicated in aging and disease-related processes such as amyloid beta processing in Alzheimer’s, huntingtin in Huntington’s, and prion protein trafficking.

MVBs are essential organelles for normal cell function, but they may also be significant for cells during vulnerable stages (development or aging) and for cells’ reaction to adverse or challenging conditions. Therefore it is not surprising that MVBs are not only present, but may participate in adaptive responses aimed at maintaining homeostasis in developing as well as mature, diseased, and aging neurons.

11.1. Development—MVBs have been described in growth cones of neurites in the PNS (Birks et al., 1972; Bunge, 1977; Estable et al., 1957; Weldon, 1975) and also the CNS (Povlishock, 1976), and in dendrites and cell bodies of developing (embryonic) neurons (Butowt and von Bartheld, 2001; Chu-Wang and Oppenheim, 1980; Rind et al., 2005; Roizin et al., 1967; Vitalis et al., 2008). Mutations and/or knock-out of various components of ESCRT complexes, the machinery necessary for MVB biogenesis and function, have been studied during development in a range of multi-cellular animal models including Drosophila, C. elegans, mouse, and Arabidopsis (Michelet et al., 2010). There are numerous and disparate developmental effects of mutations in ESCRTs, and thus MVBs, in each of these models, possibly reflecting multiple functions of MVBs in different cell types and animal models. In developing neurons of cortex and hippocampus, a specialized vesicle containing markers for recycling endosomes, but not early or late endosomes, has been implicated in growth cone formation and membrane trafficking necessary for neurite outgrowth (Alberts and Galli, 2003; Alberts et al., 2006; Hernandez-Deviez et al., 2007). An additional study identified a novel protein that interacts with Rab11 and is necessary for neuritogenesis and membrane trafficking in neurons isolated from embryonic mouse cerebral cortex (Shirane and Nakayama, 2006). Rab11 is located on recycling endosomes (Rink et al., 2005; Vonderheit and Helenius, 2005) after cargo has been sorted by the MVB, thus this work suggests a role for MVBs in neuron morphogenesis and development. As a caveat, none of these Rab studies analyzed the neurons at the ultrastructural level, and therefore the identity of these vesicles as MVBs, recycling endosomes, or as another type of endosomes is unclear.

In hippocampal neurons, the number of MVBs per spine (MVB density) was higher in younger than in older rats (Cooney et al., 2002). In the developing spinal cord, MVBs were found in myelin layers during ensheathment by oligodendrocytes, but MVBs were not present in myelin layers in the adult spinal cord (Trapp et al., 1989), suggesting a function of glial MVBs during myelin formation. Developmental functions of MVBs have been examined with most scrutiny in Drosophila for notch signaling (Kramer, 2002; Wilkin et al., 2008), and shrub signaling for dendritic arborization (Sweeney et al., 2006). Because few studies have specifically compared the number or distribution of MVBs between different developmental stages, it is not known whether MVB distribution and/or numbers differ significantly in neurons of the developing CNS/PNS as compared with the mature CNS/PNS, and whether MVBs have any specific or unique developmental roles. It is intriguing to speculate that MVBs may provide specialized signaling platforms for crucial morphogenetic events (Traub, 2010).

11.2. Dystrophic neurites—It has been known for over half a century that MVBs are particularly prominent in injured and regenerating axons (Estable et al., 1957; Holtzman and Novikoff, 1965; Inoue, 1983; Kapeller and Mayor, 1969; Smith, 1980; Yagashita, 1979). The phenomenon of MVB accumulation in PNS axons after interruption of axonal transport...
by mechanical ligature or cooling has already been discussed above (see section 3.5 and Edgar et al., 2004; Heerssen et al., 2004; Hirokawa et al., 1990). Dystrophic axons in the PNS of diabetic patients show large numbers of MVBs (Schroer et al., 1992). When CNS axons are deprived of their target, MVBs appear in large numbers in the target-deprived axon terminals as early as four days later and remain at elevated levels for at least 20 days after target ablation (Marty and Peschanski, 1994). In the CNS of genetically epilepsy-prone rat, neuronal somata, dendrites and axons display an abundance of MVBs (Roberts and Ribak, 1988) consistent with an in vivo study simulating epileptic activity (Kraev et al., 2009). This is consistent with earlier studies showing increased MVBs after prolonged (1-5 min) electrical stimulation (Kadota and Kadota, 1982; Kadota et al., 1994) or in Drosophila potassium channel mutants with dramatically increased motoneuron firing and transmitter release (Jia et al., 1993). Likewise, MVBs increase in number when a stoned mutation is introduced in Drosophila, apparently due to defective synaptic vesicle recycling and increased demands on membrane trafficking (Fergestad et al., 1999). Increase of MVB size and/or number thus appears to be a general adaptive response of neurons to injury or insult of axons (see, e.g., Birks et al., 1972; Cavalli et al., 2005; Yagashita, 1979). Our own work, which quantified MVBs in axons, supports this hypothesis by the finding that in normal, immediately fixed and processed axons, MVBs were rare, while in axons that were subjected to a period of hypoxia before fixing, MVBs were significantly increased (Altick et al., 2009).

11.3. Neurodegeneration—Neuronal cell death is the hallmark of numerous diseases, including Parkinson’s, Huntington’s, Alzheimer’s, Niemann-Pick disease, amyotrophic lateral sclerosis, and frontotemporal dementia. MVBs have been found to play a role in all of these pathologies. It is becoming increasingly clear that appropriate and efficient sorting and degradation of cellular proteins is vital to the health of neurons (Nixon et al., 2008). Mutations or disruptions that affect the endosomal-lysosomal pathway, which relies on MVB function, result in aberrant endosomal sorting and possible neuron death (Bronfman et al., 2007; Filimonenko et al., 2007; Lee and Gao, 2008; Liu et al., 2010; Parkinson et al., 2006; Rusten and Simonsen, 2008; Schindowski et al., 2008; Skibinski et al., 2005). However, it has also been postulated that the aggregation of proteins in MVBs and/or late endosomes and lysosomes is actually neuroprotective, by sequestering the aberrant protein (Truant et al., 2008), and cell death may occur via another mechanism that happens to be concurrent with aggregation. Neurons stressed by hypoxia or ischemia (Altick et al., 2009), epileptic discharges (Kraev et al., 2009; Roberts and Ribak, 1988; Schulze-Bonhage et al., 1995), or simply age (de la Roza et al., 1985; Johnson et al., 1975; Nakadate et al., 2006; Schroer et al., 1992) show an increase in size or number of MVBs. Thus, MVBs seem to be a general indicator of neuronal stress, as they are dynamic in both size and frequency. Due to their pivotal role in protein sorting, recycling and degradation, it is not surprising that many brain pathologies are associated with changes in MVBs.

11.3.1. Huntington’s disease: Huntington’s disease (HD) is a progressive neurodegenerative genetic disorder, with degeneration of neurons primarily in the corpus striatum. A role for MVBs in Huntington’s disease was first discovered in 1997, when it was revealed that huntingtin, the protein that is mutated in Huntington’s disease, accumulates in MVBs (Aronin et al., 1999; Sapp et al., 1997). Huntingtin has also been located to other membrane structures, including the plasma membrane, lipid rafts, endoplasmic reticulum, and late endosomes (Atwal and Truant, 2008; Kegel et al., 2000; Valencia et al., 2010). Over-expression of huntingtin in clonal striatal cells of rats stimulates the endosomal-lysosomal pathway as seen by increased tubulation of endosomal membranes and increases in the number, size and morphological variability of autophagic vacuoles and lysosome-like bodies (Kegel et al., 2000). Huntingtin-associated protein, HAP1, interacts with ESCRT-0,
and when over-expressed, causes enlarged endosomes and impaired trafficking of the EGF receptor through MVBs and lysosomes (Li et al., 2002). Similarly, dysfunction of Rab11, which is necessary for trafficking through the recycling endosome, is a feature of HD transgenic mouse models. Without Rab11 activity, the glutamate/cysteine transporter EAAC1 fails to be recycled to the plasma membrane, resulting in oxidative stress and neurodegeneration (Li et al., 2010). It seems that the pathology of HD is closely tied to the MVB function of recycling and sorting cellular proteins and maintaining efficient endosomal-lysosomal trafficking (Kegel et al., 2000; Li et al., 2010; Rusten et al., 2008).

11.3.2. Alzheimer’s disease: The hallmark of Alzheimer’s disease is the degeneration of neurons in the cholinergic basal forebrain as well as cortex. Although MVBs were seen in neurites of senile plaques in early studies (Kidd, 1964), an involvement of MVBs in Alzheimer’s disease was first suggested in the 1970s with the finding that MVBs were much enlarged and their number increased in cortical neurons from the forebrain of Alzheimer’s patients (Paula-Barbosa et al., 1978). Subsequently, MVBs have been found to accumulate amyloid beta 42 (Aβ42, Takahashi et al., 2002), the peptide fragment that is the main constituent of the plaques characteristic of Alzheimer’s disease. Intracellular Aβ42 accumulates in the outer membrane of MVBs prior to synaptic dystrophy (Takahashi et al., 2002; Takahashi et al., 2004), suggesting that intracellular Aβ42 accumulation may be an early and causative step prior to neuronal degeneration (Li et al., 2007). The export (Bulloj et al., 2010; Rajendran et al., 2006) or leakage of Aβ42 from MVBs is now hypothesized to be one or maybe “the” neurotoxic event involved in the pathogenesis of Alzheimer’s disease (Friedrich et al., 2010; LaFerla et al., 2007; Nixon, 2005; Nixon et al., 2005; Offe et al., 2006; Pastorino et al., 2006; Perez et al., 2005; Takahashi et al., 2002; Vingtdeux et al., 2007). Ultrastructural analysis of normal, aging, and transgenic mice shows the accumulation of amyloid-beta in MVBs of neurons (Langui et al., 2004), specifically in pre- and post-synaptic compartments (Takahashi et al., 2002), suggesting that MVBs are involved in the normal routing of amyloid-beta. In a cell culture model for Alzheimer’s disease, amyloid-beta plaques first accumulate within MVBs, then penetrate the MVB membrane to eventually invade the cytoplasm, resulting in cell death and deposition of the aggregate extracellularly (Friedrich et al., 2010). Accumulation of amyloid-beta in MVBs is known to impair protein sorting (Almeida et al., 2006), and deregulated endocytosis initiated by a Rab5 mutation causes downstream perturbations in protein sorting and degradation (Cataldo et al., 2008). The potential significance of MVBs in the development of Alzheimer’s disease may be attributed to their role in appropriate sorting and degradation of amyloid-beta. However, it is not known whether the MVB first fails to process Aβ42, or whether the rapid accumulation of amyloid-beta causes the failure of efficient sorting and degradation, and thus impairs the clearance of amyloid-beta aggregates, or if some other cellular dysfunction occurs simultaneously with amyloid-beta aggregation.

11.3.3. Frontotemporal Dementia: Frontotemporal dementia (FTD) describes a partially heritable, diverse, neurodegenerative disease that targets both the frontal and temporal lobes. Neuropathology of this disease shows an overall cortical and central atrophy, and a majority of cases have either tau or ubiquitin pathology (Urwin et al., 2010b). The correlation of a disruption in ESCRT functions with neurodegeneration has recently linked numerous neuropathologies (Lee et al., 2007; Lee and Gao, 2008; Rusten and Simonsen, 2008). ESCRTs are necessary for MVB biogenesis and function (see sections 5 and 8), and ESCRTs are necessary for proper autophagy (Filimonenko et al., 2007; Lee et al., 2007). MVBs are part of both the endosomal-lysosomal and autophagosomal systems (Fader and Colombo, 2009; Yi and Tang, 1999), thus functional MVBs are critical for neuronal viability. MVB function has been linked to FTD via a mutation in CHMP2B, a subunit of ESCRT-III (Skibinski et al., 2005). Recent work shows that CHMP2B mutations cause abnormal MVBs and prevent fusion of MVBs with lysosomes in fibroblasts from FTD.
patients (Urwin et al., 2010a), thus suggesting a direct role of MVBs in FTD pathology. Additionally, several variations of CHMP2B mutations have been found in other familial pedigrees of FTD (van der Zee et al., 2008). Studies on a human neuroblastoma cell line using mutant CHMP2B proteins (van der Zee et al., 2008) showed similar aberrant endosomal structures as seen previously in fibroblasts from FTD patients (Urwin et al., 2010a). Thus, this line of work suggests that FTD is likely the result of loss of proper MVB function.

**11.3.4. Niemann-Pick Disease:** Niemann-Pick disease is a fatal inherited metabolic disorder that affects the CNS as well as other organs. Symptoms reflect abnormal lipid storage in certain types of neurons, e.g. ataxia and dementia are seen when cerebellum and cortex are affected, respectively. Unlike Parkinson’s, Huntington’s, and Alzheimer’s disease, proteins do not tend to aggregate, instead lipids accumulate in late endosomes or MVBs and lysosomes (Chiulli et al., 2007; Liao et al., 2007; Lusa et al., 2001). Three genes are known to cause Niemann-Pick disease type C; NPC1 and NPC2 (Ko et al., 2001; Lusa et al., 2001) and sphingomyelin phosphodiesterase (SMPD1, Sikora et al., 2003; Simonaro et al., 2002). These three genes encode proteins that function in the processing of membrane components, namely cholesterol (Liao et al., 2007; Liu et al., 2010; Lusa et al., 2001) and sphingomyelin (Chiulli et al., 2007; Jones et al., 2008). For NPC1 and NPC2 the build-up of cholesterol in late endosomes and lysosomes is thought to be due to a failure to recycle LDL-cholesterol to the plasma membrane (Lusa et al., 2001; Pentchev et al., 1984). For SMPD1, which resides in membranes of late endosomes (Jones et al., 2008), the mechanism that leads to neurodegeneration has been suggested to involve a build-up of a sphingomyelin metabolite, which then activates astrocytes, creating a chain of events resulting in neurotoxicity (Chiulli et al., 2007). In Niemann-Pick pathology, the sorting system seems to be functional, but the specific carriers and enzymes responsible for the processing of cargo and membrane components malfunction, and thus MVB function is compromised due to aberrant cargo delivery, membrane recycling, and protein processing (Devlin et al., 2010).

**11.4. Toxic or drug insults**—Drugs can be neurotoxic, either when they are introduced intentionally as for chemotherapy, or inadvertently as through pesticides and occupational exposure. Ultrastructural studies on neuronal responses to drug insults commonly report a set of abnormalities that are “typical features of neurodegeneration”, which includes an increased number and/or size of MVBs (Lampert, 1967; Lieberman, 1971). Neurons contained an increased number of MVBs when they were exposed to toxic levels of aluminum (Deloncle et al., 2001), acrylamide with axonal ligation (Cavanagh and Gysbers, 1980, 1981; Schaumburg et al., 1974), to the pesticide dichlorodiphenyltrichloroethane (DDT) (Williams and Chung, 1987), or to hydrocarbon solvents (reviewed in Spencer et al., 2002). In a study of antibiotic-treated cultured astrocytes, abnormal mitochondria and MVBs were noted. In support of the hypothesis that the antibiotics added to the culture medium were responsible for the structural defects, it was found that abnormalities increased with increasing doses of antibiotics, gentamicin more so than streptomycin (Robert and Hevor, 2007). Inhibitors specific for cathepsin B and L were found to selectively kill (via apoptosis) neuroblastoma cells, and apoptosis was preceded by an accumulation of MVBs in the cytoplasm (Colella et al., 2010). When neurons are exposed to alcohol, MVBs increase in size or frequency, as documented for adult rat hippocampal cells after chronic alcohol consumption (Paula-Barbosa et al., 1986), cultured mouse sensory neurons exposed to either ethanol or acetaldehyde (Smith and Wubetu, 1991), and Purkinje cells from fetal rats whose mothers were given alcohol (Koksal et al., 2005). Ventral tegmental neurons of rats exposed to morphine, either acutely or chronically, showed reduced recycling and degradation of glutamate receptors, possibly due to decreased numbers of MVBs in postsynaptic dendrites (Lane et al., 2008). Neuronal toxicity via drug or alcohol results in cellular adaptations,
which are typical of neuronal responses to stress, injury, or disease. This includes changes in the size or number of MVBs, perhaps reflecting a compensatory mechanism for altered demand on sorting, recycling, or degradation. It remains to be determined if MVBs are a direct target of the toxin, or if the alterations of the MVB pathway reflect adaptive responses of neurons.

11.5. Virus and prion transmission—Viruses use the endosomal-lysosomal system to both enter and exit a cell (Calistri et al., 2009; Le Blanc et al., 2005; McDonald and Martin-Serrano, 2009; Morita and Sundquist, 2004). Viruses can also use cellular organelles as sites for replication (Chu et al., 2009; Dilley et al., 2010; Joshi et al., 2009; Novoa et al., 2005). The cytopathic vacuoles (CPVs), “virus factories,” harbor some of the same molecular markers that are used to identify late endosomes and lysosomes, leading to the idea that CPVs are formed from endosomal or lysosomal organelles (Kujala et al., 2001). In neurons, virus- and cell type-specific evidence indicates that viruses recruit the endocytosis machinery to gain entry into cells and then exploit ESCRTs, internal vesicle formation, and exosome release by MVBs. The alpha herpesvirus pseudorabies virus that is known to infect neurons has been located to “neurovesicles” in primary neuron cultures (Maresch et al., 2010). CVS-11, an experimental rabies virus model, causes neurodegenerative changes with incorporation of MVBs into autophagosomes in dorsal root ganglion neurons (Rossiter et al., 2009). A study on HIV-1 neuropathology has shown that the HIV-1 TAT protein binds to low-density lipoprotein receptor, the same receptor used by amyloid precursor protein, and thus uses the endosomal-lysosomal system to enter neurons via receptor-mediated endocytosis (Liu et al., 2000). HIV-1 nef protein has since been shown to actively interact with CD4 on the surface of HeLa cells and target CD4 to MVBs for degradation, thus down-regulating CD4 (daSilva et al., 2009). In rat primary hippocampal neurons, HIV-1 TAT protein promotes the secretion of Aβ42 from the cell and the release of Aβ42 aggregates from the cell surface (Aksenov et al., 2010). HIV-1 exacerbates Alzheimer’s disease (AD) pathology in a mouse model for AD (Giunta et al., 2009), and, as discussed above in section 11.3.2, AD pathology involves MVBs. Ultrastructural evidence links HIV-1 proteins with MVBs in a range of physiologically relevant cell types (but not neurons), and shows that MVBs can support virus assembly (Joshi et al., 2009). Additionally, HIV-1 neuropathology may be indirectly linked to MVBs via amyloid-beta interactions, low-density lipoprotein receptor, or by reducing the CD4 surface expression by shunting CD4 to MVBs for eventual lysosomal degradation.

Prion diseases, such as scrapie and Creutzfeldt-Jakob disease, are incurable neurodegenerative diseases. The cellular prion protein (PrP<sup>C</sup>) takes on a toxic “scrapie” conformation (PrP<sup>SC</sup>), which eventually results in neuron death, causing large spongiform vacuoles in brain tissue. In brains from normal and infected animals, electron microscopy has shown PrP<sup>C</sup> and PrP<sup>SC</sup> located in MVBs as well as other cellular membranes and structures (Ersdal et al., 2009; Laine et al., 2001; Laszlo et al., 1992). In the infected animals, the MVBs have been reported to be abnormal and increased in number (Ersdal et al., 2009; Laszlo et al., 1992). In studies that specifically track PrP<sup>C</sup> in neurons, a variety of methods have been used to locate PrP<sup>C</sup> and/or PrP<sup>SC</sup>. Ultrastructural analysis of radiolabeled PrP<sup>C</sup> showed labeled MVBs in the neuronal soma (Butowt et al., 2006), by Western blotting of neuronal cell lysates and culture media, PrP<sup>C</sup> was located to exosomes assumed to be released by MVBs (Faure et al., 2006; Fevrier et al., 2004), and fluorescent microscopy showed colocalization of specific markers of endosomal compartments, namely PrP<sup>SC</sup> in compartments positive for Rab7, a marker for late endosomal MVB, (but see discussion above) (Magalhaes et al., 2005). MVBs in mouse hippocampal neurons (Mironov et al., 2003), and mouse hippocampal cellular layers and neuropil (Godsave et al., 2008) have been shown to contain gold-labeled PrP<sup>C</sup> and/or PrP<sup>SC</sup>, however MVB abnormalities were not noted in these studies. In a similar study, a PrP<sup>C</sup> protease-resistant peptide was not
associated with MVBs when fluorescent microscopy was used to colocalize the marker Lamp-1 with the peptide in a neuroblastoma cell line (Wilson et al., 2007). Again, using endosomal compartment markers, in this case Lamp-2, to mark lysosomes, PrP<sup>Sc</sup> fibrils were identified in lysosomes in sensory neurons (Jen et al., 2010), but not within MVBs. The discrepancies in findings could be due to the form of PrP<sup>Sc</sup> being used, i.e. peptide or fibrils, or due to the ambiguity of markers used for endosomal/lysosomal compartments, as well as the difficulty in exactly determining co-localization using fluorescence. However, altogether, the data regarding PrP<sub>C</sub> and the toxic form, PrP<sup>Sc</sup>, support roles for MVBs in prion trafficking, processing, and function. The ultrastructural studies confirm that MVBs contain prion protein, both in its native conformation (Butowt et al., 2006; Laine et al., 2001; Mironov et al., 2003) as well as in its pathogenic conformation (Godsave et al., 2008; Marijanovic et al., 2009). The findings that MVBs are both abnormal and increased in the prion disease state (Ersdal et al., 2009; Laszlo et al., 1992) strongly suggest a role for MVBs in the progression of prion pathology.

11.6. Aging—Aging of normal neurons is associated with degenerative changes. An increase in MVBs is a common finding in degenerating neurons. Studies that focused on neuronal changes at the ultrastructural level have noted an increase in MVBs along with other signs of neuron degeneration. An increase in MVBs was found in aging mouse neuromuscular junctions (Boaro et al., 1998), dorsal column nuclei (Johnson et al., 1975), and axon terminals of sympathetic ganglia (Schmidt et al., 2008). Similar work in rat neurons (Jung et al., 1999; Nakadate et al., 2006) and oligodendrocytes (de la Roza et al., 1985) also found an increase or accumulation of MVBs along with other degenerative changes in cell bodies or processes. An increase in MVBs as a response to aging, specifically degeneration, was also noted in monkeys (Townes-Anderson and Raviola, 1978) and in humans (Schroet et al., 1992). However, in monkeys, the differences noted in autonomic neuromuscular junctions were not correlated to the age of the monkey, but rather to the state of degeneration or regeneration of the axon. These findings are in line with two studies that found no changes in MVBs, as measured by relative or absolute volume when comparing Purkinje cells (Monteiro, 1991) or neurons of the auditory cortex (Vaughan and Vincent, 1979) in young and old rats. Ultrastructural changes in aged brains, specifically an increase in MVBs in neurons from aged animals, are most likely related to neurodegenerative changes that occur with aging. It is becoming increasingly clear that neurodegeneration and the concurrent structural changes in the endosomal-lysosomal system inherent in the aging process are the basis for many neurodiseases in the elderly (Nixon et al., 2008).

12. Outlook

This review has summarized compelling evidence for multiple ways in which neuronal MVBs contribute to important cell biological functions related to protein transport, sorting and signaling. However, our review has also revealed numerous gaps in knowledge that require further studies. We expect to see progress in the coming years in neuronal MVB research in particular to address the following topics: In what way and to what extent are neuronal MVBs used to transport proteins – possibly in a neuron-specific fashion – between different neuronal compartments? And, in a related question, do MVB axonal and dendritic subtypes exist that have distinct morphological and functional specializations? How are the internal vesicles and their protein content released – to what extent does backfusion and exosome release contribute? Is there, and if so, to what extent, a physiological role of retrograde axonal transport for MVBs? How do neuronal MVBs contribute to prion transport and dissemination within the nervous system? What is the relevance of MVBs in Schwann cells for myelination? What is the precise role of neuronal MVBs in responses of neurons to stress and dystrophic conditions? What is their role in the sequence of
pathophysiological events, and could this be a target for therapeutic improvement? Most importantly, how do neuronal MVBs process amyloid-beta and why do they seem to fail to process this molecule in neurodegenerative diseases such as Alzheimer’s disease? How could better knowledge and appreciation of MVB’s role in this process be used to design novel therapies for this type of disease? For each of the above questions, it would be tremendously helpful to find a sensitive and truly specific marker for each functional stage of MVBs, so that these organelles can be definitively identified at the light-microscopic, and not only the ultrastructural level. We hope that our review will contribute to a better understanding of the roles that MVBs play in cellular housekeeping functions to promote neuronal integrity and function, so that neuronal MVBs do not remain the neglected stepchild among MVBs as in current research model systems.

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Abbreviation List

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>Aβ42</td>
<td>amyloid-beta 42 peptide</td>
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<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
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<tr>
<td>ESCRT</td>
<td>endosomal sorting complex required for transport</td>
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<tr>
<td>FA</td>
<td>fractional area</td>
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<tr>
<td>HD</td>
<td>Huntington’s Disease</td>
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<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
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<tr>
<td>LBPA</td>
<td>lysobisphosphatidic acid</td>
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<tr>
<td>MVB</td>
<td>multivesicular body</td>
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<tr>
<td>NGF</td>
<td>nerve growth factor</td>
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<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
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Fig. 1. **A-E Examples of multivesicular bodies (MVBs) in neurons at the ultrastructural level**

**A.** Two profiles of MVBs (arrows) in a neuronal soma. The MVBs have a single limiting outer membrane, unlike the double membrane of mitochondria (M). The MVBs contain internal vesicles of homogeneous size.  

**B.** This MVB is located near the cell nucleus (N), the endoplasmic reticulum (ER) and the Golgi apparatus (G).  

**C and D.** Dendritic MVBs in close vicinity of presynaptic terminals (S), a preferred location of MVBs (Rind et al., 2005). Note the varying amount of internal vesicles within MVBs. The internal vesicles in the dendritic MVB have a similar diameter as the synaptic vesicles (SV).  

**E.** MVB within a myelinated axon; my = myelin sheath. All images are from postnatal rat brain hypoglossal motoneurons. Scale bars A-E = 250 nm.
Fig. 2. A-D. Trophic factors accumulate in neuronal MVBs of the soma and dendrites after axonal transport

A. The radiolabeled trophic factor GDNF, visualized by an autoradiographic silver grain, localizes within an MVB of a hypoglossal motoneuron dendrite after retrograde axonal transport of the GDNF from the tongue muscle and after trans-somal transport, likely within an MVB, to reach the dendrite. B. In the soma, radiolabeled trophic factor BDNF localizes in a MVB after retrograde transport from motoneuron terminals. C. MVBs located in close vicinity of postsynaptic densities of a synapse (S) contain the radiolabeled trophic factor GDNF that arrived there by retrograde axonal transport, followed by transport across the soma and apparently along microtubules (mt) into the dendrite. D. Example of a dendritic MVB that accumulated the radiolabeled trophic factor NT-3, following internalization of this trophic factor by retinal ganglion cells and anterograde axonal transport along the optic nerve to the midbrain tectum. The radiolabeled NT-3 crossed the retinotectal synapse to accumulate in an MVB within the postsynaptic dendrite. Panel D was reproduced with permission (von Bartheld et al., 1996). Abbreviations: M, mitochondrion; mt, microtubule; S, synapse. Scale bars A-D = 200 nm.
Fig. 3. A-C. Neurotrophic factors reside primarily on the outer membrane of MVBs in dendrites, but inside MVBs located in the soma. 
A. Example of a silver grain (asterisk) representing the trophic factor BDNF residing on the outer membrane of a dendritic MVB. B. Example of a silver grain representing the trophic factor GDNF residing on the inside (asterisk) of a somal MVB. C. The location of silver grains was quantified in a histogram for a total of 119 silver grains representing either radiolabeled BDNF or GDNF that were injected in the tongue muscle and transported retrogradely to the soma and dendrites of hypoglossal motoneurons (Rind et al., 2005). The method (LaVail et al., 1983) relies on measuring the distance of the center of silver grain from the outer membrane, plotted separately for dendritic and somatic MVBs. The data for BDNF and GDNF was combined, as there was no significant difference between the two data sets. Somal and dendritic MVBs showed statistically significant differences in trophic factor location. Panel C was reproduced with permission (Rind et al., 2005). Scale bars in A, B = 250 nm.
Fig. 4. MVB biogenesis and three possible MVB sorting pathways: exosome release (1), back fusion/recycling (2), and degradation (3)

Left side: Endosome formation with membrane invagination to form internal vesicles.

Center: The MVB may progress through one of three maturation or sorting stages: (1) The limiting membrane of the MVB may fuse with the plasma membrane and release internal vesicles as exosomes. (2) The MVB may extend tubular processes formed by membranes from internal vesicles. The tubular extensions “back-fuse” to insert limiting membrane into the cell surface membrane, thereby recycling ligand/receptor to the plasma membrane. (3) MVBs can target internalized ligand/receptors for degradation in the lysosome either by fusion with lysosomes or maturation into a lysosome. None of these three pathways have been directly demonstrated in neurons, but there is suggestive evidence from non-neuronal cell types. For details on exosomes and back fusion, see Faure et al., 2005; Fevrier et al., 2004; Harding, et al., 1983; Murk et al., 2002; and Putz et al., 2008. For maturation, see Murphy, 1991; van Deurs et al., 1993; for fusion/sorting, see Gruenberg et al., 1989; Mullock et al., 1998; Vonderheit & Helenius 2005. Red square: ligand; blue dot: cytoplasmic domain of receptor; black: transmembrane domain of receptor; white circle: internal vesicle.
Fig. 5. A-D. Dynamic MVB morphology suggestive of biogenesis and recycling
Examples of neuronal MVBs with invaginations (A) or tubular extensions (B-D). The
limiting membranes and internal vesicles are outlined for clarity in the lower panel, with
arrows pointing to the invagination or tubular extension. These MVB forms indicate
biogenesis or membrane recycling according to the biogenesis and back-fusion models of
MVB functions (Murk et al., 2002). Note that the MVB is nearly devoid of internal vesicles
in the area next to the tubular extension, consistent with the notion that the tubular
extensions are generated by fusion of internal vesicles with the limiting membrane. For
other examples of neuronal MVBs in transition, see Roizin et al., 1967. Scale bar A-D = 250
nm.
Fig. 6. Synopsis of known and potential MVB trafficking pathways in neurons
Different types of MVBs may be involved in different trafficking patterns within neurons, numbered from 1 to 4 as indicated in the legend. Routes are based on presumed or known pathways (1-3, solid lines) as shown in studies tracking internalized markers and their localization within neuronal MVBs. There is no indication that MVBs move along axons in the anterograde direction (4, stippled); all other pathways are either proven or likely (solid lines). Evidence is primarily based on Altick et al., 2009; Cooney et al., 2002, LaVail and LaVail, 1974; Parton et al., 1992; and Rind et al., 2005.
### TABLE 1

History of major concepts of MVBs, focus on neurons, with major discoveries in non-neuronal cells included.

<table>
<thead>
<tr>
<th>Year</th>
<th>Authors</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1955</td>
<td>Palay &amp; Palade</td>
<td>First description of MVBs – coincidentally in neurons</td>
</tr>
<tr>
<td>1957</td>
<td>Estable et al.</td>
<td>Name “multivesicular body” (MVB) introduced</td>
</tr>
<tr>
<td>1961</td>
<td>Pappas &amp; Purpura</td>
<td>MVBs are associated with postsynaptic sites</td>
</tr>
<tr>
<td>1964</td>
<td>Rosenbluth &amp; Wissig</td>
<td>MVBs take up macromolecules</td>
</tr>
<tr>
<td>1965</td>
<td>Holtzman &amp; Novikoff</td>
<td>MVBs are prelysosomal and contain acid phosphatases</td>
</tr>
<tr>
<td>1974</td>
<td>LaVail &amp; LaVail</td>
<td>MVBs are carriers for retrograde axonal HRP transport</td>
</tr>
<tr>
<td>1979</td>
<td>Haigler et al.</td>
<td>MVBs move cargo to internal vesicles</td>
</tr>
<tr>
<td>1983</td>
<td>Harding et al.</td>
<td>MVBs release exosomes</td>
</tr>
<tr>
<td>1990</td>
<td>Felder et al.</td>
<td>MVBs sort endocytosed EGF receptor</td>
</tr>
<tr>
<td>1990</td>
<td>Chavrier et al.</td>
<td>Rabbs mark specific endosomal compartments</td>
</tr>
<tr>
<td>1991</td>
<td>Stoorvogel et al.</td>
<td>MVBs are formed by endosome maturation</td>
</tr>
<tr>
<td>1992</td>
<td>Parton et al.</td>
<td>MVBs transport between nerve terminals and cell body</td>
</tr>
<tr>
<td>1995</td>
<td>Nixon &amp; Cataldo</td>
<td>Review: Endosomal-lysosomal system involved in neuropathologies</td>
</tr>
<tr>
<td>1996</td>
<td>Futter et al.</td>
<td>MVBs fuse with lysosomes</td>
</tr>
<tr>
<td>1997</td>
<td>Saito et al.</td>
<td>KIFC2 is a neuron-specific kinesin associated with dendritic MVBs</td>
</tr>
<tr>
<td>2000</td>
<td>Denzer et al.</td>
<td>Review: Exosome – intercellular signaling device</td>
</tr>
<tr>
<td>2001</td>
<td>Piper &amp; Luzio</td>
<td>Review: MVB markers, morphogenesis and function</td>
</tr>
<tr>
<td>2001</td>
<td>Katzmann et al.</td>
<td>ESCRT-I associates with MVBs in mammalian cells</td>
</tr>
<tr>
<td>2002</td>
<td>Murk et al.</td>
<td>Review: Back-fusion pathway of MVBs in non-neuronal cells</td>
</tr>
<tr>
<td>2002</td>
<td>Cooney et al.</td>
<td>MVBs serve multiple dendritic spines by recycling cargo</td>
</tr>
<tr>
<td>2002</td>
<td>Takahashi et al.</td>
<td>MVBs accumulate amyloid beta – cause of Alzheimer’s?</td>
</tr>
<tr>
<td>2005</td>
<td>Skibinski et al.</td>
<td>ESCRT mutation causes MVB malfunction and dementia</td>
</tr>
<tr>
<td>2005</td>
<td>Rind et al.</td>
<td>MVBs recycle target-derived trophic factors at synapses</td>
</tr>
<tr>
<td>2006</td>
<td>Faure et al.</td>
<td>Cultured neurons release exosomes</td>
</tr>
<tr>
<td>2007</td>
<td>Filimonenko et al.</td>
<td>Failure of MVBs to clear protein aggregates causes neurodegenerative disease</td>
</tr>
<tr>
<td>2008</td>
<td>Nixon et al.</td>
<td>Review: MVB’s role in neurodegenerative diseases</td>
</tr>
<tr>
<td>2008</td>
<td>Hurley</td>
<td>Review: ESCRTs role in MVB biogenesis</td>
</tr>
</tbody>
</table>
### TABLE 2

MVBR fractional areas in neuronal and glial compartments in vivo and in vitro.

<table>
<thead>
<tr>
<th>Compartment</th>
<th>Fractional Area</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>in vivo</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adult Rat Motoneuron Soma</td>
<td>0.6%</td>
<td>Schwab and Thoenen, 1976</td>
</tr>
<tr>
<td>Adult Rat Adrenergic Ganglion Soma</td>
<td>0.2% - 0.4%</td>
<td>Schwab and Thoenen, 1977</td>
</tr>
<tr>
<td>Adult Rat Pyramidal Cell Soma</td>
<td>0.2-0.6%</td>
<td>Paula-Barbosa et al., 1986</td>
</tr>
<tr>
<td>Adult Lizard Schwann Cell Soma</td>
<td>0.5-0.9%</td>
<td>Paula-Barbosa et al., 1986</td>
</tr>
<tr>
<td>Adult Lizard Schwann Cell Soma</td>
<td>0.4%</td>
<td>Pannese et al., 1988a</td>
</tr>
<tr>
<td>Adult Lizard Schwann Cell Soma</td>
<td>0.2%</td>
<td>Pannese et al., 1988b</td>
</tr>
<tr>
<td>Adult Cat PNS Axon</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Internodal</td>
<td><em>ROA= 0</em></td>
<td>Berthold et al., 1993</td>
</tr>
<tr>
<td>Paranode-node-paranode</td>
<td><em>ROA=60</em></td>
<td>Berthold et al., 1993</td>
</tr>
<tr>
<td>P5 Rat Motoneuron Soma</td>
<td>1.5% - 1.7%</td>
<td>Rind et al., 2005</td>
</tr>
<tr>
<td>P5 Rat Motoneuron Dendrite</td>
<td>0.5% - 1.4%</td>
<td>Rind et al., 2005</td>
</tr>
<tr>
<td>P7 Rat Motoneuron Axon</td>
<td>0.013% - 0.039%</td>
<td>Altick et al., 2009</td>
</tr>
<tr>
<td><strong>in vitro</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neonatal Rat SCG Soma</td>
<td>0.35% - 0.4%</td>
<td>Claude et al., 1982a</td>
</tr>
<tr>
<td></td>
<td>0.5% - 1.8%</td>
<td>Claude et al., 1982b</td>
</tr>
<tr>
<td>Neonatal Rat SCG Soma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>With 0.05 mM Chloroquin</td>
<td>0.04%</td>
<td>Claude et al., 1982b</td>
</tr>
<tr>
<td>With 10 mM Methylamine</td>
<td>3.6%</td>
<td>Claude et al., 1982b</td>
</tr>
<tr>
<td>PC12 Cell Soma in vitro</td>
<td>0.12% - 0.29%</td>
<td>Stieber et al., 1984</td>
</tr>
</tbody>
</table>

*ROA, relative organelle abundance within specific axonal region SCG, superior cervical ganglion; PC12, pheochromocytoma 12 cell line
### TABLE 3
Accumulation of endogenous and exogenous cargo molecules in neuronal MVBs.

<table>
<thead>
<tr>
<th></th>
<th>ENDOGENOUS</th>
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</thead>
<tbody>
<tr>
<td><strong>Trophic factors/receptors</strong></td>
<td></td>
</tr>
<tr>
<td>Neurotrophin receptors</td>
<td>Pioro et al., 1991; Martinez-Murillo 1991; Valdez et al., 2005</td>
</tr>
<tr>
<td>Neurotrophic factors</td>
<td></td>
</tr>
<tr>
<td>NGF</td>
<td>Claude et al., 1982^a,b, Sandow et al., 2000; Butowt and von Bartheld, 2009</td>
</tr>
<tr>
<td>BDNF</td>
<td>Butowt and von Bartheld, 2001, 2009</td>
</tr>
<tr>
<td>NT3</td>
<td>von Bartheld et al., 1996; Schnapp, 1997</td>
</tr>
<tr>
<td>NT4</td>
<td>Butowt and von Bartheld, 2009</td>
</tr>
<tr>
<td>IGF</td>
<td>Aguado et al., 1992</td>
</tr>
<tr>
<td>GDNF</td>
<td>Rind et al., 2005</td>
</tr>
<tr>
<td><strong>Receptors for transmitters</strong></td>
<td></td>
</tr>
<tr>
<td>Acetylcholine receptors</td>
<td>Bernard et al., 1998; Liste, 2002</td>
</tr>
<tr>
<td>Ion channels/Glutamate receptors</td>
<td>Baude et al., 1994</td>
</tr>
<tr>
<td>Adrenergic receptors</td>
<td>Milner et al., 1998</td>
</tr>
<tr>
<td>Cannabinoid receptors</td>
<td>Vitalis et al., 2008</td>
</tr>
<tr>
<td><strong>Transporters (dopamine/monoamine)</strong></td>
<td>Nirenberg et al., 1996; Hersch et al., 1997; Dehnes et al., 1998; Agulhon et al., 2003; Bloch et al., 2003; Grimes and Miettinen, 2003;</td>
</tr>
<tr>
<td><strong>Neuropeptides</strong></td>
<td></td>
</tr>
<tr>
<td>Neurotensin</td>
<td>Castel et al., 1992; Delle Donne et al., 1996</td>
</tr>
<tr>
<td>CGRP (Calcitonin-gene-related-protein)</td>
<td>Caldero et al., 1992</td>
</tr>
<tr>
<td>Substance P</td>
<td>Pickel et al., 1988</td>
</tr>
<tr>
<td><strong>Calcium</strong></td>
<td>Csilik and Knyihar-Csilik, 1980; Fifkova et al., 1983; Zareba-Kowalska et al., 1990; Buchs et al., 1994</td>
</tr>
<tr>
<td><strong>Miscellaneous Proteins</strong></td>
<td></td>
</tr>
<tr>
<td>Interphotoreceptor retinoid-binding protein</td>
<td>Hollyfield et al., 1985</td>
</tr>
<tr>
<td>NEEP21 (endosomal protein)</td>
<td>Utvik et al., 2008</td>
</tr>
<tr>
<td>LR11 (lipoprotein receptor)</td>
<td>Offe et al., 2006</td>
</tr>
<tr>
<td>Amyloid (Abeta 40, 42)</td>
<td>Takahashi et al., 2002, 2004</td>
</tr>
<tr>
<td>Prion proteins</td>
<td>Laine et al., 2001; Mironov et al., 2003; Vella et al., 2008</td>
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<tr>
<td>SV2 (synaptic vesicle protein)</td>
<td>Wittich et al., 1994</td>
</tr>
<tr>
<td>Synaptophysin</td>
<td>Kadota et al., 1994</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>EXOGENOUS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tracers</strong></td>
<td></td>
</tr>
<tr>
<td>HRP (horseradish peroxidase)</td>
<td>Holtzman and Peterson, 1969; LaVail and LaVail, 1974; Bunge, 1977; LaVail et al., 1980; Chu-Wang and Oppenheim, 1980; Berthold, 1982; Graffstein, 1980</td>
</tr>
<tr>
<td>Lectins</td>
<td>Broadwell 1985; Steindler and Cooper, 1986</td>
</tr>
<tr>
<td>Ferritin</td>
<td>Rosenbluth and Wissig, 1964; Birks et al., 1972; Bunge, 1977; Richardson et al., 1997</td>
</tr>
<tr>
<td>Thorium dioxide</td>
<td>Birks et al., 1972; Weldon, 1975</td>
</tr>
<tr>
<td><strong>Toxins</strong></td>
<td></td>
</tr>
<tr>
<td>Tetanus toxin</td>
<td>Schwab and Thoenen, 1976, 1977; Parton et al., 1987</td>
</tr>
<tr>
<td>Substance</td>
<td>Reference/Year</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>----------------</td>
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<tr>
<td>Ethanol</td>
<td>Paula-Barbosa et al., 1986; Koksal, 2005</td>
</tr>
<tr>
<td>Capsaicin</td>
<td>Hiura, 1989</td>
</tr>
<tr>
<td>Mutant huntingtin</td>
<td>Sapp et al., 1997; Aronin et al., 1999</td>
</tr>
<tr>
<td>Aminoglycoside antibiotics</td>
<td>de Groot et al., 1990</td>
</tr>
<tr>
<td>Viruses*</td>
<td>Uchil, 2005; Morita and Sunquist, 2004; Ashok and Hegde, 2006; Chu et al., 2009</td>
</tr>
</tbody>
</table>

*Viruses that can affect neurons, but examples are from non-neuronal cells.