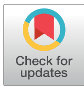

thematic review series

Thematic Review Series: Exosomes and Microvesicles: Lipids as Key Components of their Biogenesis and Functions

Role of sphingolipids in the biogenesis and biological activity of extracellular vesicles

Claudia Verderio,[1,*,†] Martina Gabrielli,[*] and Paola Giussani[§]

Consiglio Nazionale delle Ricerche (CNR) Institute of Neuroscience,* 20129 Milano, Italy; Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Humanitas,† 20089 Rozzano, Italy; and Department of Biotechnology and Translational Medicine,§ University of Milano, 20090 Segrate, Italy

ORCID ID: 0000-0001-7216-5873 (C.V.)

Abstract Extracellular vesicles (EVs) are membrane vesicles released by both eukaryotic and prokaryotic cells; they not only serve physiological functions, such as disposal of cellular components, but also play pathophysiologic roles in inflammatory and degenerative diseases. Common molecular mechanisms for EV biogenesis are evident in different cell biological contexts across eukaryotic phyla, and inhibition of this biogenesis may provide an avenue for therapeutic research. The involvement of sphingolipids (SLs) and their enzymes on EV biogenesis and release has not received much attention in current research. Here, we review how SLs participate in EV biogenesis by shaping membrane curvature and how they contribute to EV action in target cells. First, we describe how acid and neutral SMases, by generating the constitutive SL, ceramide, facilitate biogenesis of EVs at the plasma membrane and inside the endocytic compartment. We then discuss the involvement of other SLs, such as sphingosine-1-phosphate and galactosyl-sphingosine, in EV formation and cargo sorting. Last, we look ahead at some biological effects of EVs mediated by changes in SL levels in recipient cells.—Verderio, C., M. Gabrielli, and P. Giussani. **Role of sphingolipids in the biogenesis and biological activity of extracellular vesicles.** ***J. Lipid Res.*** **2018.** 59: **1325–1340.**

Supplementary key words exosomes • ectosomes • microvesicles • ceramide • sphingosine-1-phosphate • psychosine • sphingomyelinase • endocannabinoids

Extracellular vesicles (EVs) are membrane vesicles released by both eukaryotic and prokaryotic cells, such as Gram-positive and -negative bacteria, mycobacteria, and Archaea (1). EVs are highly heterogeneous in size, structure, and molecular content (2). While the release of bacterial EVs is predominantly by direct outward "blebbing" and pinching of the external cellular membranes (2) in eukaryotic cells, EVs are generated both at the plasma membrane [ectosomes, also called shed microparticles or microvesicles (MVs)] or inside multivesicular bodies (MVBs) (exosomes) through two membrane inversion processes: the inward budding of the limiting membrane of the multivesicular endosome to form intraluminal vesicles and the fusion of MVBs with the plasma membrane. As a result, both ectosomes and exosomes have the same membrane topology of donor cells. However, EVs, especially ectosomes, often lose transmembrane lipid asymmetry and have phosphatidylserine (PS) residues externalized in the outer leaflet of the vesicle (3–5).

Plasma membrane-derived EVs are on average larger than exosomes in size, ranging from 100 to 300 nm up to 1 μm, while exosomes are generally 30–100 nm in diameter and are isolated by centrifugation at higher speeds compared with ectosomes (100,000 *g* vs. 10,000 *g*). Both types of EVs are recognized as mediators of cell-to-cell communication due to their ability to transfer biomolecules among cells (6–9) and to influence the extracellular microenvironment

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Abbreviations: AD, Alzheimer's disease; AEA, *N*-arachidonoylethanolamine (anandamide); a-SMase, acid SMase; CB_1, endocannabinoid type 1 receptor; CDase, ceramidase; Cer, ceramide; CS, cigarette smoke; eCB, endocannabinoid; ER, endoplasmic reticulum; ESCRT, endosomal sorting complex required for transport; EV, extracellular vesicle; GSL, glycosphingolipid; HOG, human oligodendroglioma; IL, interleukin; mEPSC, miniature excitatory postsynaptic current; MV, microvesicle; MVB, multivesicular body; n-CDase, neutral ceramidase; n-SMase, neutral SMase; NOE, *N*-oleoylethanolamine; PC, phosphatidylcholine; PLP, proteolipid protein; PS, phosphatidylserine; RBC, red blood cell; SK, sphingosine kinase; SL, sphingolipid; SMPD, SM phosphodiesterase; S1P, sphingosine-1-phosphate; $S1P_{1–4}$, sphingosine-1-phosphate types 1–4 receptor; Sph, sphingosine; TRPV1, transient potential receptor vanilloid type 1.

[1] To whom correspondence should be addressed.
e-mail: c.verderio@in.cnr.it

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via regulation of critical nutrients (10, 11). EVs not only serve several physiological functions, such as neurotrophic support (12, 13) and disposal of unwanted cellular components (14), but also play pathophysiological roles in inflammatory and degenerative diseases (15–17). Therefore, inhibiting the biogenesis and release of EVs may be an important therapeutic goal, as recently outlined by Tricarico, Clancy, and D'Souza-Schorey (18).

The mechanism of EV biogenesis has been a matter of intense research in the recent years. For discussion of the role of proteins, including members of the endosomal sorting complex required for transport (ESCRT), small GTPases, and glutaminase, in both exo- and ectosome biogenesis and fission, we refer readers to these other excellent reviews (18, 19). Here, we focus on the involvement of sphingolipids (SLs) and their metabolic enzymes in the biogenesis and release of ectosomes and exosomes, which has received less attention.

SLs are a class of lipids located in the plasma membrane and at intracellular organelle membranes, which have not only structural roles but also signaling function. Ceramide (Cer) and sphingosine-1-phosphate (S1P) are the main SLs that act as signaling molecules, controlling a vast number of cellular processes, such as cell growth, adhesion, migration, senescence, cell death, and inflammatory response (20–22). In these cellular activities, dynamic balance between S1P and Cer levels is crucial. This process is known as "SL rheostat" (23).

SLs are constituted by a sphingoid base. In bilayer-forming SLs, a fatty acid is tethered/covalently linked to the sphingoid base. All SLs may be modified at the first hydroxyl group with a variable polar head group, and also by an esterified fatty acid tethered to the amino group of the sphingosine (Sph).

Cer, composed of a long-chain amino alcohol, known as Sph, linked to an amide bond to a long-chain fatty acid, is the structural unit of membrane-forming SLs (not present in Sph and S1P), which represent the most abundant SLs in cells. Cer, can originate from "de novo synthesis," degradation of complex SLs (24), or recycling of long-chain bases through a salvage pathway (21, 25). The "de novo" biosynthesis of SLs begins with the condensation of palmitoyl-CoA with L-serine to form sphinganine and dihydroceramide, respectively, after reduction and acylation. In the de novo biosynthesis, dihydroceramide is then desaturated with the consequent formation of Cer. All of the enzymes involved in the de novo biosynthesis of Cer are localized in the endoplasmic reticulum (ER) membrane and act on the cytosolic surface of this subcellular organelle. The products of the reactions that are catalyzed by these enzymes remain anchored to the ER. In the salvage pathway, Cer is synthesized from Sph (26). During the degradation of complex SLs, glycosphingolipids (GSLs) are constitutively degraded to monosaccharides and Cer (27, 28), while SM is degraded to Cer and phosphocholine (29).

Once generated, Cer can be cleaved by a ceramidase (CDase) to generate Sph that, in turn, can be phosphorylated for the synthesis of the crucial bioactive SL, S1P. On the other hand, Cer is a substrate for various enzymes, mainly by the addition of phosphocholine to produce SM or by the addition of monosaccharides to produce GSLs. Among GSLs, gangliosides are a particular class characterized by the presence of one or more sialic acid residues (26). The GSLs, together with simple SLs, are located in the plasma membrane, where they interact with growth factor receptors, integrins, and other key molecules that participate in cell-cell and cell-matrix interactions (30). Importantly, several SL metabolites have been associated with development of several pathologies, including diabetes, cancer, microbial infections, neurological syndromes, and cardiovascular disease (21, 25, 26, 31–35).

In this review, we will critically summarize current knowledge on the action of SL-metabolizing enzymes in the formation and release of the two main types of EVs (ectosomes and exosomes) as well as past and current efforts in targeting these enzymes pharmacologically or genetically for therapeutic purposes. We then focus on the SL content of EVs and discuss the molecular mechanisms underlying the capability of EVs to promote SL metabolism in target cells.

ROLE OF SLs IN EXOSOME AND ECTOSOME RELEASE

Cer-dependent exosome biogenesis: role for neutral SMase

Plasma and endosomal membranes display an asymmetric lipid distribution, with SM and phosphatidylcholine (PC) enriched on the noncytosolic (luminal) side, and PS and other lipid classes enriched in the leaflet facing cytosol (36). Consolidated evidence indicates that changing the local lipid composition is a way to alter the membrane fluidity and curvature. For example, breakdown of SM, which has a high affinity for membrane cholesterol (37), results in increased efflux of cholesterol from the membrane and increased membrane fluidity (38, 39), while generation of Cer, which has a cone-shaped structure, can give a spontaneous curvature to the membrane (40) (**Fig. 1A**).

SM is hydrolyzed into phosphorylcholine and Cer by the action of SMases, a family of enzymes that possesses acid, neutral, or alkaline optimal pH (41). Neutral SMase (n-SMase) 2, also known as SM phosphodiesterase (SMPD)3, mostly localizes to the Golgi and ER (42), while n-SMase 1, also called SMPD2, is localized to the Golgi and nucleus (43, 44). However, plasma membrane-associated n-SMases have also been characterized (45, 46), and n-SMase 2 has been reported to recycle via the endosomal/recycling compartment (42). Activity of n-SMases is present at the cytosolic leaflet of the membrane (45). By contrast, acid SMase (a-SMase) activity is present in lysosomes and on the outer plasma membrane leaflet following lysosome fusion to the cell membrane (47).

Membrane destabilization driven by SM hydrolysis was initially reported to play a key role in membrane blebbing and release of apoptotic bodies during the late phase of cell damage (48). In 2002, Nurminen et al. (49) visualized the macroscopic consequence of SM breakdown by SMases on

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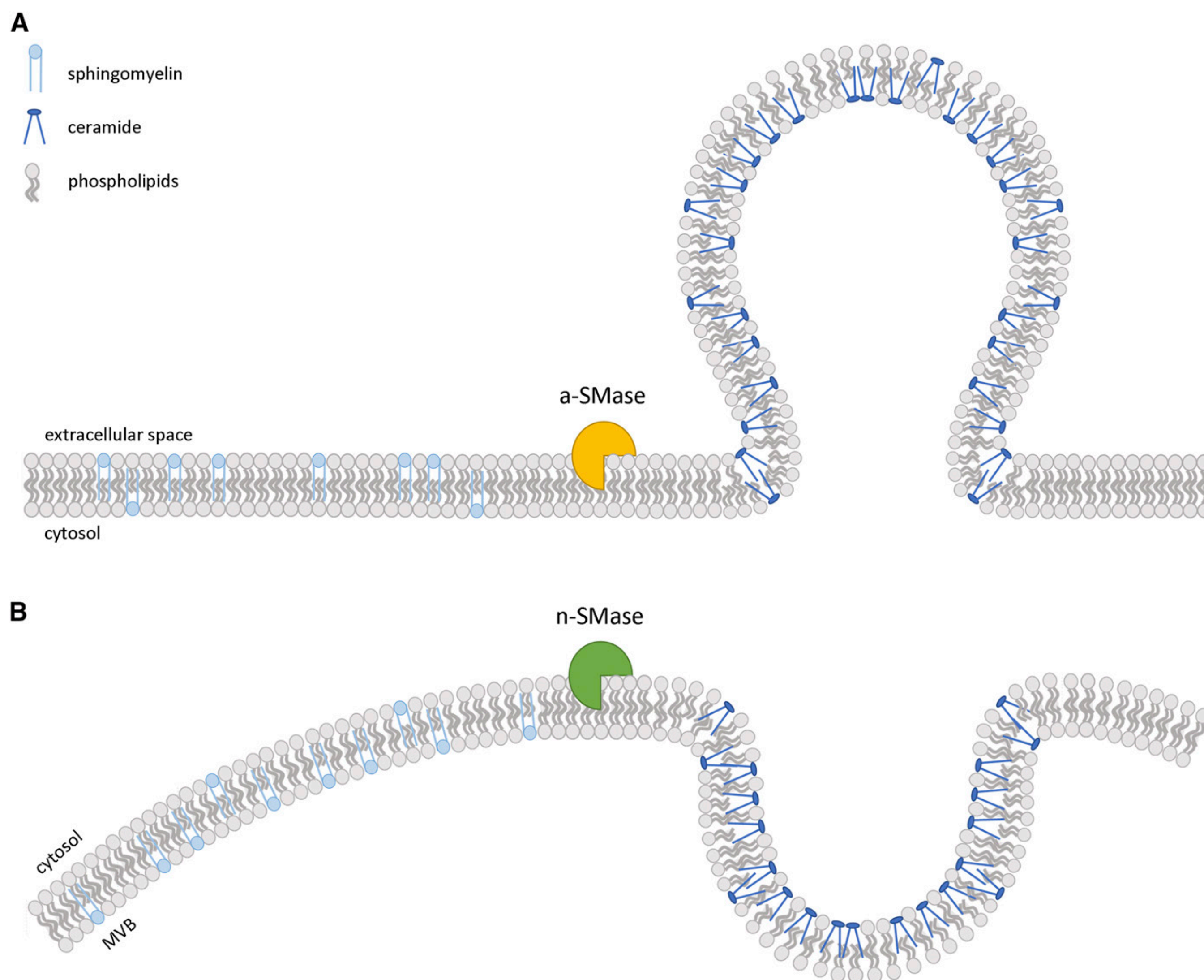

Fig. 1. MV and exosome biogenesis: the role of SMase and its product Cer. A: On the left, SM (cylinder shaped) stabilizes membrane structure; on the right, when a-SMase translocates from the lysosomal compartment to the outer leaflet of the plasma membrane, SM hydrolysis and Cer accumulation into the inner leaflet of the plasma membrane favor blebbing and MV evagination, thanks to the inverted cone shape of Cer. B: n-SMase resides on the cytosolic leaflet of endosomes/MVBs. As in A, before n-SMase action SM stabilizes membrane structure, while Cer production by n-SMase induces spontaneous negative curvature to the membrane of endosomes, thus promoting the formation of internal vesicles inside MVBs.

lipid membrane using fluorescence and differential interference contrast microscopy. SMase was immobilized onto synthetic microspheres and brought into contact with a giant liposome membrane by a micromanipulator: the contact caused formation of membrane microdomains and subsequent shedding of small vesicles from the membrane into the interior of the giant liposome (49). This elegant study was followed in 2008 by the seminal work by Trajkovic et al. (50), who first described that SM hydrolysis and Cer formation participate in ESCRT-independent biogenesis of intraluminal vesicles inside MVBs, the vesicles that are released as exosomes upon MVB fusion with the plasma membrane. While investigating sorting mechanisms of protein cargo into exosomes in an oligodendrocyte cell line (oli-Neu), Trajkovic et al. (50) observed that total Cer was enriched more than 3-fold in a subpopulation of exosomes containing proteolipid protein (PLP). The role of Cer in the biogenesis and release of PLP-positive exosomes was analyzed by treating Oli-neu cells with GW4869, an inhibitor of n-SMase 1 and n-SMase 2, and two structurally unrelated n-SMase blockers, spiroepoxide and glutathione. Exosome release was markedly reduced after treatment of the cells with all the n-SMase inhibitors. siRNA depletion of n-SMase 2 confirmed the involvement of the enzyme in the formation and secretion of PLP-positive exosomes. On this basis, Trajkovic et al. (50) proposed a lipid-based mechanism for membrane bending and exosome formation. They proposed that biogenesis of PLP-positive exosomes occurs at membrane domains enriched in SLs through formation of Cer microdomains and their coalescence into larger domains, which promote inward vesicle budding (50) (Fig. 1A). The cone-shaped structure of Cer induces spontaneous negative curvature to the membrane of endosomes and favors formation of internal vesicles inside MVBs.

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Subsequent in vitro works reported that, while exogenous cell permeable C6 Cer dose-dependently increases the number of exosomes released from multiple myeloma cells (51), pharmacological or genetic block of n-SMase inhibited packaging of the prion protein into exosomes (52) and reduced exosome release among others in HEK cells (53), T cells (54), N2a cells (55), astrocytes (56), microglia (57), macrophages (58), hepatocytes (59), and multiple myeloma cells (51). Also, in vivo, in the brain and serum of 5XFAD mice, a transgenic model of Alzheimer's disease (AD), the n-SMase inhibitor, GW4869, decreased exosome concentration (60). Importantly, decreased exosome production was reported by the same group in 5XFAD mice crossed with fro/fro mice, which lack n-SMase 2 (61), confirming the enzyme involvement in exosome release. Conversely, elevation of Cer caused by block of Cer conversion to SM by the SM synthase inhibitor, D609, promoted exosome secretion (55, 60).

In agreement with these studies, it has recently been shown that basal exosome production can be influenced by cellular components that control n-SMase activity. For example, in hepatocytes, the chemokine receptor, CXCR2, negatively regulates n-SMase activity and cells lacking CXCR2 expression produce significantly more exosomes than wild-type hepatocytes (62). Interestingly, inhibition of exosome release by CXCR2 is unrelated to ligand binding to the receptor and may occur in endosomal membranes where n-SMase localizes and exosomes are generated (62).

Collectively, these studies led to the idea that Cer is important for exosome release in general. However, exosomes are a heterogeneous population of vesicles and it is important to remember that blocking of n-SMases does not block release of all exosomes or impair exosome biogenesis in all the cells tested (63–65), as recently highlighted by Skotland, Sandvig, and Llorente (66).

S1P-dependent cargo sorting in exosomes

Trajkovic et al. (50) revealed a role for Cer in exosome formation by inward budding inside MVBs. However, the mechanism underlying cargo sorting remained unanswered. A recent discovery by Nakamura's group provided new insights into mechanisms of both Cer-dependent exosome formation and cargo sorting (67). Kajimoto et al. (67) initially showed that exosomal formation inside MVBs depends on S1P, the Cer metabolite generated by sequential activity of CDase and Sph kinase (SK). Specifically, they demonstrated that the S1P type 1 receptor ($S1P_1$), an inhibitory G protein-coupled receptor, is present on MVBs and its sustained activation controls exosomal maturation and cargo sorting in HEK cells. In fact, downregulation of either $S1P_1$ or the S1P synthesizing enzyme, SK-2, decreased CD63, CD81, and flotilin-2 content in exosomes (67). In a subsequent study, the same group further dissected the molecular mechanism necessary for cargo sorting by showing that downstream signaling of $S1P_1$, mediated by the β and γ subunits of the inhibitory G protein ($G_i\beta\gamma$), constantly activates the Rho family GTPases, Cdc42 and rac1, on MVBs. Through this mechanism, S1P promotes formation of F-actin networks that are essential for cargo sorting in exosomes. This is indicated by decreased content of CD63 in exosomes released under pharmacological inhibition of both F-actin and S1P formation (68).

Does n-SMase control biogenesis of MVs at the plasma membrane?

Until very recently, the action of n-SMase activity had been explored in the biogenesis of exosomes, but not in a larger EV population, such as MVs (69). The only study that analyzed the impact of n-SMase inhibitors on MV secretion from the plasma membrane was conducted on primary microglia under stimulation with ATP (70), a stimulus particularly effective in promoting release of both ectosomes and exosomes from brain immune cells (57, 71). This work showed no changes in ATP-induced MV shedding under pharmacological block of n-SMase by GW4869 or manumicyn, another n-SMase inhibitor, revealing that n-SMase activity is not required for ATP-induced MV shedding at the cell surface (70).

Recently, Menck et al. (69) investigated the effects of n-SMase on basal MV production from epithelial cells. Using pharmacological and genetic inhibitors of n-SMases, they observed an increase rather than a decrease in ectosome release from the cell surface. Consistently, overexpression of n-SMases (SMPD2 or SMPD3) decreased MV shedding (69). Although further work is necessary to define the mechanisms underlying enhanced MV production under block of n-SMase, this study clearly showed that n-SMase differentially controls the release of exosomes and MVs in epithelial cells. Enhanced basal MV production in cells treated with GW4869, a common tool to block exosome release, may have important consequences: specific proteins may be redirected from exosomes to MVs for secretion when exosome production is blocked. Consistent with this possibility, Menck et al. (69) showed that exosome-enriched proteins, such as CD81 and Wnt, are released in association with MVs under n-SMase inhibition. Importantly, MVs and exosomes derived from many cells share a number of common proteins. For example, both MVs and exosomes released from brain cells in the course of neurodegenerative diseases carry misfolded proteins (Aβ, tau, PrP, and α-synuclein), which form pathological aggregates (72). Although prolonged treatment of AD mice with GW4869 has been shown to reduce exosome levels in brain and serum and to decrease Aβ and tau aggregates (57, 60), how the n-SMase inhibitor impacts release of tau/Aβ in association with MVs has yet to be defined.

Cer-dependent MV biogenesis: role for a-SMase

A specialized type of EV release exists for cells that express the ATP receptor, $P2X_7$, which sheds MVs formed by evaginations of the surface membrane when the receptor is activated. $P2X_7$ receptor-positive cells include monocytes, macrophages, mast cells, dendritic cells, T cells, microglia, and astrocytes. In myeloid or lymphoid cells, the $P2X_7$ receptor functions as an ATP-gated nonselective cation channel and its activation shapes the intensity or duration of innate immune and inflammatory responses (73). The most relevant consequence of $P2X_7$ channel opening is net

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K^+ efflux and assembly of the NLR family pyrin domain containing 3 (NLRP3) inflammasome complexes. These complexes enable activation of caspase-1 protease, which in turn leads to proteolytic cleavage and release of the proinflammatory cytokines, interleukin (IL)-1β, and IL-18 (74).

Before the mechanism of NLRP3 inflammasome assembly was discovered (75), the pioneering work by MacKenzie et al. (5) provided evidence that $P2X_7$-induced MV shedding acts as a secretory pathway for IL-1β release. They reported that, in THP-1 monocytes, the $P2X_7$ receptor induces formation of blebs, which are subsequently shed as MVs into the extracellular space within the first few minutes of receptor activation. Importantly, membrane blebs accumulate IL-1β, which is then packaged into MVs and released extracellularly. $P2X_7$-induced membrane blebbing is preceded by loss of plasma membrane asymmetry and PS externalization, a commonly accepted marker for cell apoptosis. However, alterations of membrane asymmetry are reversible after brief ATP stimulation, dissociating bleb formation and MV shedding from apoptotic cell death. Subsequent studies reported that a similar mechanism for IL-1β and IL-18 release occurs in microglial cells (71), dendritic cells (76), and macrophages (77), suggesting a general role for $P2X_7$-dependent MV shedding in rapid secretion of cytokines lacking a secretory sequence. In macrophages, MVs shed in response to $P2X_7$ receptor activation also bear active tissue factor and have high procoagulant activity (78).

However, an unsolved question was how signaling by the $P2X_7$ receptor led to alterations of the biophysical properties of the plasma membrane, which, together with actin-cytoskeleton reorganization, is a prerequisite for membrane blebbing and vesiculation. In 2009, Bianco et al. (70) identified a-SMase as the key enzyme responsible for $P2X_7$-dependent MV biogenesis at the surface of glial cells (microglia and astrocytes). They showed that following $P2X_7$ receptor activation, a src-protein tyrosine kinase interacts with the C terminus of the receptor (79) and promptly phosphorylates P38 MAP kinase. P38 phosphorylation, in turn, induces translocation of a-SMase from lysosomes to the plasma membrane outer leaflet, where it catalyzes Cer formation from SM (70). As mentioned above, SM to Cer conversion perturbs membrane curvature and fluidity, favoring budding of MVs. Specifically, redistribution of inverted cone-shaped Cer molecules, synthesized extracellularly, into the inner membrane leaflet probably drives membrane evagination (80) (Fig. 1B).

ATP-induced translocation of a-SMase to the plasma membrane and SM hydrolysis were recently confirmed by an independent group in macrophages (81), the peripheral counterpart of brain resident microglia. Immunostaining with an anti-a-SMase antibody revealed the presence of the enzyme on the surface of ATP-stimulated macrophages, while labeling was limited primarily to the cytoplasm in untreated cells. ATP-dependent SM hydrolysis was indicated by the release of the [^{14}C]-labeled phosphorylcholine head group into the supernatant medium. Importantly, inhibition of a-SMase expression or activity not only attenuated ATP-induced SM hydrolysis but also attenuated release of tissue factor-positive MVs, which exhibit pro-coagulant activity (81).

Of note, a-SMase-dependent MV formation likely occurs at SM-enriched domains of the plasma membrane, where the $P2X_7$ receptor localizes (82). As a consequence, cytoskeleton/membrane proteins interacting with the long cytoplasmic C terminus of the $P2X_7$ receptor can be recruited and sorted into MVs. Consistent with this sorting mechanism, MVs released from ATP-stimulated microglia contain several proteins previously shown to bind to the $P2X_7$ receptor C terminus, which are not present in MVs constitutively released by microglia (83).

Recent works suggest that a-SMase can be recruited to the plasma membrane to rapidly enhance EV release upon stimulation of other surface receptors besides $P2X_7$. Initially, Truman et al. (84) found that a-SMase is activated in response to engagement of surface Fcγ receptors by oxidized LDL-containing immune complexes and plays a role in the release of IL-1β in association with exosomes in the human macrophage cell line, U937. This evidence was documented by reduced exosome and IL-1β secretion from cells in which a-SMase was pharmacologically (desipramine) or genetically inhibited (a-SMase siRNA) (84). More recently, Marrone et al. (85) reported that a brief stimulation of the transient potential receptor vanilloid type 1 (TRPV1), also known as vanilloid receptor 1, promotes shedding of MVs to the same extent as $P2X_7$ receptor activation in primary murine microglia. TRPV1 receptor is a nonselective cationic channel highly expressed in brain immune cells, which is exogenously activated by capsaicin (86) and endogenously activated by inflammatory molecules, such as the signaling lipid, lysophosphatidic acid (87). Importantly, p38 MAPK, which is essential for $P2X_7$-dependent a-SMase translocation onto the plasma membrane, is activated downstream of TRPV1 receptors (88) and its inhibition blocks capsaicin-induced MV shedding from microglia in vitro and in cortical brain slices (85), consistent with a role of a-SMase in TRPV1-dependent MV production.

Besides the consolidated role of a-SMase in MV shedding from myeloid cells, the enzyme has also been implicated in the release of EVs from red blood cells (RBCs), at least under pathological conditions. In 2014, Awojoodu et al. (89) reported that plasma membrane stresses and alterations in sickle cell disease, an inherited RBC disorder, enhance the activation of a-SMase and result in increased EV production from erythrocytes. Although erythrocytes do not possess SMase activity of their own (90) and SMases have been reported at low levels in erythrocytes (91, 92), RBCs can be exposed to a-SMase secreted by myeloid cells or by vascular endothelium (93). RBC-derived EVs display pro-inflammatory activities and promote monocyte adhesion to endothelial cells (89). Importantly, treatment with amitriptyline, an indirect inhibitor of a-SMase, reduces EV generation in in vitro and in vivo models of sickle cell disease, thereby representing a possible treatment to mitigate inflammatory processes (93). A very recent study corroborated the role of a-SMase in the formation of EVs from RBCs under stressful conditions, i.e., during packed storage (94). Standard

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blood banking practice allows storage of RBCs up to 42 days of age. However, as stored human packed RBCs age, they release a heterogeneous population of EVs, which cause lung inflammation and worse clinical outcomes in patients with anemia or hemorrhage. Hoehn et al. (94) showed that a-SMase activity is increased in aged compared with fresh RBC-derived EVs, whereas a-SMase activity is decreased on stored erythrocytes as compared with fresh. This suggests that a-SMase changes location and is concentrated in EVs over the course of RBC storage. Interestingly, the authors demonstrated that inhibition of a-SMase by amitriptyline reduces EV production during storage, supporting a primary role of the enzyme in EV biogenesis, and leads to a change in the composition of EVs that ultimately prevents lung inflammation. In fact, less lung inflammation was observed in the recipient mice transfused with equal numbers of EVs produced from RBCs treated with amitriptyline, revealing that a change in a-SMase content and/or Cer concentration in EVs likely preserves the integrity of endothelial cells in the lung and prevents endothelial cell apoptosis (94).

Another recent study implicated a-SMase in the release of EVs evoked by cigarette smoke (CS) from endothelial cells (95). Following exposure to CS, endothelial cells contract their bodies and release EVs from the tips of retracting filopodia-like structures without appreciable cell death. Given that Cer production is activated by CS in lung endothelial cells and a-SMase is rapidly activated by stress in the cells, Serban et al. (95) interrogated the role of a-SMase in the mechanism of CS-induced EV release. They found that treatment with imipramine, a functional inhibitor of a-SMase, markedly decreased CS-induced EV production and that a-SMase-null endothelial cells produced fewer EVs compared with wild-type cells. The role of a-SMase in EV production was corroborated by the observation that mice lacking a-SMase ($Smpd1^{-/-}$) are characterized by reduced levels of EVs in plasma following CS exposure, while mice overexpressing a-SMase in endothelial cells display increased levels of circulating EVs. Besides pointing to a key role of a-SMase activation in CS-induced EV release, this work showed that EVs derived from endothelial cells are Cer rich, suggesting that EVs may act as a vehicle for the transport of Cer, a highly lipophilic molecule, in aqueous environments, such as plasma or bronchoalveolar lavage fluid. Also, in human macrophages, CS may promote MV shedding through an a-SMase-dependent pathway. This is suggested by the work of Li et al. (96) who found that production of MVs from human macrophages relies on a series of regulated steps that include activation of p38 MAPK, the enzyme that drives a-SMase translocation onto the plasma membrane (70).

While the role of a-SMase in enhancing MV shedding in response to danger signals (ATP, TRPV1 agonists) or stressful membrane conditions (CS, sickle cell disease, packed storage) is established (**Table 1**), the enzyme may not play a role in the constitutive shedding that occurs without any stimulation. Indeed, a very recent study showed that long exposure of microglial cells or monocytes to pro-inflammatory or pro-regenerative cytokines (24 h treatment)

TABLE 1. a-SMase involvement in EV release

	Reference						
	(70)	(81)	(84)	(85)	(93, 94)	(95)	(96)
Cell type	Glial cells	Macrophages	Macrophage cell line U937	Primary microglia	RBCs	Endothelial cells	Macrophages
Species	Rat, mouse	Human	Human	Mouse	Human, mouse	Mouse	Human
EV subpopulation	MVs	MVs	Exosomes	MVs	EVs	EVs	MVs
Stimulus	ATP on $P2X_7$ receptor	ATP on $P2X_7$ receptor	oxLDL-IC on Fcγ receptor	Capsaicin on TRPV1 receptor	Stressful membrane conditions (sickle cell disease, packed storage)	CS	CS
Evidence	Pharmacological and genetic	Pharmacological and genetic	Pharmacological and genetic	Pharmacological	Pharmacological	Pharmacological and genetic	Pharmacological

oxLDL-IC, oxidized LDL-containing immune complex.

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enhances basal production of MVs, but pharmacological inhibition of a-SMase by imipramine or by the structurally unrelated indirect inhibitor, siramesine, fails to reduce cytokine-induced MV release. Intriguingly, both constitutive release of MVs and cytokine-induced MV release were efficiently blocked by a sub-toxic concentration of actinomycin D, a strong inhibitor of transcription. While ruling out a role for a-SMase in constitutive release of MVs, this study suggests that cytokines may strengthen constitutive MV production through modulation of the transcriptional activity in myeloid cells (97).

Galactosyl-Sph-dependent EV biogenesis

A recent study revealed a role for the GSL, galactosyl-Sph, and its metabolic enzyme, galactosyl-CDase, in the control of plasma membrane structure and EV shedding. D'Auria et al. (98) highlighted the involvement of galactosyl-Sph in alteration of membrane organization and microvesiculation from oligodendrocytes, the glial cell type that deposits myelin along neuronal axons in the central nervous system.

Galactosyl-Sph, also known as psychosine, is an inverted cone GSL composed of a hydrophobic sphingoid base and a hydrophilic polar head group that accumulates in brain membranes of Krabbe's disease, a genetic sphingolipidosis caused by mutations in the psychosine-degrading enzyme, galactosyl-CDase. Different from SM, psychosine is not ubiquitously present, but generated quite specifically in oligodendrocytes, where it is considered the main SL responsible for demyelination and oligodendrocyte loss in Krabbe's disease. Indeed, psychosine accumulates in lipid rafts and disrupts their structures, altering signaling pathways important for oligodendrocyte differentiation and survival (99). However, until recently, no clear insights have been provided into how psychosine causes demyelination. D'Auria et al. (98) studied psychosine-mediated membrane disorganization in RBCs and showed that psychosine, due to the quite large size of its polar head group, affects the organization of the SM-enriched domain and elicits outward membrane blebbing and EV shedding. It should be noted, however, that, in this study, specificity of psychosine action has not been assessed by the use of an equal concentration of similarly structured lipids, e.g., octyl glucoside or hexadecylglycerol. Findings obtained in RBCs were confirmed in oligodendrocytes established from Twitcher mice, a model of Krabbe's disease, which were more prone to produce MVs than wild-type cells upon exposure to the psychosine. Vesiculation was accompanied by lateral mobility of SM and cholesterol toward areas in the plasma membrane with high focal rigidity, which may introduce focal weak points in the myelin sheath contributing to myelin disruption. Importantly, this model of MV biogenesis implies that MVs do not originate from membrane blebs, which progressively bud from the plasma membrane and are then pinched off from the cell surface, a process that requires membrane bending and increased membrane fluidity. However, no mechanistic explanation of MV formation is provided by the author.

Independent of the underlying mechanisms, psychosine-dependent MV production may spread disease from sick to healthy cells. Indeed, while clearing some of the accumulated SL material via EV secretion, brain cells may shuttle pathogenic SLs [e.g., psychosine or proapoptotic Cer (56)] to cells that are not intrinsically affected, contributing to establishing non-cell-autonomous defects (100).

SL CONTENT OF EVs

Comparative analysis of SL composition in exosomes/MVs and in parental cells

To date, high resolution lipidomic analyses of EVs have been reported for a limited number of cell lines (101), thus limiting current knowledge of EV lipid composition and complexity. However, it is well-established that EVs contain lipids, such as aminophospholipids and Cer, which, along with GSLs and SM, are the most enriched lipids in EVs of different cellular origin (**Table 2**) (66). The SL content of less represented SL species is still very uncertain.

There are major differences in the lipid composition of exosomes/MVs compared with whole cell lipid extract of parental cells, demonstrating the existence of specific mechanisms of lipid sorting into EVs. Interestingly, SM, GSLs, and Cer are among EV-enriched lipids compared with most donor cells (Table 2) (4, 50, 66, 102–105).

However, Llorente et al. (102) demonstrated that exosomes released from reticulocytes and rat basophil leukemia cells exhibit a lower phospholipid-to-protein ratio than parental cells. Specifically, they found that most lipid classes were more abundant in exosomes, and that the relative amounts of specific lipids, such as GSLs, cholesterol, SM, and PS, were highly enriched in exosomes (102). Other studies confirmed enrichment from 1.3- to 2.8-fold for SM (4, 50, 66, 102–105) and from 2.0- to 3.8-fold for GSLs (50, 102, 103) in exosomes depending on the types of donor cells. Exosomes derived from mouse Oli-neu cells show less enrichment in SM and GSLs and a much higher enrichment in Cer, which is crucial for exosome biogenesis inside MVBs (50). Specifically, SM is 1.5 times enriched in exosomes isolated from Oli-neu cells, compared with parental cells, while it is 2.4 times enriched in PC-3 cell-derived exosomes (102), 2.3 times enriched in human B-cell-derived exosomes (104), 2.8 times enriched in mast cell RBL-2H3-derived exosomes (4), and 2.2 times enriched in exosomes released from dendritic cells with respect to donor cells (105). GSLs are 2.0 times enriched in exosomes of Oli-neu cells, compared with their parental cells (50), while the enrichment in GSLs is of about 3.8 times in exosomes from PC-3 cells (102) and even 18 times in those produced from murine neuroblastoma Neuro2a cells (106).

Different methods have been used to analyze the SL content of EVs, including TLC, gas LC, and MS. Wubbolts et al. (104) were the first group to use MS (in addition to TLC) to study the SL content of exosomes released from human B-cells. By this approach, they described a large

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TABLE 2. SL content in exosomes and enrichment from the originating cells

Lipids	PC-3 Cells (102)		Oli-neu Cells (50)		B-Cells (104)		Mast Cells (4)		Dendritic Cells (4)		Reticulocytes (105)		Mesenchymal Stem Cells (101)		U87 Cells (101)		Huh7 Cells (101)		FEMX1 Cells (153)		DU145 Cells (154)		VCaP (154)		LNCaP (154)	
	%	F	%	F	%	F	%	F	%	F	%	F	%	F	%	F	%	F	%	F	%	F	%	F	%	F
SM	16.3	2.4	8.2	1.5	23.0	2.3	12	2.8	20	2.2	8.4	1.31	5	1	20	2.35	4	0.8	—	4	—	—	—	—	—	—
GSLs	0.78	3.8	—	2.0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1.8	—	3.1	—	3
Cer	0.32	1.3	—	3.3	—	—	—	—	—	—	—	—	0.04	1	0.2	1	0.04	0.25	—	—	—	—	—	—	—	—

Data are based on the EV network database, Vesiclepedia. Only the reports that allowed us to calculate the changes of SLs in EVs compared with the parental cells are reported. %, percent of total lipid quantified; F, factor of enrichment from cells to exosomes or MVs.

SL enrichment in exosomes and also highlighted similarities between the lipid classes abundant in exosomes and in detergent-resistant membranes, the membrane regions from which EVs are generated. The use of different methodologies could account for the slightly different SL composition of EVs produced by different cell types. However, the lipid content may actually vary depending on source cell type and even among exosomes and MVs released by the same donor cells. A few studies support this possibility: *i*) The comprehensive analysis of lipid composition of exosomes, MVs, and source cells in three different cell types by Haraszti et al. (101) showing specific enrichment in SMs in MVs derived from Huh7 hepatocellular carcinoma cells or mesenchymal stem cells with respect to MVs released by U87 glioblastoma cells (**Table 3**). Importantly this study also revealed that the MVs and exosomes differ in their types of lipid contents. Enrichment in glycolipids and free fatty acids characterized exosomes, whereas enrichment in Cers and SMs characterized MVs derived from Huh7 hepatocellular carcinoma cells as well as mesenchymal stem cells (101). *ii*) The work by Dang et al. (107) reporting significant differences in the lipid composition of exosomes secreted across the apical compared with the basolateral plasma membrane of epithelial cells. In particular, this study showed that SM is more represented in exosomes secreted across apical compared with the basolateral plasma membrane (107). *iii*) SL profiling of human oligodendroglioma (HOG) cell-derived exosomes, revealing differences in the SL composition, in particular of C16-, C24-, and C24:1-Cer species; C16-, C24-, and C24:1-dihydroCer species; and C16-, C24-, and C24:1-SM species, between exosomes released constitutively or under stimulation with inflammatory cytokines (108). *iv*) The work by Carayon et al. (109) showing that the exosomal content of Cer versus SM is significantly increased during reticulocyte maturation. *v*) The study by Baig et al. (110) demonstrating that GM3 levels are significantly downregulated in MVs derived from syncytiotrophoblasts of preeclampsia patients.

SL species in EVs as potential biomarkers

EVs are attracting increasing interest as a source of noninvasive biomarkers for several diseases, including cancer and multiple sclerosis, because these vesicles contain pathogenic molecules of donor cells that can be analyzed in biofluids (111–113). Interestingly, SLs have recently been identified among EV components that are relevant for disease diagnosis.

In depth lipidomic analysis of exosomes secreted by a colorectal cancer cell line first revealed selective changes in SL abundance in EVs compared with parent colon cancer cells (114) and suggested that specific exosome SL(s), i.e., Cer and SM, could serve as effective diagnostic or prognostic biomarkers for colon cancer. The diagnostic potential of exosome SL(s) was then demonstrated by Skotland et al. (115), who found that levels of lactosylceramide (d18:1/16:0) in urinary exosomes are different between prostate cancer patients and healthy controls. Importantly, measurements of lactosylceramide in combination with other exosomal

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TABLE 3. SL content in MVs and enrichment from the originating cells

	Bone Marrow-Derived Mesenchymal Stem Cells (101)		U87 Cells (101)		Huh7 Cells (101)		Platelets (155)	
Lipids	%	F	%	F	%	F	%	F
SM	10	2	15,2	0.97	17	2	17	3.4
GSLs	—	—	—	—	—	—	—	—
Cer	0.48	2	—	—	0.68	2.4	0.24	1.5

Data are based on the EV network database, Vesiclepedia. Only the reports that allowed us to calculate the changes of SLs in EVs compared with the parental cells are reported. %, percent of total lipid quantified; F, factor of enrichment from cells to exosomes or MVs.

lipids were shown to have the capacity to distinguish cancer patients from healthy subjects with high sensitivity (93%) and specificity (100%) (115).

Another recent work revealed that a distinct SL (C16:0 sulfatide), a sulfated galactosylceramide with long-chain fatty acid, in blood EVs represents a unique indicator of multiple sclerosis, a chronic neuroinflammatory disease (116). Specifically, using ultra-high-performance LC tandem MS, Moyano et al. (116) detected a significant increase in C16:0 sulfatide in EVs isolated from multiple sclerosis patients when compared with healthy subjects.

SL-MEDIATED EV ACTION ON RECIPIENT CELLS

Several lines of evidence point to the involvement of exosome GSLs in the pathogenic aggregation of amyloidogenic peptide Aβ (106) that typically forms extracellular aggregates in the brain of subjects affected by AD, the most common neurodegenerative disease. GSLs present on the exosome surface of Neuro2a cells were shown to form clusters and bind to Aβ peptide. Aβ-GSL complexes, in turn, were shown to act as templates for further Aβ aggregation and fibril formation (106). In agreement with this study, Yanagisawa et al. (117) found that the monosialoganglioside, GM1, associates with Aβ in the brain of AD patients. On these bases, Yuyama et al. (106) proposed that there may be multiple GSLs on exosome membranes that organize themselves into specific areas that bind to Aβ and induce its assembly. Moreover, cholesterol and SM, which were highly enriched in exosomes compared with their parent cells, were also shown to promote Aβ assembly via the lateral packing of gangliosides on membranes (106, 119, 120).

Emerging evidence indicates that the SL cargo of EVs may be responsible for detrimental EV action. In 2012, an interesting study by Bieberich's group revealed the involvement of Cer-enriched exosomes in Aβ-dependent apoptosis, which critically contributes to AD (56). The authors of this study showed that Cer levels are regulated by n-SMase 2 in astrocytes and are critical for astrocyte apoptosis caused by Aβ, which is not observed in n-SMase 2-deficient cells. Astrocyte apoptosis was accompanied by release of Cer-enriched exosomes, which propagated a death signal into recipient astrocytes not exposed to Aβ protein (56).

A subsequent study confirmed that Cer-enriched exosomes mediate cytotoxic effects in recipient cells. Specifically, exosomes released from HOG cells treated with a combination of inflammatory cytokines were found to be Cer-enriched and to induce cell death in fresh (naïve) HOG cells (108). Interestingly, Cer levels did not increase much in donor HOG cells under cytokine treatment, suggesting that Cer is immediately exported out of the cells by exosomes after generation.

Another recent work highlighted the involvement of the Cer metabolite, S1P, in exosome-mediated neoplastic transformation. Exosome-like particles produced by the intestinal mucosa in response to enterobacteria were shown to contain S1P, which favors the recruitment and proliferation of inflammatory Th17 T cells in the intestine and promotes tumor growth in animal models of colorectal cancer (121).

Finally, Wang et al. (122) identified S1P as the primary mediator of endothelial cell exosome actions on hepatic stellate cells, responsible for the pathological migration and aberrant phenotype of hepatic stellate cells during liver fibrosis. Mechanistically, this study revealed that exosome-induced hepatic stellate cell migration is dependent on exosome adhesion to target cells, which facilitates exosome uptake through dynamin-dependent endocytosis (122).

Involvement of SL enzymatic machinery or SL receptors in the biological activity of EVs

Accumulating evidence indicates that EVs can influence SL metabolism in receiving cells by providing them with key SL metabolic enzymes, among other enzymes of lipid metabolism borne by EVs (123).

The presence of the S1P-generating enzymes, neutral CDase (n-CDase) and SK-1, was first described in EVs shed by hepatocarcinoma and carcinoma cultured cells and was suggested to contribute to the proangiogenic activity of EVs (124). Later on, SK-1 was detected within exosomes derived from endothelial cells and, together with its product, S1P, identified as the active agent responsible for pathological migration on hepatic stellate cells, as mentioned above (122). In addition, Zhu et al. (125) found that active n-CDase is also released in association with exosomes by β-pancreatic INS-1 cells exposed to a low concentration of inflammatory cytokines. Exosome-packaged n-CDase inhibited INS-1β-cell destruction induced by a high concentration of inflammatory cytokines and the protective action was mediated by S1P generation and activation of S1P type 2 receptor ($S1P_2$). These findings revealed a novel n-CDase-$S1P_2$-dependent mechanism by which exosomes produced in response to a low level of inflammatory cytokines protect pancreatic β-cells from apoptosis (125).

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In agreement with this work, hepatocyte-derived exosomes were recently shown to be incorporated into target hepatocytes and deliver both n-CDase and SK-2 to the cells, increasing S1P levels. By this mechanism, exosomes carrying S1P metabolic enzymes induce dose-dependent proliferation of hepatocytes in vitro and contribute to liver repair and regeneration after an ischemia/reperfusion injury, a major clinical problem that affects liver transplantation and other hepatic surgeries (59). Accordingly, exosomes produced by mesenchymal stem cells differentiated from human pluripotent stem cells were recently shown to exert protective and proliferative effects on primary hepatocytes and their beneficial action was also ascribed to activation of the synthetic machinery for S1P production in hepatocytes upon exosome internalization (126). Indeed, either the SK inhibitor, SKI-II, or the $S1P_1$ antagonist, VPC23019, completely abolished the proliferative effect of exosomes on primary hepatocytes.

Not only the transfer of SL(s) and/or their enzymatic machinery but also the transfer of SL receptors may participate in the biological action of EVs on target cells. This hypothesis has recently been proposed by Pyne et al. (127), who found that $S1P_2$ is released from breast cancer cells through exosomes, and it is cleaved into a smaller constitutively active form lacking the N terminus (red) into recipient fibroblasts, where the receptor stimulates the ERK-1/2 pathway and promotes proliferation. However, to the best of our knowledge, experimental data supporting the role of exosomal $S1P_2$ cargo in cross-talk between cancer cells and fibroblasts are still unpublished. A previous work revealed a specific enrichment in S1P types 3 and 4 receptors ($S1P_3$ and $S1P_4$) in MVs and exosomes derived from a mammary luminal epithelial cell line overexpressing HER2/ERBB2 oncogene. Their presence may indeed be relevant for the biology of HER2+ tumors, by controlling tumor invasion and cell migration. However, their functional significance has yet to be evaluated (128).

EV-dependent activation of SL metabolism in target cells

In the last years, evidence has been provided that EVs can stimulate SL metabolism in receiving cells not only via the transfer of SL enzymatic machinery but also through activation of SL metabolic enzymes in recipient cells. Data from our laboratory showed that lipid species housed on the membrane of microglia-derived MVs, through activation of contact-mediated signaling pathways, act on neuronal a-SMase promoting both Cer and Sph production. The latter SL increased spontaneous release from excitatory terminals in cultured hippocampal neurons by enhancing glutamate release probability (129, 130) (**Fig. 2B**). The stimulation of SL metabolism in neurons required the action of neuronal a-SMase and was not due to the enzyme transfer from microglia in association with MVs. In fact, hippocampal cultures established from a-SMase KO mice exposed to MVs did not show any significant alteration in miniature excitatory postsynaptic current (mEPSC) frequency relative to untreated transgenic cultures, whereas wild-type cultures treated with MVs displayed an increase in mEPSC frequency compared with control neurons (Fig. 2B). Neuronal Sph was accountable for MV-mediated potentiation of excitatory transmission, as the blockade of Sph production through the CDase inhibitor, *N*-oleoylethanolamine (NOE), strongly prevented MV effects on mEPSCs, whereas the blockade of S1P synthesis from Sph by the Sph kinase inhibitor, SKI-1, was ineffective (Fig. 2A, B). However, in a subsequent study, we found that the SL cascade induced by microglial MVs in neurons does not stop at Sph, but proceeds to S1P, which contributes to enhance glutamate transmission by targeting synapsin I, the phosphoprotein controlling synaptic vesicle availability for exocytosis (131). The key role of S1P and its receptor, $S1P_3$, in controlling synapsin I activity was defined by the use of pharmacological inhibitors of the SL cascade (i.e, the a-SMase inhibitor, imipramine, the CDase inhibitor, NOE, and the SK inhibitor, SKI-1) as well as S1P receptor antagonists (the pan receptor antagonist, S-FTY720-vinylphosphonate, the $S1P_3$ antagonist, CAY1044, and the $S1P_1$ antagonist, W146) (Fig. 2A, B). These findings were corroborated by a recent study reporting that the inhibition of a-CDase by the potent inhibitor, ARN14988, completely prevents the increase of mEPSC frequency induced by microglial-derived MVs in cortical slices (85). In this case, MV release from microglia was triggered by capsaicin acting on TRPV1 receptors, a receptor coupled to P38-MAPK, the enzyme that drives a-SMase (see also above). Given that TRPV1 receptors are antagonized by the broadly used a-CDase inhibitor, NOE, the authors employed the CDase inhibitor, ARN14988, instead of NOE in order to block a-CDase activity. ARN14988 prevented the enhancement of mEPSC frequency caused by MVs without interfering with capsaicin-dependent MV release or MV function on mEPSCs (85).

Importantly, a recent study showed that EVs have the epigenetic machinery to target SL metabolism in recipient cells. Schatz et al. (132) observed that EVs released by a cosmopolitan alga (*Emiliania huxleyi*) during infection with its specific virus (EhV, Phycodnaviridae) have a unique lipid composition that differs from that of viruses and infected host cells, highly enriched in triacylglycerols. In addition, RNA isolation and analysis revealed that EV cargo is composed of specific microRNAs that are predicted to target SL metabolism (132–134). Together with our findings, this evidence suggests that SL metabolism may be a highly conserved pathway activated in recipient cells by EVs either through their lipid or miRNA cargoes.

ENDOCANNABINOID-MEDIATED EV ACTION ON RECIPIENT CELLS

Another intriguing aspect of lipid involvement in EV biological activity concerns the role of endocannabinoids (eCBs).

eCBs are lipid mediators that regulate a large number of physiological functions in the central and peripheral nervous system (135–137). The two major eCBs, 2-arachidonoylglycerol and *N*-arachidonoylethanolamine (AEA), also called anandamide, primarily act as retrograde messengers: typically produced by neurons, eCBs are synthesized on demand in response to high synaptic activity and rapidly

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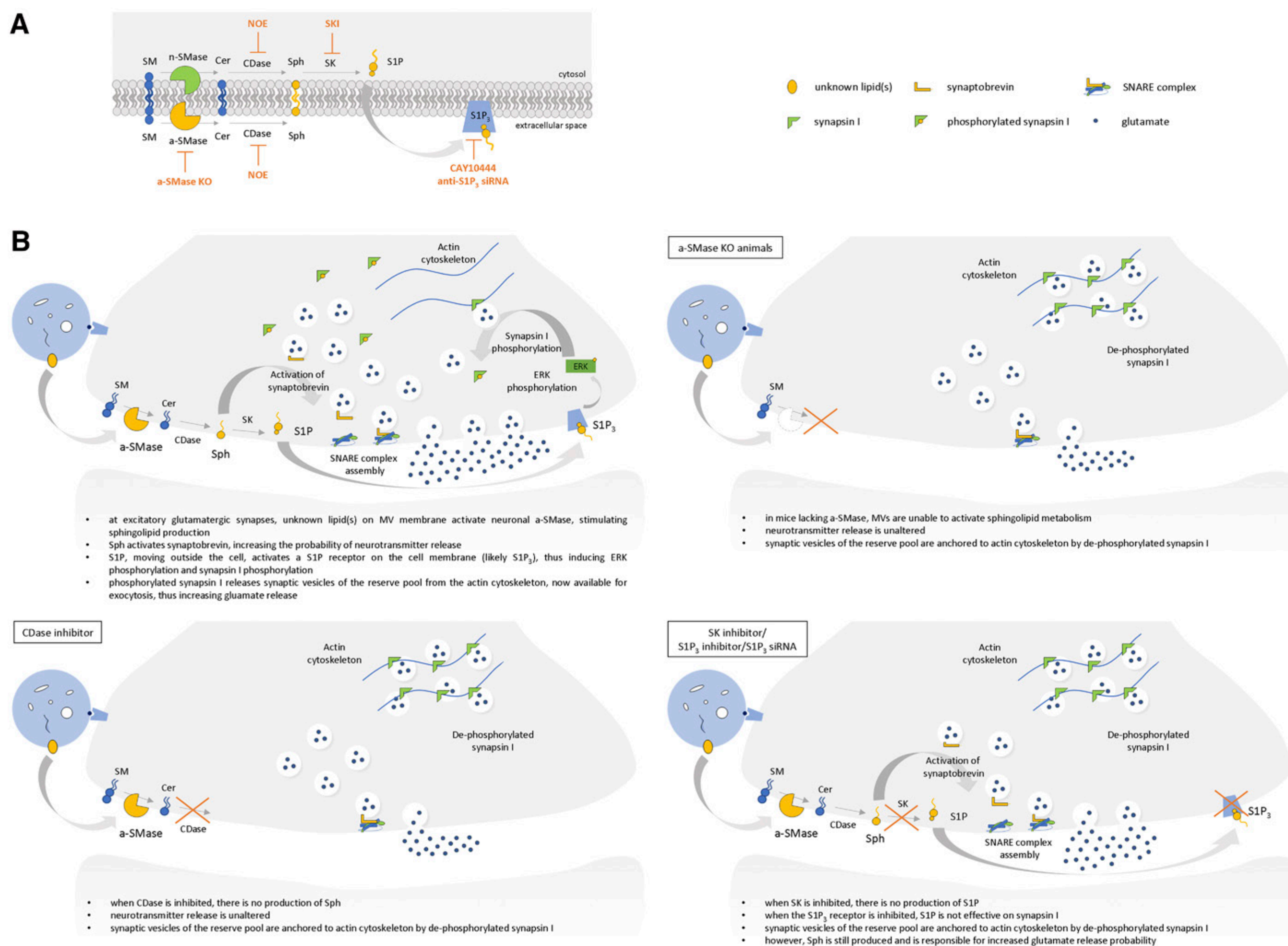

Fig. 2. MV-dependent activation of SL metabolism in neurons. A: The SL cascade on the plasma membrane and the targets of genetic/pharmacological inhibitors. B: The cartoons elucidate the mode of action of MV released by microglial cells on excitatory glutamatergic synapses and the effects of genetic/pharmacological inhibitors. Microglial MVs interact with the plasma membrane of neurons through surface contact facilitated by receptor-ligand interaction between PS residues (typically exposed on the MV membrane) and PS neuronal receptor (in blue). Subsequently, unidentified lipid(s) on the surface of MVs activate a-SMase (translocated to the outer leaflet of plasma membrane from lysosomes), thus stimulating SL metabolism in neurons. Sph and S1P have been identified as the SLs responsible for the MV effects on the excitatory presynaptic terminal (see also the "a-SMase KO animals," "CDase inhibitor," and "SK inhibitor/$S1P_3$ inhibitor/$S1P_3$ siRNA" panels). Sph acts on the synaptic vesicle-associated SNARE protein, synaptobrevin, activating it and thereby promoting the assembly of the SNARE complex (which is necessary for synaptic vesicle fusion at the presynaptic terminal and, therefore, neurotransmitter release). This event increases the probability of neurotransmitter release, empowering excitatory synaptic transmission in neurons targeted by MVs. On the other hand, S1P, a SL able to move outside the membranes, travels extracellularly to engage its receptor on the presynaptic terminal (likely the $S1P_3$ receptor). $S1P_3$ receptor activation translates into downstream signaling in the target neuron, inducing ERK activation and synapsin I phosphorylation. Synapsin I is a presynaptic protein responsible for anchoring synaptic vesicles of the reserve pool to the actin cytoskeleton, therefore preventing their exocytosis. Dephosphorylated synapsin I binds synaptic vesicles and the actin cytoskeleton, tying the two of them together. When phosphorylated, synapsin I breaks the link between vesicles and actin, leaving synaptic vesicles free to migrate to the presynaptic terminal, ready for exocytosis upon stimulation. This increases the number of synaptic vesicles that undergo exocytosis when neurotransmitter release is triggered. The actions of genetic/pharmacological inhibitors are described in the a-SMase KO animals, CDase inhibitor, and SK inhibitor/$S1P_3$ inhibitor/$S1P_3$ siRNA panels.

released extracellularly. In the extracellular space, eCBs move toward the presynaptic membrane and activate eCB type 1 (CB_1) receptors to inhibit neurotransmitter release (135, 138–140). However, eCBs are also produced by astrocytes and microglial cells (141–143), and control glia-neuron interaction (137, 144) with microglia being the major provider of eCBs under inflammatory conditions (145).

A long-standing question in the field of eCB research has been how these highly hydrophobic lipids are transported through the aqueous extracellular environment to engage their targets (146, 147). Studies conducted in our laboratory uncovered the role of MVs in the transport of eCB species outside brain cells (148). We found that EVs released from primary microglial cells are enriched in the eCB, AEA (while 2-arachidonoylglycerol was under the limit of detection), which likely localizes on the vesicle surface. This was indicated by the affinity of a biotinylated analog of AEA (biotinyl-AEA) (149) to the MV external membrane. We also found that vesicular eCBs are biologically active: they bind presynaptic CB_1 receptors, activate CB_1

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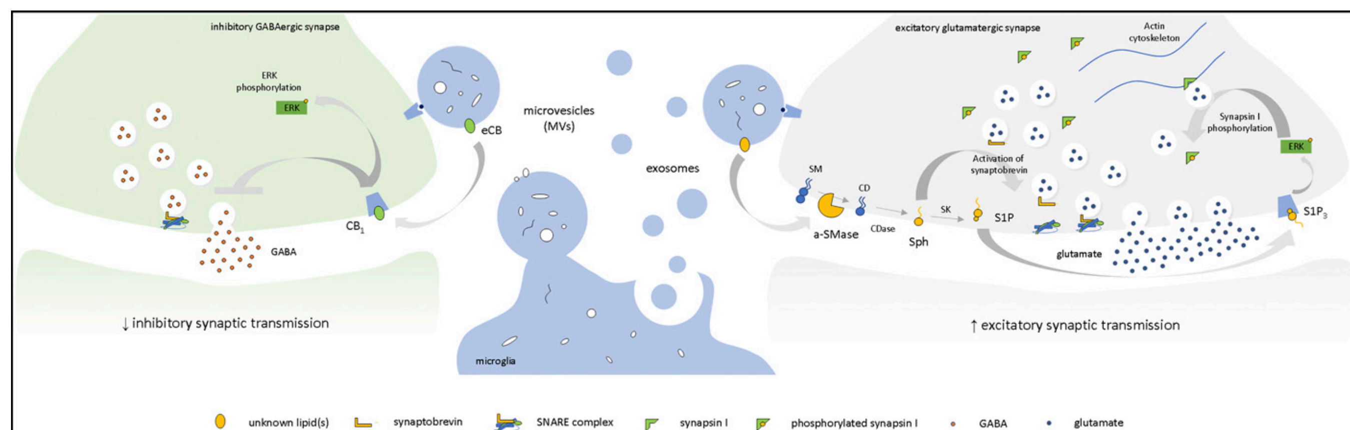

Fig. 3. Effects on inhibitory/excitatory synapses of MV-associated bioactive lipids. Center: Microglial cells release EVs (MVs and exosomes). MV interaction with the neuronal surface is mediated by PS residues, typically exposed on the MV membrane, which bind their specific neuronal receptor (in blue). Left: On inhibitory synapses, vesicular eCB(s) (likely AEA exposed on the surface of MVs) interact with presynaptic CB_1 receptors. CB_1 receptor activation induced by vesicular eCBs causes an increase in phosphorylated ERK levels and a down-regulation of inhibitory synaptic transmission. Right: As already described in Fig. 2, on excitatory synapses, microglial MVs activate SL metabolism. Sph and S1P mediate MV action, inducing an increase in excitatory transmission.

receptor downstream signaling, and induce an impairment of inhibitory synaptic transmission (**Fig. 3**). In fact, primary hippocampal neurons exposed to MVs and recorded through patch-clamp electrophysiological technique displayed reduced miniature inhibitory postsynaptic current frequency compared with control untreated cells, revealing that MVs reproduce the action of the CB_1 receptor agonist, WIN 55,212-2 (150, 151), on inhibitory hippocampal synapses (152). Furthermore, the involvement of vesicular AEA in this phenomenon was inferred from the ability of the CB_1 receptor antagonist, SR141716A (150–152), to block the depression of miniature inhibitory postsynaptic current frequency evoked by MVs. Finally, MV treatment also determined an increase in phosphorylated ERK levels in neurons, an event downstream CB_1 receptor activation, which was again abolished in the presence of SR141716A.

CONCLUSIONS AND FUTURE DIRECTIONS

Progress has been made in the last 10 years in elucidating how EVs are formed and a fundamental role for Cer during formation of membrane negative curvature (away from the cytoplasm) has been established, which is necessary for both exosome and MV biogenesis. In the examples of EV biogenesis discussed above n- and a-SMase have been extensively studied and shown to mediate release of exosomes and MVs, respectively. In particular, a clear role for a-SMase has been demonstrated in MV release evoked by stimulation of various surface receptors or occurring under stressful conditions, while a more general role for n-SMase in exosome biogenesis has been shown, despite the fact that the enzyme does not mediate exosome release in all the systems tested. However, a more complex scenario is emerging with the n-SMase inhibitor, GW4869 (a common tool to block exosome release), enhancing rather than blocking production of MVs from the cell surface of epithelial cells.

Consolidated evidence indicates that SLs and their enzymatic machinery are enriched in EVs, despite the SL content of less represented SL species is still uncertain.

Besides attracting interest as potential disease biomarkers in cancer and inflammatory diseases, vesicular SLs and their metabolic enzymes have been shown to contribute to EV action by influencing SL levels in recipient cells. Interestingly EVs may also influence SL activity in receiving cells via transfer of microRNAs targeting SL metabolism, as indicated by transcriptomic analysis of EVs produced by prokaryotic cells.

We expect that future characterization of EVs in diverse cell physiology will have a wide impact in understanding biogenesis and signaling functions of these highly conserved mediators of intercellular communications in mammals.

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