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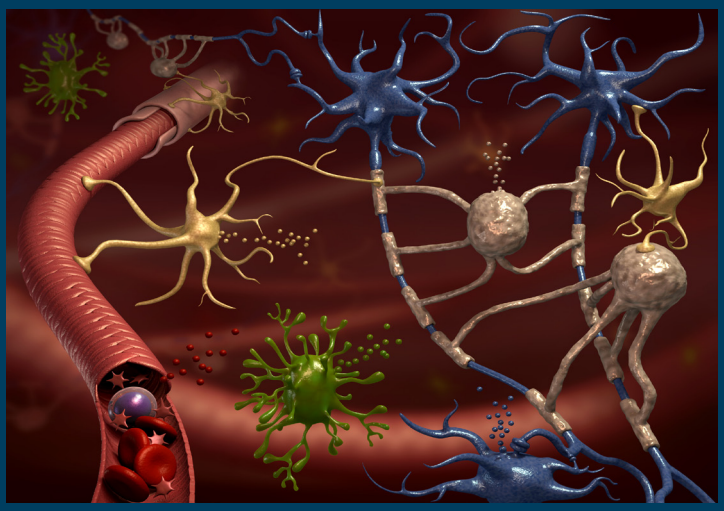

EXTRACELLULAR MICROVESICLES AND NANOTUBES IN THE BRAIN:
UNDERSTANDING THEIR NATURE AND FUNCTION IN CELL-TO-CELL COMMUNICATION, THEIR ROLE IN TRANSCELLULAR SPREAD OF PATHOLOGICAL AGENTS AND THEIR THERAPEUTIC POTENTIAL

Topic Editor
Claudia Verderio

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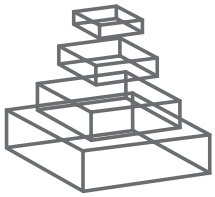

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EXTRACELLULAR MICROVESICLES AND NANOTUBES IN THE BRAIN: UNDERSTANDING THEIR NATURE AND FUNCTION IN CELL-TO-CELL COMMUNICATION, THEIR ROLE IN TRANSCELLULAR SPREAD OF PATHOLOGICAL AGENTS AND THEIR THERAPEUTIC POTENTIAL

Topic Editor:
Claudia Verderio, University of Milan, Italy

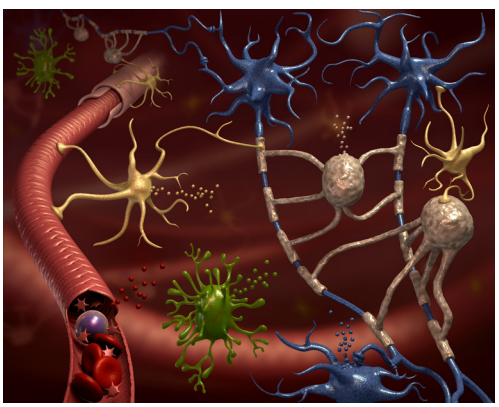

Image created by Bruno Borgiani.

Recent reports convey the intriguing idea that nanotubes and microvesicles represent novel pathways for cell-to-cell communication. Nanotubes are exceedingly thin protrusion up to several micrometer long that can connect cells from several cell diameters apart and provide membrane continuity between connected cells. Extracellular vesicles include exosomes, which are secreted as a result of multivesicular bodies fusion, and shed microvesicles/ectosomes, which bud directly from the cell plasma membrane. In this Research Topic we will discuss mechanisms involved in nanotubes and microvesicles formation, in microvesicles secretion and interaction with target cells. The role of nanotubes and microvesicles in the intercellular transfer among different brain cells will be also discussed as well as their involvement in brain development, cancer, inflammation,

dissemination of pathogens or proteins associated to neurodegenerative disorders and their potential role as markers of brain tissue damage. The goal of this Research Topic is to give a summary of the current knowledge of microvesicles and nanotubes in the brain by describing their nature and revealing data about their biological roles and to stimulate theories and opinions on their possible implication in brain functions not yet identified. For example hypothesis on the potential use of microvesicles in diagnostic and for delivering therapeutic agents into the central nervous as compared to artificial nanoparticles, and a full discussion on the methodology currently available to isolate and quantify microvesicles derived from brain cells are encouraged.

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EDITORIAL
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Extracellular membrane microvesicles and nanotubes in the brain: understanding their nature, their function in cell-to-cell communication, their role in transcellular spreading of pathological agents and their therapeutic potential

Claudia Verderio [1,2]*

[1] *Institute of Neuroscience, Consiglio Nazionale delle Ricerche, Milan, Italy*
[2] *IRCCS Humanitas, Rozzano, Italy*
**Correspondence: c.verderio@in.cnr.it*

Edited by:
Mario L. Diaz, Universidad de La Laguna, Spain

Although initially perceived as cellular artifacts, nanotubes and extracellular membrane vesicles (EMVs), have been recently recognized as novel pathways for cell-to-cell communication. Membrane nanotubes are long and thin protrusions formed from the plasma membrane that can connect different cells over long distances, up to several micrometers, providing membrane continuity. EMVs are small membranous vesicles which bud directly from the plasma membrane (ectosomes), or result from exocytosis of multivesicular bodies (exosomes). EMVs are capable of transferring specific proteins, lipids, (micro)RNAs and DNAs between cells, thus serving as a unique mechanism for the intercellular trafficking of complex biological messages.

The present Special Issue encompasses thirteen reviews and original articles which outline recent progresses in understanding the biogenesis, biophysical properties and the possible role of nanotubes and EMVs in the healthy and diseased brain. The impressive number of studies accumulated so far indicates that EMVs and nanotubes are becoming of key importance in brain physiology.

The issue opens with a comprehensive review on formation, structure and role of nanotubes in brain cell-to-cell transfer of various signals and materials, including pathogens (Marzo et al., 2012). The attention then turns toward EMVs secreted by neural cells, especially exosomes, their biogenesis and role in inter-neuronal signaling at the synapse, where exosomes may strongly influence plasticity phenomena (Chivet et al., 2012). Three additional review articles broaden the role of neural EMVs to neuron-glia communication in the central and peripheral nervous system. Particular attention is paid to exosomes released by oligodendrocytes and their potential implication in myelin diseases (Frühbeis et al., 2012), to microglia-derived ectosomes and their modulation of excitatory neurotransmission (Turola et al., 2012), and to Schwann cell-derived vesicles and their function in axonal growth and regeneration (Lopez-Verrilli and Court, 2012).

The intriguing capacity of neural EMVs to modulate the immune system activity, either stimulating or repressing, depending on their origin (stem cells, endothelial cells, or tumor cells) is addressed with attention to details by Cossetti et al. (2012). Conversely, the functional activity of EMVs produced by the immune resident brain cells, i.e., microglia, is the focus of a contribution from our own laboratory (Turola et al., 2012).

Among the most relevant effects of EMVs in the diseased brain, D'Asti et al. (2012) highlight the oncogenic properties of tumor-derived EMVs, also called oncosomes, by comprehensively reviewing transformation-related molecules found in their cargo and describing how these effector molecules impact the tumor microenvironment of the central nervous system.

Intriguingly, Bellingham et al. (2012) put forward the hypothesis that neural EMVs may represent a molecular mechanism for the spreading of key proteins involved in neurodegenerative diseases such as, Creutzfeldt-Jakob, Parkinson's and Alzheimer's diseases and amyotrophic lateral sclerosis. The concept that EMVs can deliver and propagate pathogens and misfolded proteins is broaden by Vingtdeux et al. (2012), who specifically discuss the potential contribution of exosomes to amyloid and tau pathologies.

A common issue to the articles focused on brain pathologies is the potential use of EMVs for diagnostics (Bellingham et al., 2012; Colombo et al., 2012; D'Asti et al., 2012). For instance, in brain tumors it is postulated that EMVs circulating in blood or cerebrospinal fluid may be used to decipher molecular features (mutations) of the underlying malignancy and to monitor responses to therapy (D'Asti et al., 2012). While, in neurological disorders with a vascular or ischemic pathogenic component, detection of endothelium- or platelet-derived EMVs in plasma or serum reflects disease activity, and represents a very useful marker to support therapeutic choices (Colombo et al., 2012).

The therapeutical potential of EMVs is instead dual. Indeed blocking EMV secretory pathways could represent a potential therapeutic in neurodegenerative and inflammatory diseases, and in brain tumors (Bellingham et al., 2012; Colombo et al., 2012; D'Asti et al., 2012). On the other hand, the possibility emerges to take advantage of EMVs spreading capability and specificity for drug delivery and therapeutic purposes. This is clearly outlined by Lai and Breakefield (2012), who report emerging EMV-mediated therapies, such as cancer immunotherapy, RNA-interference (RNAi) and drug therapies that could be applied in the foreseeable future to counter brain diseases.

An impressive progress has been recently made in the knowledge of the cellular and molecular mechanisms of EMVs, but still many questions remain to be answered with respect to different

aspects of EMV biology. Most EMV functions arise from *in vitro* data obtained in pathological conditions. To evaluate EMV's physiological role during development and adult functions, the field will greatly benefit from the creation of genetic models in which EMV production can be inducibly regulated (Frühbeis et al., 2012; Turola et al., 2012). Moreover, defining EMVs' cargos and understanding EMVs' half-life and circulation *in vivo* will shed light into the intricate intercellular communication system within the body (Lai and Breakefield, 2012). Improvement of isolation protocols, i.e., higher grade of standardization and quality control, and more sensitive and reliable quantification methodologies need to be established by the research community in order to achieve these goals. In this respect, Momen-Heravi et al. (2012a) critically discuss the latest developments in technology concerning methods for EMV isolation and characterization. In addition, the Special Issue ends with an original article describing how viscosity of biological fluids influences isolation efficiency of EMVs by ultracentrifugation, which still represents the "gold standard" method for isolating EMVs (Momen-Heravi et al., 2012b).

Hopefully this Special Issue will encourage/foster innovative studies for the years to come and will stimulate future implications of EMVs and nanotubes in brain functions not yet investigated.

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Multifaceted roles of tunneling nanotubes in intercellular communication

Ludovica Marzo[1,2†], Karine Gousset[1†] and Chiara Zurzolo[1,2]*

[1] *Unité de traffic membranaire et pathogenèse, Institut Pasteur, Paris, France*
[2] *Dipartimento di Biologia e Patologia Cellulare e Molecolare, Università Federico II, Napoli, Italy*

Edited by:
Claudia Verderio, Consiglio Nazionale delle Ricerche, Italy

Reviewed by:
Hans-Hermann Gerdes, University of Bergen, Norway
Yan Zhang, Peking University, China

***Correspondence:**
Chiara Zurzolo, Unité de traffic membranaire et pathogenèse, Institut Pasteur, 25 rue du docteur Roux, 75074 Paris Cedex 15, France.
e-mail: chiara.zurzolo@pasteur.fr

[†] *Ludovica Marzo and Karine Gousset have contributed equally to this work.*

Cell-to-cell communication and exchange of materials are vital processes in multicellular organisms during cell development, cell repair, and cell survival. In neuronal and immunological cells, intercellular transmission between neighboring cells occurs via different complex junctions or synapses. Recently, long distance intercellular connections in mammalian cells called tunneling nanotubes (TNTs) have been described. These structures have been found in numerous cell types and shown to transfer signals and cytosolic materials between distant cells, suggesting that they might play a prominent role in intercellular trafficking. However, these cellular connections are very heterogeneous in both structure and function, giving rise to more questions than answers as to their nature and role as intercellular conduits. To better understand and characterize the functions of TNTs, we have highlighted here the latest discoveries regarding the formation, structure, and role of TNTs in cell-to-cell spreading of various signals and materials. We first gathered information regarding their formation with an emphasis on the triggering mechanisms observed, such as stress and potentially important proteins and/or signaling pathways. We then describe the various types of transfer mechanisms, in relation to signals and cargoes that have been shown recently to take advantage of these structures for intercellular transfer. Because a number of pathogens were shown to use these membrane bridges to spread between cells we also draw attention to specific studies that point toward a role for TNTs in pathogen spreading. In particular we discuss the possible role that TNTs might play in prion spreading, and speculate on their role in neurological diseases in general.

Keywords: tunneling nanotubes, intercellular communication, long-range connections, vesicular transport, signal spreading, pathogen spreading, organelle transfer

INTRODUCTION

The ability of cells to communicate with each other is essential for the life of a multicellular organism and is evolutionarily conserved between species (Gurke et al., 2008). Without cell-to-cell communication, processes such as remodeling of tissues and organs, differentiation during development, growth, cell division, and responses to stimuli could not take place. Therefore, a great number of cellular genes and their products are implicated in intercellular communication and their misregulation leads to the establishment of pathological conditions associated with many diseases.

Chemical signaling by secretion of small molecules toward distant cells is the classical form of cell-to-cell communication and does not involve physical contact. It includes chemical mediators with paracrine effects on cells nearby, release of synaptic vesicles containing neurotransmitters between neurons (chemical synapses; Süudhof, 2008), and hormones, which travel in the blood stream after their release and can reach and stimulate distant target cells.

In cases of close proximity, cells can interact with each other through gap junctions or synapses. Gap junctions connect the cytoplasm of two neighboring cells by clustering tens to thousands of intercellular channels, allowing the transfer of ions and small, hydrosoluble molecules (Maeda and Tsukihara, 2011). They mediate electrical and metabolic coupling of cells and are implicated in a wide range of biological processes such as muscle contraction or electrical synapses in neurons (Connors and Long, 2004). Immunological synapses, established at the interface between a T-cell and an antigen-presenting cell (APC), are rather mediated by membrane receptors (Rechavi et al., 2007; Tarakanov and Goncharova, 2009) and are essential for the adaptive immune response (Dustin et al., 2010). Structurally similar to the immunological synapse are the virological synapses. These supramolecular structures are cytoskeleton-dependent adhesive junctions induced by virus-infected cells and used by these pathogens to directly transfer to non-infected cells (Jolly and Sattentau, 2004). Human immunodeficiency virus type 1 (HIV-1) and human T-cell leukemia virus type 1 (HTLV-1) can spread using virological synapses between T cells (Tarakanov and Goncharova, 2009).

Recently, long-range forms of intercellular communication consisting of different types of membrane bridges have been described in a wide variety of cell types in *in vitro* cell culture systems (Gerdes et al., 2007; Abounit and Zurzolo, 2012; **Figure 1**). Similar connections have also been found *in vivo* and in tissue explants (Wolpert and Gustafson, 1961; Miller et al., 1995; Ramírez-Weber and Kornberg, 1999; Demontis and Dahmann, 2007; Chinnery et al., 2008). The discovery of these new types of

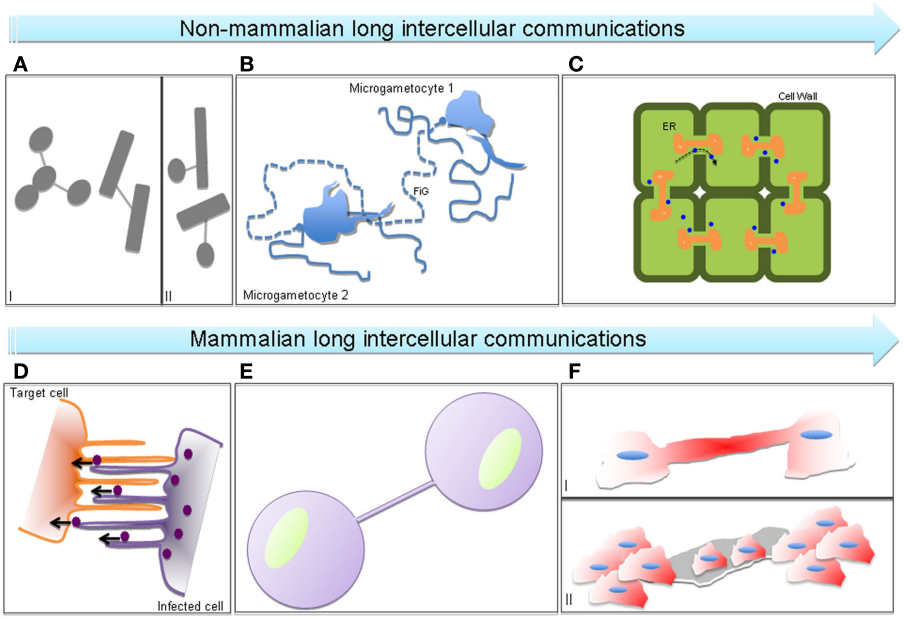

FIGURE 1 | Schematic representation of non-mammalian and mammalian long intercellular communications. (A) Nanotubes formed between bacteria of the same **(I)** and distinct **(II)** species, **(B)** filamentous connection (FiG) between extra flagellating microgametocytes (malaria sexual stage parasites), **(C)** plasmodesmata connecting neighboring plant cells, constituted by a membrane-lined cytoplasmic channel traversing the cell wall with an endoplasmic reticulum (ER) tubule passing through the middle and allowing the passage of molecules (blue dots), **(D)** viruses (violet dots) spreading on filopodial bridges or inside viral cytonemes formed between virus-infected and target cells, **(E)** tunneling nanotubes connecting mammalian cells, **(F)** type I **(I)** EP (epithelial) bridge connecting human bronchial EPs and type II EP bridge **(II)** formed between two EP islands of human bronchial EPs and allowing the passage of entire cells.

communication highways has opened up new ways of viewing how cells interact with one another, leading to the reconsideration of the traditional view of the cell as a basic unit of structure, function, and organization originally postulated by Schwann and Schleyden (1847).

Tunneling nanotubes (TNTs; **Figure 1E**) were initially described by Rustom et al. (2004) as long thin actin-containing bridges connecting PC12 cells in culture that do not contact the substratum, extending up to 100 μm in length with diameters ranging from 50 to 200 nm. Since then, TNTs have been found in many cell types in culture, from immune to neuronal cells and primary cells, acting as conduits for cytosolic and membrane-bound molecules, organelles and spreading of pathogens (Gerdes et al., 2007; Gousset and Zurzolo, 2009).

Filopodial bridges (**Figure 1D**), also called viral cytonemes for their similarity with cytonemes (e.g., filopodial protrusions described in *Drosophila* imaginal disks; Ramírez-Weber and Kornberg, 1999), are instead cellular extensions observed in different cell types (Cos-1, HEK293, DFJ8, XC cells) and induced by some retroviruses before their entry into the cell (Sherer et al., 2007). It has been shown that murine leukemia virus (MLV) and HIV-1 can be unidirectionally transported on the surface of these structures, using them for cell–cell transmission and spreading (Lehmann et al., 2005; Sherer et al., 2007; Mothes et al., 2010). Vesicular clusters containing VP16, a structural protein of herpes simplex virus (HSV), and US3 kinase of the pseudorabies virus have been found in similar cellular projections, mainly at the contact site with neighboring cells, respectively in Vero cells (La Boissière et al., 2004) and RK13 cells (Favoreel et al., 2005).

In addition, Zani et al. (2010) have described two different types of cellular bridges (**Figures 1FI,II**), called epithelial bridges (EP bridges) that connect primary human bronchial epithelial cells. Differently from TNTs, EP bridges are more stable, longer (from 25 μm up to 1 mm), and with a diameter ranging from 1 to 20 μm (Zani and Edelman, 2010). Structurally, they contain both F-actin and microtubules, similar to the TNTs found in primary human macrophages (Onfelt et al., 2006), rat cardiac myoblast cells (He et al., 2010), and cardiomyocytes/cardiofibroblasts co-culture system (He et al., 2011). While type I EP bridges (**Figure 1FI**) seamlessly allow the bi-directional transfer of different cellular components (e.g., lysosomes and Golgi), the type II structures (**Figure 1FI**) might represent a new way of cell migration since it can transfer an entire cell from one multicellular EP island to another (Zani and Edelman, 2010).

The discovery of mammalian bridges is more recent compared to plant conduits, called plasmodesmata (PDs; **Figure 1C**), because they are more fragile and more difficult to observe. For example, they are sensitive to prolonged light excitation, mechanical stress, and chemical fixation and are close to the optical limit of resolution (Hurtig et al., 2010). PDs share some structural characteristics with TNTs. They are thin membrane structures with a diameter around 50 nm but are shorter than TNTs as their length is determined by the thickness of the cell walls between neighboring cells. Moreover, PDs allow an actin-mediated transfer of small

molecules, transcription factors, and also spreading of viruses, creating a sort of continuity between the cytoplasm of connected cells (symplast; Lucas et al., 2009). Even more similarities between the mammalian TNTs and the plant PDs are found regarding the mechanisms of formation and transfer (e.g., passive diffusion of small molecules and gated-mechanisms for bigger components) although the nature of the transported molecules can vary (Rustom, 2009; Abounit and Zurzolo, 2012). This highlights a possible common origin during evolution of TNTs and PDs that can allow a better understanding of the newly discovered mammalian bridges by comparing them with the better-known PDs.

Interestingly, along the same line of thinking, recent findings of bacterial networks (**Figure 1A**) and parasite protrusions (**Figure 1B**) make us wonder how evolutionally conserved these kinds of intercellular communications can be. Indeed, Dubey and Ben-Yehuda (2011) have recently shown that *Bacillus subtilis* grown on a solid surface can establish nanotube-mediated networks with neighboring bacteria of the same or different species (**Figures 1AI,II**), as *Staphylococcus aureus* or *Escherichia coli*, pointing toward a common way of communication shared between phylogenetically distant bacterial species. These linking structures and their mammalian or plant counterparts facilitate transfer of cytoplasmic components and non-conjugative plasmids, allowing the exchange of hereditary traits for the acquisition of new features between connected bacteria (Dubey and Ben-Yehuda, 2011). Sometimes in nature similarities in structure do not reflect related functions. This could be the case of the cell-to-cell connections formed by the malaria pathogen during reproduction in the mosquito midgut (Rupp et al., 2011; **Figure 1B**). In this paper, the authors have described the presence of filamentous structures containing F-actin, that they called "filaments of gametes" or FiGs, in the activated gametocytes. Multiple FiGs are generated on the surface of the cell a few minutes after activation and can extend up to 180 μm. A closer look at these structures revealed that they possess closed-ends and they do not transfer material. Interestingly, FiGs exhibit adhesion molecules on their surface that can instead mediate contact and recognition with the right mating partners for the *Plasmodium*, allowing clustering of gametocytes and facilitating the process of reproduction (Rupp et al., 2011).

This and the other examples of intercellular contacts established by different types of cells reported here reveal a high heterogeneity in both structure and functions of these fascinating new routes of communication that need further characterization and classification (**Figure 1**). Furthermore in order to better understand their physiological relevance more efforts will be needed to identify these structures *in vivo*. To this aim the identification of specific TNT markers by using *in vitro* models is of fundamental importance.

This review will focus on mammalian TNTs, their possible mechanism of formation and their various functions, giving particular attention to their implication in prion spreading.

MECHANISMS OF TNT FORMATION AND PROTEINS INVOLVED

In two-dimensional cultures, TNT-like structures were first discriminated from filopodia from their structural space. Contrary to filopodia, they formed long bridges between cells and were not attached to the substratum (Rustom et al., 2004). In addition to their spatial differences, TNTs, and filopodia appear to serve different purposes. While filopodia act as important environmental sensors and play key roles in cell motility, the main role of TNTs appears to be as a direct conduit for cell-to-cell communication, specifically in the transport of material from one cell to another. As stated above, numerous membrane bridges have been described in a multitude of cell types. Even within TNT-like structures, it became quickly evident that these various structures were distinct from one another both in their structures and functions.

TNT FORMATION AND STRUCTURAL COMPONENTS

Tunneling nanotube-like structures were first described in PC12 neuronal cells (Rustom et al., 2004). In these cells, *de novo* actin-driven formation of TNTs was observed. Further examination of PC12 cells and TNT formation suggested that while the majority of tubes formed via directed filopodia-like protrusions, a small subset (7%) were also able to form after cells previously in contact detached from one another (Bukoreshtliev et al., 2009) (for review, see Abounit and Zurzolo, 2012). In the mouse neuronal CAD cell line, we were also able to observe both types of TNT formation (data not shown). However, the significance and the differences between these two modes of formation and whether they lead to various structures remain unclear. Similar to other cell types, we observed a high degree of heterogeneity in the diameters of TNT-like structures (Gousset et al., 2009). Furthermore, as previously described in PC12 cells (Rustom et al., 2004), neuronal TNTs formed between CAD cells contained actin filaments but no microtubules, even in the tubes with larger diameters (Gousset et al., 2009). The fact that most TNTs in neuronal cells arise from the extension of filopodia-like protrusions toward neighboring cells suggested that actin polymerization plays an important role in this type of TNT formation. Rustom et al. (2004) demonstrated that using the F-actin depolymerizing drug latrunculin, no TNTs were detected in PC12 treated cells. This type of treatment could thus be used to selectively block TNT formation and look at the effect of the presence or absence of nanotubes in various cultures. In our lab, we took advantage of this treatment to highlight the importance of the presence of TNTs in the transfer of infectious prion aggregates in neuronal cells (Gousset et al., 2009). Using nanomolar concentrations of Cytochalasin D (CytoD), another actin-depolymerizing drug, Bukoreshtliev et al. (2009) went further and examined the effects of this drug during the lifetime of TNTs. They showed that as expected, low levels of CytoD abrogated both filopodia formation and TNT formation. Interestingly, they also demonstrated that once formed, CytoD had little effects on the stability of these tubes or their ability to transfer material from one cell to another. Thus, most neuronal TNTs arise from filopodia-like structures, detached from the substratum. Once formed however, they are no longer sensitive to low levels of actin-depolymerizing drugs, demonstrating that functional TNTs are distinct from filopodia in both structure and function. Interestingly, recent experiments with primary rat astrocytes and neurons also showed actin to be the major cytoskeleton component of TNTs formed between these cells (Wang et al., 2011). Indeed, these

authors showed that treatment with latrunculin or CytoD abrogated their formation, thus further validating the use of neuronal cell lines as models for neuronal TNTs.

Tunneling nanotube-like structures have also been described in immune cells, such as B-cells, Natural killer cells, and macrophages (Onfelt et al., 2004). In macrophages, two types of nanotubes were described (Onfelt et al., 2006). The thin nanotubes were found to contain actin filament only, whereas thicker nanotubes, with diameters larger than 0.7 μm, contained both F-actin and microtubules. These different structures appeared to have distinct functions, with the thicker structures being able to transport in a bi-directional manner vesicles and various organelles in a microtubule dependent mechanism. Similarly, long nanotube connections between Jurkat T cells and primary T cells were also described (Sowinski et al., 2008). In these cells, F-actin but no microtubules were detected in TNTs. In addition, while these tubes were not open-ended, they still allowed for the transfer of HIV-1 via a receptor-dependent mechanism. Finally, numerous networks of TNT-like structures were observed between dendritic cells and THP-1 monocytes (Watkins and Salter, 2005). These connections varied greatly in length and diameter but were able to quickly transfer calcium fluxes and small dyes to interconnected cells.

Thus, while numerous TNT-like structures have been described in immune cells, these tubes are clearly distinct from one another both in their structural components as well as in their means of transfer. The one characteristic consistent for all types of immune cells is their formation that appears to rely primarily on cell-to-cell attachment and formation of immunological synapses prior to cell separation and tube formation (Sowinski et al., 2008).

In urothelial cell lines, two types of TNT-like structures were observed (Veranic et al., 2008). The shorter but more dynamic structures, described as Type I nanotubes, were found to contain actin and to connect with neighboring cells by an anchoring type of intercellular junctions. By using time-lapse phase-contrast microscopy the authors observed that these structures did not collapse after micromolar concentrations of CytoD suggesting that after anchoring actin was no longer necessary (Veranic et al., 2008). On the other hand, the longer and more stable structures, or type II nanotubes, no longer contain actin filaments but were composed of cytokeratin filaments. Although the authors have observed vesicles on both these types of structures, further investigation is necessary to understand if these structures are involved in transferring materials, thus fulfilling the TNT definition.

These examples show the disparity in the various cytoskeleton requirements and formation mechanisms in naturally occurring TNT-like structures in neuronal, immunological, or epithelial cells. The type of formation however (*de novo* actin-driven vs detachment after cell-to-cell contact) might arise from the nature and role that these cells play *in vivo*. Indeed, mobile cells, which can more easily come into contact with other cells, might be more prone to form tubes from a previous cell-to-cell contact, whereas more immobile cells might be more adept at creating and extending tubes *de novo* toward distant cells. Because of the increasing number of studies on different and highly heterogeneous TNT-like structures in several *in vitro* systems a more systematic classification is needed.

SIGNALS AND MOLECULES INVOLVED IN TNT FORMATION: IS STRESS A MAJOR PLAYER?

In order to better understand the role that TNTs may play in intracellular transfer of materials, a better characterization of the initiation steps of TNT formation, the signals that guide the extension of these structures toward a neighboring cell and the mechanisms of binding and fusion need to be elucidated.

Recently, the effects of stress on TNT formation have been analyzed in different cell types (Wang et al., 2011). In their studies, Wang and colleagues have shown that stress induced by hydrogen peroxide (H_2O_2) treatment led to an increase in TNT formation in both astrocytes and neurons. They also observed the transfer of various organelles, such as ER, Golgi, endosomes, and mitochondria via TNTs in astrocyte cultures. For both astrocytes and neurons, it was always the cells undergoing stress that developed TNTs and transferred cellular materials in a unidirectional fashion to the non-activated cells, suggesting that TNT formation might be directly induced by stress and may represent a defense mechanism of the stressed cells. Interestingly, they found that p53 activation, which is critical in apoptosis, led to an increase in TNT formation. Conversely, down regulation of p53 blocked TNT formation (Wang et al., 2011). Subsequently, they showed that EGF receptor up-regulation was also necessary for TNT initiation using different conditioned media and that the initiation of TNT formation was likely dependent on the initiating cells and not the receiving cells. Finally, since the EGF receptor can activate the Akt/PI3K/mTOR pathway, they used various mutants and inhibitors to selectively block or activate each protein and found that this pathway was indeed up regulated in H_2O_2 activated cells, leading to an increase in TNT development (Wang et al., 2011). In another study, using a macrophage cell line and HeLa cells, it was demonstrated that the interaction between m-Sec and the Ral/exocyst complex was also critical for TNT formation (Hase et al., 2009). Therefore, to understand if m-Sec might also be important for TNT formation in astrocytes, Wang et al. (2011) analyzed by RT-PCR the levels of m-Sec in astrocytes and found a positive relationship between H_2O_2 treatment and the levels of m-Sec expression. Interestingly, their data indicated that m-Sec might be regulated by p53 activation. Thus, the authors suggest that the initiating cells control TNT formation in a p53 and Akt/PI3K/mTOR pathway activation-dependent manner, but they do not exclude that some guidance cues might be originating from the receptor cells (Wang et al., 2011). Further studies are required in order to explore other potential molecular targets downstream of p53 and Akt/PI3K/mTOR pathways that might represent key elements involved in TNT formation.

In another study Yasuda et al. (2010) analyzed the transfer of mitotracker labeled vesicles via TNTs between endothelial progenitor cells (EPC) and human umbilical vein endothelial cells (HUVEC). They observed both TNT formation between the two-cell types and transfer of mitochondrial material from the EPC to the HUVEC. Upon treatment of the HUVEC with adriamycin, they observed a large increase in the transfer of mitotracker particles from the non-stressed EPC to the adriamycin-stressed HUVEC. In addition, the transfer was unidirectional since the reverse loading and transfer experiments were not significant (Yasuda et al., 2010). While it was not clear in these experiments

which cell type initiated the formation of the nanotubes, contrary to what was found in neuronal and astrocyte cultures (Wang et al., 2011), the transfer of material occurred from the non-stressed cells to the stressed cells. These observations raised the question of how these cells initiated TNTs. Further characterization in these co-cultures could determine whether the stressed cells might release some signals that might attract filopodia-like protrusions from the EPC to the HUVEC or whether the HUVEC cells might initiate formation and allow for a reverse transfer of material from the receptor cell to the initiator cell.

This is exactly what the authors next set out to demonstrate. Indeed, in a follow-up study, they analyzed more precisely the TNT formation mechanisms between these cells. First they showed that co-cultures of EPC with stressed HUVEC led to a rescue of HUVEC viability. However, when the EPC were pre-treated with nanomolar levels of CytoD to block TNT formation prior to co-culture with the HUVEC, the rescue effects were almost entirely abrogated, pointing toward the importance of TNT formation from EPC to HUVEC for cell survival. Using both fluorescence microscopy and FACS analyses they observed basal levels of transfer of lysosomes between the two-cell types in a bi-directional manner under non-stressed conditions. However, the transfer was much more efficient as it increased in speed and frequency and was found preferentially between non-stressed EPC and GC-stressed HUVEC, suggesting that the stressed cells were able to signal and guide filopodia-like protrusions for the formation of *de novo* TNTs to occur (Gerdes et al., 2007; Yasuda et al., 2010, 2011). Further examination suggested that surface-exposed phosphatidylserines (PS) in HUVEC might be able to guide TNT formation from the EPC to the stressed HUVEC. Indeed, when PS on HUVEC were blocked by binding of Annexin V, the selective TNT formation and transfer from EPC to HUVEC was also blocked (Yasuda et al., 2011).

Overall, these studies suggest that transfer of materials via TNTs in most cell types occurred from the cell type that initiated TNT formation to the receptor cell. However, while certain stress conditions might increase the formation of TNTs between cells, it does not affect all cells the same way. Indeed, while in astrocytes and neurons, stress appears to increase TNT formation in the stressed cells leading to an increase in transfer of material, in endothelial cells stress increase the guidance signals from the stressed cells leading to an increased formation of TNTs from the non-stressed cells. Thus, once more the analysis of these two studies brings forward the disparities that exist in formation and nature of TNTs between different cell types. It suggests that even within an identical type of TNT formation (i.e., *de novo* extension of filopodia-like protrusions) the mechanisms might be very distinct from one another (activation of attractive guidance signals vs activation of initiation of filopodia-like protrusions). However, these studies implicate the involvement of more general signaling pathways in TNT formation. For example, the role of m-Sec, which was found to be important in macrophages, HeLa cells, and astrocytes (Hase et al., 2009), could be of general importance in TNT formation, independent of cell type. In addition, since filopodia-like protrusions are critical for TNT formation in neuronal cells (Bukoreshtliev et al., 2009), our lab, has turned its attention to the role that the actin molecular motor protein Myosin-X might play in both the formation of TNT-like structures and its function in transfer of materials in neuronal cells. We found that over-expression of Myosin-X (Berg and Cheney, 2002) increased the number of TNTs observed in our cell cultures (data not shown). In addition, similar to what Wang and colleagues (Wang et al., 2011) have found with stress signals, we observed a unidirectional transfer of vesicles occurring from the cells over-expressing Myo-X to the acceptor cells (data not shown).

Finally the search for guidance signals and the role that lipids might play in TNT formation might provide further information about TNT formation.

MECHANISMS INVOLVED IN OPEN-ENDEDNESS OF TNTs

As previously stated, in T cells no membrane continuity or transfer of cytosolic material have been observed (Sowinski et al., 2008), suggesting different types of tubular structures between T cells and other cell types that allowed for the transfer of cytosolic materials such as neuronal cells, astrocytes, myeloid cells, or endothelial cells. Recently, however, Arkwright et al. (2010) have shown that specific stimulation could lead to an increase of TNTs in T cells along with the transfer of cytosolic material. First, they showed that FAS activation resulted in an increase in TNT formation and that both toxin B of *Clostridium difficile* (an inhibitor of actin Rho-GTPases) and secramine A (an inhibitor of CDC42) specifically blocked FAS stimulated TNT formation in T cells. They also analyzed the bi-directional exchange of labeled membranes in T-cell co-cultures. As expected, they only found a negligible number of TNTs with both markers in control cells, whereas upon FAS stimulation they observed a 20-fold increase in the number of TNTs labeled with both membrane markers. The transfer of cytosolic materials, including fluorescent cytosolic proteins as well as labeled vesicles, was also observed upon FAS-stimulation between T cells. These experiments demonstrated that the nanotubular structures initiated by FAS-stimulation were different from the TNTs previously described in non-activated T cells and did not contain an immunological synapse (Sowinski et al., 2008). These connections were similar to the connections observed in other cell types and demonstrate the complexity and dynamism of the various TNT-like structures that have been described to date. While this study demonstrates that within the same cells, different activation can quickly lead to the formation of different types of TNTs with distinct functions; the mechanisms involved in the gating of these tubular structures remain undetermined. Overall, these recent studies on TNTs have shown the diversity of these structures but also their ability to transfer numerous signals upon specific activation.

CAUSE AND CONSEQUENCES OF TNT-MEDIATED TRANSFER, FROM SIGNAL TO ORGANELLES AND PATHOGENS

Tunneling nanotubes have revealed a high degree of heterogeneity also from a functional point of view, as different components seems to be selectively transferred by different cell types. What determines this selectivity remains unknown.

First, further investigation is needed to understand why some cargoes are unidirectionally or bi-directionally transported. Unilateral transfer occurs in the case where a donor cell transfers material to an acceptor cell, whereas bi-lateral transfer happens

when both cells mutually exchange materials. The reasons for these different transport mechanisms can depend on the structural components (actin only vs actin + microtubules containing TNTs) or on specific signals that stimulate nanotube formation and are responsible for directing the traffic in one or two ways.

As already mentioned above, bi-directional transfer is found when both actin and microtubules are present (Onfelt et al., 2006; Arkwright et al., 2010; He et al., 2010, 2011), while it appears to be unidirectional when TNTs contain actin only (Rustom et al., 2004; Koyanagi et al., 2005; Gurke et al., 2008; Eugenin et al., 2009; Gousset et al., 2009; Domhan et al., 2011). A recent work by Plotnikov et al. (2010) shows that unidirectional transfer from rat renal tubular cells (RTC) to bone marrow multipotent mesenchymal stromal cells (MMSC) was observed in this co-culture system (Plotnikov et al., 2010). However, passage of molecules in the opposite direction was also detected, albeit at a lower rate. Additionally, it has been shown that lysosome exchange (Lysotracker-labeled) between endothelial progenitor cells (EPC) and endothelial cells (HUVEC) in co-cultures occurs at a basal level and that this transfer selectively increases in one direction, from EPC to HUVEC cells, upon injury of the latter (Yasuda et al., 2010). These two reports suggest that a shift from a bi-directional basal level of transfer to a selective unidirectional transfer toward a specific cell population might take place by means of intercellular thin connections resembling TNTs between cells upon specific treatment, as is the case for differentiation signal flow toward MMSC cells (Plotnikov et al., 2010) and stress signal deriving from damaged organelles (Yasuda et al., 2010). What remains to be determined is how transfer occurs within TNTs and whether common molecular motors might be involved during this process. Furthermore, the fact that TNT structures contain F-actin as backbone suggests that an acto-myosin-dependent mechanism could be responsible for organelles or pathogens transfer mediated by TNTs (Rustom et al., 2004; Gerdes et al., 2007; Hurtig et al., 2010). It has been reported that organelle transfer through TNTs is an active process that depends on actin and ATP (Onfelt et al., 2006; Gurke et al., 2008; Bukoreshtliev et al., 2009; Gousset et al., 2009). Indeed the use of F-actin depolymerizing drugs and ATP-depletion experiments resulted in an almost complete block of organelle transfer (Onfelt et al., 2006; Gurke et al., 2008; Bukoreshtliev et al., 2009; Gousset et al., 2009). Furthermore by measuring the trajectory of the organelles transferring from one cell to another Gurke et al. (2008) demonstrated that the vesicle movement inside TNTs of NRK cells was due to active transport and not to free diffusion. Similar conclusions were obtained by measuring the mean square displacement of PrP containing vesicles in TNTs (Gousset et al., 2009). In addition, vesicular traffic on actin- and microtubules-containing TNTs in macrophages was shown to be sensitive to ATP-depletion, indicating that independently of the cytoskeleton components transfer through TNTs occurs as an active process (Onfelt et al., 2006). Finally the actin-binding motor Myosin Va is present in TNTs and partially localizes with endocytic organelles (Rustom et al., 2004; Gerdes et al., 2007). A more detailed analysis on the role of Myosin Va and the screening of myosin motors involved in endocytic vesicles traffic or pathogens spreading will be necessary to further dissect the mechanism of transfer occurring *via* TNTs.

SIGNAL TRANSFER

Up to now several reports have shown that calcium signals could propagate between remote cells through TNTs (Watkins and Salter, 2005; Hase et al., 2009; Smith et al., 2011; **Table 1**). This is especially important for remote cells that are unable to propagate calcium-mediated signaling to cells in close proximity using gap junctions (Wang and Gerdes, 2011). Initially, Watkins and Salter (2005) demonstrated that myeloid cells can respond to stimulation through soluble factors or mechanical stress and are able to amplify the cellular response by calcium signaling through membrane connections. Since then, propagation of calcium flux has been shown in many other cell types able to make connections between each other (Hase et al., 2009; Smith et al., 2011; **Table 1**). More recently, the transfer of IP3 receptor (IP3R) and endoplasmic reticulum has been described along TNTs in SH-SY5Y neuroblastoma and HEK cell lines (Smith et al., 2011). The authors made a comparison between the current produced at the end of a TNT (typically 30 μm in length and 200 nm in diameter) and single inositol trisphosphate receptor (IP3R)-channels. While the first produces a current <1 fA, corresponding to calcium flux propagated from an activated cell, the opening of a single channel results in ∼100 fA. Considering that a single opened IP3R-channel generally fails to induce Ca^{2+} signaling, the passive diffusion of Ca^{2+} within TNTs appears quite inefficient. However, since IP3R is able to transfer along TNTs, it could overcome the limit of passive diffusion of calcium by amplifying calcium signaling within a population. Finally, a recent study has reported the formation of electrically coupled nanotubes that do not allow diffusion of calcium or IP3R, but are instead involved in the bi-directional spread of electrical current between distant cells through gap junctions (Wang et al., 2010). These type of TNTs are immuno-positive for connexin-43, at one end of the connection and allow the passage of electrical signals which in turn leads to the activation of low voltage gated channels that allow a local influx of calcium in the connected cell. Electrical coupling-competent TNTs, distinguished from those that do not possess gap junctions, have been found in different cell types and represent a selective way for transferring electrical signals compared to gap junctions coupling (Wang et al., 2010; Wang and Gerdes, 2011; Abounit and Zurzolo, 2012).

Overall, calcium spreading through nanotubes appears to be a good option for different types of cells to quickly spread calcium signals under physiological conditions, leading to fast responses in connected neighboring cells (for review see Abounit and Zurzolo, 2012; **Table 1**).

Particularly fascinating and newly discovered is the spreading of death signals by nanotubes occurring in Jurkat and primary T cells (Arkwright et al., 2010; **Table 1**). Fas-mediated signaling is important for peripheral deletion of activated T lymphocytes (Green et al., 2003). Mutations in the cytoplasmic domain of the Fas receptor are responsible for a rare genetic disease, the autoimmune lymphoproliferative syndrome (the type Ia ALPS; Martin et al., 1999). As stated previously, Arkwright et al. (2010) have shown that stimulation of the Fas receptor leads to an increase in the number of TNT-connected cells and this is critically dependent on Rho GTPase activation. Accordingly, the authors also demonstrated that primary T cells deriving from ALPS patients were not able to form networks of TNTs. This points toward a pivotal

Table 1 | Overview of the different cargos found in TNT-like structures.

Functions of TNTs	Cargo detection	Cell type	References
SPREADING OF SIGNALS			
Calcium signaling	IP3R	SH-SY5Y neuroblastoma, HEK cells	Smith et al. (2011)
	Ca^{2+}; Fura-2	THP-1 monocytes and dendritic cells	Watkins and Salter (2005)
	Ca^{2+}	Raw264.7 macrophages, HeLa cells	Hase et al. (2009)
	Electrical coupling through gap junction at the TNT end	Normal rat kidney (NRK), HEK, HUVEC, NCC and rat pheochromocytoma (PC12) cells	Wang et al. (2010)
Death signals	FasL, caspase-3	Jurkat and primary T cells	Arkwright et al. (2010)
	Cytotoxicity	NK cells	Chauveau et al. (2010)
ORGANELLE EXCHANGE			
Endosomes	Purified mouse anti-EEA1 antibodies	CMs and FBs co-culture system*	He et al. (2010)
	DiD (1,1′-dioctadecyl-3,3,3′,3′-tetramethylin dodicarbocyanine perchlorate)	NRK cells	Gurke et al. (2008)
	Qtracker®	Human renal proximal tubular epithelial cells (RPTEC)	Domhan et al. (2011)
	DiD (1,1′-dioctadecyl-3,3,3′,3′-tetramethylin dodicarbocyanine perchlorate)	Human monocyte-derived macrophages	Onfelt et al. (2006)
	Endosomes-related organelles (DiI and DiO)	PC12 cells	Rustom et al. (2004)
Lysosomes	Lysotracker®	PC12 cells	Rustom et al. (2004)
	Qtracker®	Human renal proximal tubular epithelial cells (RPTEC)	Domhan et al. (2011)
	Lysotracker®	Mouse catecholaminergic neuronal cell line, Cath.a-Differentiated (CAD)	Gousset et al. (2009)
	Lysotracker®	EPC and HUVEC co-culture system (rescue from injuries)*	Yasuda et al. (2011)
	Mouse anti-LAMP1 antibodies	Human monocyte-derived macrophages	Onfelt et al. (2006)
Mitochondria	Mitotracker®	EPC or CD34+ cells and neonatal rat cardiomyocytes co-culture system (Differentiation)*	Koyanagi et al. (2005)
	Mitotracker®	MMSC and RTC*	Plotnikov et al. (2010)
	Mitotracker®	H9c2 Cardiomyoblasts and MMSC (rescue from injuries)*	He et al. (2011)
	TMRE	Jurkat and primary T cells	Arkwright et al. (2010)
	MitoTracker	Human monocyte-derived macrophages	Onfelt et al. (2006)
Membrane components	CD81, CD59	Jurkat and primary T cells	Arkwright et al. (2010)
	c-HA-Ras	PC12	Rustom et al. (2004)
	Surface receptors (HLA-A,B,C class I MHC)	Myeloid cells	Watkins and Salter (2005)
	DiO	MMSC and RTC*	Plotnikov et al. (2010)
	GPI-anchored GFP, TM-proteins (ICAM-I, HLA-Cw7)	Jurkat T cells, primary mouse T cells	Sowinski et al. (2008)
	GFP-PrP	CAD neuronal cells	Gousset et al. (2009)
	MHC-I	Immune cells	Onfelt et al. (2004)
Golgi and Endoplasmic reticulum	Bodipy FL glibenclamide (ER-tracker)	Human monocyte-derived macrophages (MDM)	Kadiu and Gendelman (2011b)
	Bodipy FL C5-ceramide (Golgi-tracker)		
Cytoplasmic components	Cytosolic GFP	CMs and FBs co-culture system*	He et al. (2010)
	Calcein	MMSC and RTC	Plotnikov et al. (2010)
	Cytosolic GFP	EPC or CD34+ cells and neonatal rat cardiomyocytes co-culture system*	Koyanagi et al. (2005)
	Cytosolic stain CFSE	Jurkat and primary T cells	Arkwright et al. (2010)
	Lucifer yellow	Myeloid cells	Watkins and Salter (2005)
Nanoparticles	Nanoparticles quantum dots (CdSe/ZnS)	CMs and FBs co-culture system*	He et al. (2010)

(Continued)

Table 1 | Continued

Functions of TNTs	Cargo detection	Cell type	References
PATHOGENS SPREADING			
Bacteria	*Mycobacterium bovis* BCG	Human monocyte-derived macrophages	Onfelt et al. (2006)
Virus	Gag and Env (antibodies), GFP-Gag	Jurkat T cells, activated primary human or primary mouse T cells	Sowinski et al. (2008)
	HIV particles, HIV-p24	Primary human macrophages infected by HIV	Eugenin et al. (2009)
	Env and Gag proteins	Human monocyte-derived macrophages	Kadiu and Gendelman (2011a), Kadiu and Gendelman (2011b)
Proteinaceous aggregates	PrP^{Sc}	CAD neuronal cells, GCN and DC co-culture system	Gousset et al. (2009)
	A–b fusion proteins	Astrocytes and neurons	Wang et al. (2010)

*The table summarizes all the cargo detected in TNT-like structures by classifying them according to their nature (signals, organelle, and pathogens) and the cell type in which they were found. *Exchange of cargos observed in co-culture of different cell type.*

EPC, endothelial progenitors; HUVEC, stressed endothelial cells; MMSC, bone marrow multipotent mesenchymal stromal cells; RTC, rat renal tubular cells; CM, rat ventricular cardiomyocytes; FB, cardiofibroblasts.

role of the Fas-mediated pathway in promoting TNT formation and transfer in T cells. Additionally, transfer of both membrane (detected by CD59 and CD81 staining) and cytoplasmic components was detected in Fas-induced TNTs. Interestingly, FasL and active caspase-3 passage from Fas-activated cells in neighboring non-activated ones was detected, thus resulting in the spreading of apoptosis through fratricide, highlighting that this might be an efficient way to shut down cellular responses (Arkwright et al., 2010). Moreover, it has been reported that FasL is upregulated in cancer cells (O'Connell et al., 1996) and this could confer a double advantage to these cells in "counterattacking" the immune system and stimulating their own proliferation. In this light, TNTs could act as conduits for diverse signals between tumor cells (for their own survival) and from tumor cells to immune cells (for death), thus leading to opposite effects.

Finally, Chauveau et al. (2010) have recently observed that also Natural Killer immune cells (NK cells) can easily form intercellular nanotubes, particularly upon activation. NK cells are important immune cells implicated in defense against a range of infections (Herberman and Ortaldo, 1981). The authors demonstrated that human primary NK cells are able to connect with different cell types by intercellular bridges and use them to mediate cytotoxicity (**Table 1**) and, therefore, help lyse remote target cells leading to cell death (Chauveau et al., 2010).

ORGANELLE TRANSFER

Tunneling nanotubes can in certain cases be highways for diverse organelle transfer (**Table 1**). Labeling with membrane-specific dyes, markers of the endo-lysosomal pathway, or other dyes specific to organelles such as mitochondria, has revealed subcellular organelles traveling between cells along these connections (**Table 1**). A range of cell types, including T cells, macrophages, NRK, stem cells, epithelial cells, myocardial cells have exhibited transfer of mitochondria (**Table 1**). Differentiation of embryonic endothelial progenitor cells (EPC) in myocyte-like phenotype was observed when EPC were co-cultured with neonatal rat cardiomyocytes suggesting that TNT-mediated transfer of mitochondria could have a reprogramming function in these cells (Koyanagi et al., 2005). Moreover, Spees et al. (2006) have observed the passage of mitochondria from adult non-hematopoietic stem cells (from human bone marrow hMSCs) or skin fibroblasts to A549 ρ° epithelial cells that were defective or deleted in mtDNA rescue aerobic respiration. However, the authors could only hypothesize an involvement of tubular connections between the two-cell types without demonstrating it. A closer look at some recent work involving the use of co-culture systems shows that TNT-mediated mitochondrial transfer could indeed rescue injured cells for pathological conditions (Cselenyák et al., 2010). For example, Cselenyak and coworkers set up a co-culture system of H9c2 cardiomyoblasts and mesenchymal stem cells (MSC) mimicking ischemic damage in H9c2 cells by using oxygen glucose deprivation (OGD). They were able to show passage of functionally active mitochondria (labeled with Mitotracker dye) in the damaged cells specifically when nanotubular connections between the cells were present (Cselenyák et al., 2010). In addition, selective bi-directional transfer of mitochondria in between connected rat ventricular cardiomyocytes (CMs) and cardiofibroblasts (FBs) was observed in tubular structures (He et al., 2011). These connections were enriched in actin and microtubules and allowed for the traffic of soluble cytosolic dyes as well, suggesting continuity between the membranes. The authors also explored a possible physiological significance of the nanotubular structures found in CMs–FBs co-culture system *in vitro* by culturing mouse heart tissue slices. By labeling CMs and FBs with WGA and other specific markers, the authors were able to detect thin structures between the two-cell types, reminiscent of the connections observed *in vitro* (He et al., 2011).

A rescue function of TNT-mediated organelle transfer might be associated with other cell types that undergo injuries as well (**Table 1**). Accordingly, the observation cell-to-cell contacts established between RTC and MMSC leads to the hypothesis that the exchange of cytoplasmic and organelle components could be involved in restoring functions of damaged cells following acute renal failure (Plotnikov et al., 2010). Indeed, endothelial cells

presenting lysosomal dysfunction after exposure to AGE-modified collagen I (Yasuda et al., 2010) appeared to be rescued by transferring normal lysosomal pool from endothelial progenitors to stressed cells (Yasuda et al., 2011) This suggests a role for organelle TNT-mediated transfer in restoring functions and tissue repair, which needs to be further characterized (**Table 1**).

Smaller particles, named nanoparticles, have also been shown to travel within nanotubes (He et al., 2011). Particularly, Streptavidin-coated CdSe/ZnS Quantum Dots (QDs) were detected along membrane nanotubes of rat cardiac myoblast cells (H9c2) at a speed compatible with movement of DiD-labeled vesicles associated with dynein/kinesin motors walking on microtubules (Onfelt et al., 2006), thus suggesting that nanoparticles can be transported inside vesicles within these structures (He et al., 2011). In fact, when WGA was used to label membrane vesicles, QDs colocalized with it inside TNTs, confirming the vesicular transport of these molecules. Moreover, like thicker TNTs described in macrophages (Onfelt et al., 2006) the nanotubes of H9c2 cells contained both actin and microtubules and allowed a bi-directional transfer of membrane vesicles (He et al., 2010). Use of nanoparticles, such as QDs, is an emerging research field for diverse medical applications, such as therapies and diagnostics (Youns et al., 2011). For example, these small compounds could be used to selectively deliver drugs to cancer cells or for other infectious diseases (Singh and Nalwa, 2011). The fact that cells can establish membrane nanotubes together with the new finding that nanoparticles could pass from one cell to another by these means of communication open up new ways for diffusing small therapeutics inside target "cell communities."

PATHOGEN SPREADING

Tunneling nanotubes could be either actively hijacked from different pathogens or transport them as "Trojan horses," along the membrane or inside, leading to the spreading of infection (**Table 1**). Hijacking of these structures can be preceded by induction of TNT formation, thus optimizing pathogen transfer, as has been shown for HIV particles spreading, both surfing on or inside TNTs in primary macrophages (Eugenin et al., 2009). The HIV virus can use these highways to spread as an alternative to the other means already mentioned above.

Recently, a more detailed characterization of HIV-carriers mediating the transfer of the virus along TNTs bridging macrophages has been made that the authors called bridging conduits (BCs; Kadiu and Gendelman, 2011b). In this work, the authors first observed an increase in the number of connections in macrophages, as previously described (Sowinski et al., 2008). They then identified the composition of BCs by proteomic analysis following isolation from cell bodies. Although the approach used to isolate intercellular connections could not totally exclude the presence of other cellular protrusions, the work gives some insights on the possible compositions of BCs in the context of HIV spread. Indeed, they found several organelle markers including endo-lysosomal compartment (14%), ER (9%), and Golgi (4%) inside BCs, the majority of which were regulators of different steps within the HIV life cycle. They were also able to confirmed by confocal microscopy that 72% of Golgi and 32% of ER colocalize in TNTs with the viral protein Env; similar results were also obtained for the viral protein Gag, suggesting a role for these intracellular compartments in HIV intracellular trafficking (Kadiu and Gendelman, 2011a). Indeed, Golgi and ER represent sorting stations for the virus prior to reaching endosomal vesicles and before spreading. Additionally, they observed that Golgi and ER undergo morphological changes upon HIV infection (Kadiu and Gendelman, 2011a). Overall these observations shed light on a possible new role for the Golgi and ER in TNT-mediated transfer of diverse cellular components and their regulation mechanisms that need to be further investigated.

Additional observations on the trafficking of HIV have shown that HIV specifically traffics in TNTs associated with endocytic compartments and so these organelles could be responsible for viral spread between macrophages (Kadiu and Gendelman, 2011a). Moreover, the acto-myosin machinery used by the cell to move virus-containing cargoes within TNTs is 25 times faster than the surfing process seen for HIV and other retroviruses on filopodial protrusions (Sherer et al., 2007). In particular, HIV preferentially associates in TNTs with recycling endosomes and MVB (Kadiu and Gendelman, 2011a). Whether viral particles spreading in vesicles through BC results in a productive infection of a recipient cell and how the flow of these carriers is regulated and intersects with the intracellular pathway remain to be investigated. Comparing intra- and inter-cellular trafficking with our current knowledge in the HIV field could improve our understanding and help in characterizing intercellular spreading of other pathogens that manipulate host intracellular components for their own survival, leading to progressive loss of cellular identity.

One of the best known mechanisms of cell-to-cell spread, common in some pathogenic bacteria such as *Listeria, Shigella*, and *Salmonella*, is their ability to polymerize the host actin cytoskeleton to escape the host and keep infecting new targeted cells (Cossart and Sansonetti, 2004). While little was known about other atypical cytoplasmic bacteria spreading, recently, new "unusual" ways of bacterial spreading have been observed. For example, it has recently been shown that *Cryptococcus neoformans* is able to laterally transfer from an infected macrophage to an uninfected one allowing a latent persistency in the host for long periods before causing meningoencephalitis in the central nervous system (CNS; Ma et al., 2007). The authors observed an actin-dependent transfer of the bacterium in both immortalized cell lines and human primary macrophages by a mechanism not yet understood. More recently, it has been reported that the obligate intracellular bacterium *Ehrlichia chaffeensis* associates with filopodia of infected DH82 monocytes and increases their numbers and lengths (Thomas et al., 2010). The authors hypothesized that the transport of *Ehrlichia* through filopodia could be a potential mechanism for the pathogen to pass from one cell to another without contacting the extracellular environment. Another unusual way of spreading recently highlighted is the formation of an actin barrel (Hagedorn et al., 2009), the "ejectosome," induced by *Mycobacterium marinum* and used by it to pass within infected *Dictyostelium discoideum* ameba as host. This mechanism is an alternative to the formation of a protrusion containing the pathogen created by actin polymerization that is then engulfed by adjacent cells (Carlsson and Brown, 2009). Onfelt et al. (2006) have shown that *M. bovis* BCG or clusters of several bacteria can surf on thin

membrane nanotubes between macrophages before being internalized by receptor-mediated endocytosis (Onfelt et al., 2006), pointing toward a possible role of these structures in bacterial infection by concentrating the pathogen on the entry site for a more efficient invasion.

Additionally, one could also envisage a role for these newly discovered highways in the spreading of some obligatory intracellular bacteria, unable to surf along TNT membranes that could use them to escape from the immune response. As already mentioned above, different sub-cellular organelles are found to shuttle in between cells by TNTs. Bacteria can use different endocytic compartments and modulate them to escape lysosomal degradation (Ham et al., 2011). In particular, vacuoles-containing bacteria deriving from fusion of the pathogen with intracellular organelles were found to be positive for several endosomal proteins (Bonazzi and Cossart, 2006). A problem for nanotubes in transporting these bigger cargoes along their tracks could be overcome by the presence of expansions along the tunnel, known as gondolas (Hurtig et al., 2010). Veranic et al. (2008) have observed that these dilatations of the membrane can move for 5–15 μm with an average speed of 40 nm/s (Veranic et al., 2008). This "pearling" phenomenon seen along some TNT structures might be due to the redistribution of lipids and cytoskeleton components localized in discrete areas and could be compatible with a vesicular transport of pathogen as well.

SPREADING OF PRIONS AND PRION-LIKE NEURODEGENERATIVE DISEASES

The mechanisms of prion spreading from the periphery to the CNS, and subsequently within the CNS, remain questionable. A number of mechanisms, such as cell-to-cell contact, exosomes, and GPI-painting, have been proposed (Baron et al., 2002; Kanu et al., 2002; Fevrier et al., 2004). We have recently demonstrated the presence of TNTs in neuronal CAD cell cultures (Gousset and Zurzolo, 2009; **Table 1**). In addition, we showed that these TNTs were able to transfer lysosomal organelles, the cellular GPI-anchored prion protein PrP^{C}, as well as fluorescently labeled infectious prion particles, PrP^{Sc}. Using various co-culture conditions, we demonstrated that these infectious particles were efficiently transferred to non-infected cells only in the presence of TNTs (Gousset et al., 2009).

Since the prion protein is a GPI-anchored protein, it has the possibility of traveling via TNTs either along their surface or inside the tube within vesicular structures (**Figure 2F** and enlarged box). Recently we have further analyzed the presence of PrP^{Sc} and various organelles inside TNTs. Overall, we observed that similar to what can be found in the cell body, PrP^{Sc} travels in TNTs in early endosomes and lysosomes but it is preferentially enriched in the endosomal recycling compartment. Additionally, increasing the number of TNTs formed, by over-expression of Myosin-X, also increases the spreading of PrP^{Sc} to non-infected cells (data not shown). These data further demonstrate how efficient these structures are in allowing the passage of infectious prions from one cell to another.

Finally, we have also demonstrated that the transfer via TNTs of infectious prion particles resulted in the transmission of infectivity to the recipient cell. This transfer was not confined to neuronal co-cultures but was also efficient between loaded bone marrow derived dendritic cells and primary neurons (Gousset et al., 2009; Langevin et al., 2010). Thus, our studies suggested that TNTs might play a critical role *in vivo* in the spreading of prions within the CNS and at the periphery (Gousset and Zurzolo, 2009).

In vivo, the players involved in the spreading of prions from the gastrointestinal tract, to the lymphoid system and to the peripheral nervous system (PNS) are still unclear (Mabbott and Bruce, 2001). Dendritic cells could bring infectious prion particles from the gut to Follicular dendritic cells, and subsequently pick up prions particles from FDCs and deliver them to the PNS. Thus, analyzing the interactions between these two-cell types might reveal important clues about prion spreading in general. We have started to address these issues. Interestingly, co-culturing DCs and FDC cell lines (Nishikawa et al., 2006) we were able to detect formation of TNT-like structures between the two-cell types (data not shown).

Overall, our studies suggest that TNTs might play a pivotal role in the spreading of prion diseases. Moreover, protein aggregation represents a common neuropathological hallmark for most other neurodegenerative disorders, including Alzheimer's, Parkinson's, Huntington's diseases, and amyotrophic lateral sclerosis (ALS) and each of them is characterized by the misfolding, followed by aggregation, of a specific protein. In particular, β-amyloid (βA) and tau for Alzheimer's, α-synuclein (α-syn) for Parkinson's, huntingtin (htt) for Huntington's disease, and superoxide dismutase-1 (SOD1) for ALS. Interestingly, it has been shown that these misfolded proteins can be transmitted experimentally in animal or cellular models (Krammer et al., 2009) where they can act as "seeds" to recruit endogenous protein into aggregates (seeding process; **Figure 2G**) as it is the case for PrP^{Sc} (Gousset et al., 2009; Langevin et al., 2010). For example, it has been shown that α-syn oligomers once internalized can trigger aggregation of endogenous cytosolic α-syn in cultured primary cortical neurons as well as in neuronal cell lines (Danzer et al., 2007, 2009). Also, extracellular aggregated tau has been shown to enter cells and transmit a misfolded state to intracellular tau (Frost et al., 2009). In this work, the authors have been able to demonstrate that exogenous tau aggregates following their uptake readily induced fibrillation in cells over-expressing a fluorescently labeled form of tau (Full-length Tau-YFP). Interestingly, the resulting aggregated form of endogenous Tau-YFP is able to seed the fibrillation of tau monomer *in vitro* and can transfer between cells (Frost et al., 2009). Taken together, these findings support the idea that other neurodegenerative diseases linked to protein misfolding could be considered prion-like disorders, possibly extending some features of prions to other protein pathologies (reviewed in Frost and Diamond, 2009; Brundin et al., 2010; Dunning et al., 2011; Lee et al., 2011). Furthermore, considering that these diseases follow anatomical pathways for their propagation in the brain (Brundin et al., 2010), it is tempting to speculate the possible common spreading mechanisms of different proteinaceous aggregates that might contribute to the progression of neurodegeneration (**Figure 2**).

As already mentioned before, transfer of prion-like aggregates between cells has been shown in *in vitro* cell culture models and different mechanisms of transfer have been proposed including endo/exocytosis, exosomes, trans-synaptic transmission at axonal terminals (Aguzzi and Rajendran, 2009). Consistently, α-syn can move between neurons in culture (Desplats et al., 2009). In this work, a co-culture system consisting of SH-SY5Y cells

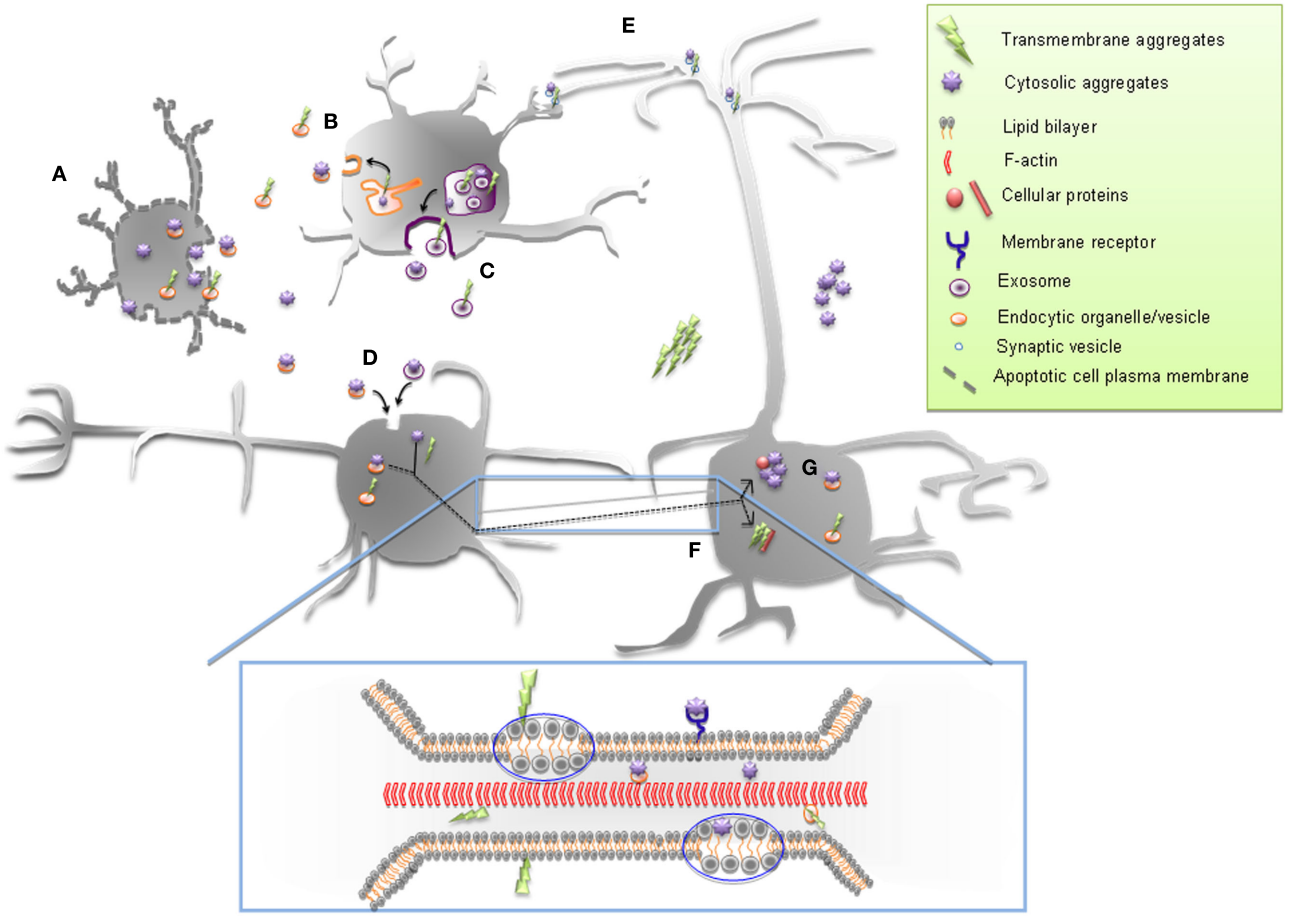

FIGURE 2 | Possible mechanisms of cell-to-cell spreading of cytosolic and transmembrane proteinaceous aggregates. Both cytosolic and transmembrane protein aggregates can be released in the extracellular space from apoptotic cell **(A)**, by exocytosis **(B)** and through exosomes **(C)** and endocytosed by neighboring cells **(D)**. They could also move between cells trans-synaptically **(E)** and through tunneling nanotubes (TNTs) **(F)**. TNT-mediated transfer of both types of protein aggregates (enlarged box) can occur within endocytic vesicles or as aggresomes. "Surfing" on the TNT membrane could also occur: for transmembrane aggregates through their membrane attachment and for cytosolic aggregates either within the cytosolic leaflet of the TNT or along the external leaflet in association with a membrane-receptor. Once inside the recipient cell, proteinaceous aggregates can then seed aggregation of the cytosolic or transmembrane cellular counterpart **(G)**.

over-expressing a myc-tagged version of α-syn (donor population) and SH-SY5Y cells differently labeled (acceptor population) was established. In these conditions the detection of α-syn in the acceptor cell population was proportional to its expression level in the donor cell population. Of interest, no membrane leakage was detected suggesting that cell-to-cell α-syn transmission occurs without cellular membrane damage and implies viable cells (Desplats et al., 2009). More recently, it has been reported that exogenous aggregates of SOD1 deriving from highly purified recombinant SOD1 protein efficiently enter Neuro-2a neuronal (N2a) cells by macropinocytosis and rapidly escape from this compartment to reach the cytosol (Münch et al., 2011). Once there, SOD1 aggregates are able to self-propagate by converting the soluble endogenous counterpart and to spread continuously between cells (Münch et al., 2011).

Although these experiments indicate the propagation of these different proteinaceous aggregates between cells, the mechanism of transfer has not been addressed yet. Recently, Wang et al. (2011) have analyzed whether intracellular Aβ particles could spread through TNTs in astrocytes and neurons. Microinjection experiments demonstrated that intracellular Aβ-fusion proteins were able to quickly spread from cell-to-cell via TNTs (**Table 1**). In addition they showed that increasing the number of TNTs between the cells by H_2O_2 treatment led to an increase in neuronal cell death in co-cultures with pEGFP-Aβ over-expressing astrocytes compared to pEGFP controls (Wang et al., 2011). These data suggest that Aβ particle spreading via TNTs within the cultures resulted in an increase in neuronal toxicity leading to cell death. Such observations are very similar to what we found with PrP^{Sc} spreading and propagation in primary neurons (Gousset et al., 2009; Costanzo and Zurzolo, data not shown) and suggest that other protein aggregates like prions might use TNTs as one possible spreading mechanism (**Figure 2**).

Despite that, one should also take into account the different nature of the protein implicated in each neuropathology. For example, differently from PrP^{Sc} and Aβ, that are amyloids

anchored to the membranes, tau, htt, SOD1, and α-syn are cytosolic (Aguzzi and Rajendran, 2009; Münch and Bertolotti, 2011), thus raising the question of how these cytosolic aggregates might spread between cells by TNTs (**Figure 2F**).

For example, it has been reported that α-syn aggregates internalization was sensitive to temperature and required dynamin-1, pointing toward a role for the endocytic pathway in its entry mechanism (Desplats et al., 2009). Similarly, internalized tau partially colocalizes with dextran, indicating also an involvement of the endocytic pathway in this process (Frost et al., 2009). Thus internalized aggregates of α-syn and tau are likely packaged into endocytic vesicles from where they have to escape by a mechanism not yet understood in order to reach the cytosol. It might therefore be possible that endocytic vesicles could "shuttle" these aggregates in TNTs connecting cells, prior to their escape from this compartment (**Figure 2B**). Then once they reached the recipient cell they could be released in the cytosol where they could seed the misfolding of endogenous cytosolic proteins. On the other hand, a cytosolic passage as aggresomes through TNTs could also be envisaged since TNTs have been shown to transfer cytosolic components between connected cells (Watkins and Salter, 2005; He et al., 2010; **Figure 2F** and enlarged box). Interestingly α-syn and htt can interact with acidic phospholipids enriched on the cytoplasmic membrane leaflet (Kegel et al., 2005, 2009; van Rooijen et al., 2008). Therefore a "surfing" process of the "membrane-associated" protein inside TNT membranes could also be possible (**Figure 2**, enlarged box).

Further investigations in this direction are intriguing and can potentially open up new ways of looking at these diseases and could potentially lead to new therapeutical approaches to selectively block misfolded protein aggregates spreading with the ultimate aim of fighting them.

CONCLUDING REMARKS

Since their discovery in 2004, an enormous amount of work has been done on the characterization of TNTs in a multitude of cell types. Here, we have reviewed recent studies and highlighted advances that have been made more specifically with respect to TNT formation, the role of specific molecules and signaling pathways, as well as their different physiological roles in the spreading of various molecules, signals, and pathogens.

What has become evident from these studies is that long distance intercellular connections between cells are not artifacts, as they were first perceived. Indeed, they have become commonly observed features found in most cell types examined. Although discovered only recently, TNT-like structures are becoming more and more a part of mainstream cell biology. The biggest hurdle however might be the large heterogeneity that exists within these structures. This is in part due to their high dynamicity. Indeed, TNTs can form quickly and have short lifetimes. They can be induced by different signals leading to different transport mechanisms. Thus, as more molecules and signaling pathways are being described as important players in both TNT formation and/or function (Abounit and Zurzolo, 2012), it will be necessary to determine whether a general mechanism might exist for most cell types or whether each cell system might have evolved its own set of mechanisms for TNT formation, stability, and function. However, because of the disparity in the requirements of specific cytoskeleton components or specific proteins, more attention might have to be put on the role of specific lipids or lipid pathways. Indeed, while most naturally occurring nanotubes require some type of cytoskeleton components, artificially made nanotubes can be pulled from synthetic vesicles. Thus, the lipid environments and their subsequent interactions with specific proteins might bridge some of the differences observed between each cell type. For example, the determination that PI3K might play a role in TNT formation (Wang et al., 2011) suggests that phosphoinositides such as PIP2 and PIP3 might play important roles. To this aim it will be important to use biophysical approaches and model membranes to determine the role that certain lipids might play in the membrane flexibility and ability to curve. Furthermore whether common membrane domains enriched in specific lipids and proteins bring important components at the base and within TNTs for both formation and transfer needs to be analyzed.

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Emerging role of neuronal exosomes in the central nervous system

Mathilde Chivet[1,2], Fiona Hemming[1,2], Karin Pernet-Gallay[2,3], Sandrine Fraboulet[1,2] and Rémy Sadoul[1,2]*

[1] *U836, Equipe 2, Neurodégénérescence et Plasticité, INSERM, Grenoble, France*
[2] *Grenoble Institut des Neurosciences, Université Joseph Fourier, Grenoble, France*
[3] *U836, Plateforme de microscopie, INSERM, Grenoble, France*

Edited by:
Claudia Verderio, CNR Institute of Neuroscience, Italy

Reviewed by:
Eva-Maria Krämer-Albers, Johannes Gutenberg University Mainz, Germany
Frank Kirchhoff, University of Saarland, Germany

***Correspondence:**
Rémy Sadoul, Grenoble Institute of Neuroscience, Chemin Fortuné Ferrini, BP 170, F-38042 Grenoble, France.
e-mail: remy.sadoul@ujf-grenoble.fr

Exosomes are small extracellular vesicles, which stem from endosomes fusing with the plasma membrane, and can be recaptured by receiving cells. They contain lipids, proteins, and RNAs able to modify the physiology of receiving cells. Functioning of the brain relies on intercellular communication between neural cells. These communications can modulate the strength of responses at sparse groups of specific synapses, to modulate circuits underlying associations and memory. Expression of new genes must then follow to stabilize the long-term modifications of the synaptic response. Local changes of the physiology of synapses from one neuron driven by another, have so far been explained by classical signal transduction to modulate transcription, translation, and posttranslational modifications. *In vitro* evidence now demonstrates that exosomes are released by neurons in a way depending on synaptic activity; these exosomes can be retaken by other neurons suggesting a novel way for inter-neuronal communication. The efficacy of inter-neuronal transfer of biochemical information allowed by exosomes would be far superior to that of direct cell-to-cell contacts or secreted soluble factors. Indeed, lipids, proteins, and RNAs contained in exosomes secreted by emitting neurons could directly modify signal transduction and protein expression in receiving cells. Exosomes could thus represent an ideal mechanism for inter-neuronal transfer of information allowing anterograde and retrograde signaling across synapses necessary for plasticity. They might also allow spreading across the nervous system of pathological proteins like PrPsc, APP fragments, phosphorylated Tau, or Alpha-synuclein.

Keywords: microvesicles, exosomes, inter-neuronal communication, synaptic plasticity, neurodegeneration, CNS neurons

INTRODUCTION

The development and function of mammals, like that of any multicellular organism, depends on intercellular communication. Classically, this occurs either through direct cell-to-cell interaction bringing together cell surface proteins or, at a distance, through secreted soluble molecules binding to cell surface receptors. These interactions lead to transduction of intracellular signals from the cell surface to the nucleus, where regulation of gene expression might occur. A breach of this dogma, which is based on the impermeable property of biological membranes, came from the demonstration that lipid vesicles containing RNAs and proteins released by mammalian cells, can modify the biological activity of non-contacting cells (Simons and Raposo, 2009). Microvesicles can be shed directly by budding from the plasma membrane. They can also form through budding into the lumen of endosomes and be released after fusion of the limiting membrane of endosomes to the plasma membrane. Once secreted in extracellular milieu the endosomal intraluminal vesicles (ILVs) are referred to as exosomes. Our review will stick to this strict definition of exosomes to discuss the most recent findings indicating the potential role of neuronal exosomes in intercellular communication within the normal and pathological central nervous system.

WHY NEURONS COULD MAKE GOOD USE OF EXOSOMES

It is now widely accepted that exosomes represent a way of intercellular exchange of effector molecules, which allows emitting cells to modify gene and protein expression in receiving cells. They allow transfer of membrane and cytoplasmic proteins (Thery et al., 2002; Morelli et al., 2004), as well as lipids involved in signal transduction (Laulagnier et al., 2004; Subra et al., 2010) or RNAs. Exosomal mRNAs can be translated (Valadi et al., 2007), and small RNAs, including microRNAs (miRNAs) mediate gene silencing in receiving cells (Kosaka et al., 2010; Pegtel et al., 2010; Montecalvo et al., 2012).

Functioning of the brain relies on the capacity of neurons to locally modulate each other at the level of synapses. Chemical synapses are made of a presynaptic part filled with neurotransmitter (NT) – containing vesicles and a post-synaptic part in which NT receptors are anchored at the level of the post-synaptic density (PSD). Specific patterns of stimulation of the presynaptic cell can durably increase or decrease the strength of synaptic responses, thereby reinforcing circuits underlying associations and memory. Changes in synaptic efficacy are based on modifications of the number of post-synaptic NT-receptors or of the amount of NT released pre-synaptically for a given stimulus. Changes of one

neuron driven by another, have so far been explained by ways of classical signal transduction: NTs, lipids, or proteins secreted from one side of the synapse bind to receptors of the opposite surface. Pre-synaptic-activity substances can also be released by cell bodies and dendrites (Regehr et al., 2009). This leads to modulations of second messengers and enzymatic activities acting on effectors of the synaptic changes (adhesion molecules, neurotransmitter receptors, cytoskeleton anchors. . .; Malenka and Bear, 2004). Signal transduction also leads to changes in gene expression and translation, which are needed for long-lasting synaptic modifications (Bullmore and Sporns, 2009). The efficacy of such mechanisms must deal with the extreme compartmentalization of the parenchyma. Indeed, control of transcription occurs in the nucleus far away from synapses undergoing plastic changes. Transcripts can be specifically transported along dendrites to synapses undergoing specific patterns of activation, where they are translated into proteins modifying synaptic strength. Translation of targets mediating dendritic growth can also be regulated by miRNAs, which are expressed within dendrites (Schratt et al., 2006; Siegel et al., 2009). We have recently observed that exosomes secreted by neurons contain miRNAs (unpublished observations). Given that single miRNAs have multiple targets, the impact of exosome-mediated local transfer of miRNA on the pattern of translated mRNAs in receiving neurons may be quite extensive. Confined exchange of RNAs at synapses would thus certainly represent an efficient mechanism for long-term modifications of specific synapses. Therefore, the exosomal pathway may constitute a well designed mechanism for local and systemic inter-neuronal transfer of information within functional brain networks, with a complexity superior to that of direct cell-to-cell contacts or secreted soluble factors (Belting and Wittrup, 2008). The dark side would be that exosome transfer might also represent a privileged way for propagating pathological alterations throughout the brain (Fevrier et al., 2005; Aguzzi and Rajendran, 2009).

ENDOSOMES IN NEURONS CONTROL SYNAPTIC PLASTICITY

Endosomes are intracellular compartments collecting plasma membrane proteins, which are constantly renewed by constitutive or selective endocytosis (**Figure 1**). The first compartments to accept incoming proteins after their endocytosis are early endosomes. From there, the majority of the proteins are recycled back to the membrane. Other proteins, classically those meant for degradation, are selectively entrapped in vesicles budding from the endosomal membrane into the lumen of endosomes. Maturation of endosomes leads to individualization of multivesicular bodies (MVBs), which are large vacuoles delimited by a single membrane and containing a varying number of 50–80 nm membrane vesicles (**Figure 2**; Gruenberg and Stenmark, 2004; van der Goot and Gruenberg, 2006). Invagination of the endosomal membrane leading to the formation of MVBs also allows selective microautophagy of cytoplasmic proteins (Sahu et al., 2011). Membrane and cytoplasmic proteins entrapped in vesicles will be hydrolyzed after fusion of MVBs with lysosomes. They can also be expelled from cells after fusion of MVBs with the plasma membrane leading to the release of exosomes into the extracellular milieu (**Figure 1**; Simons and Raposo, 2009).

In neurons, endosomes are present in both pre- and post-synaptic compartments. Electron microscopy (EM) observations of the adult hippocampus revealed the presence of MVBs and sorting endosomes in dendritic shafts and inside a limited number

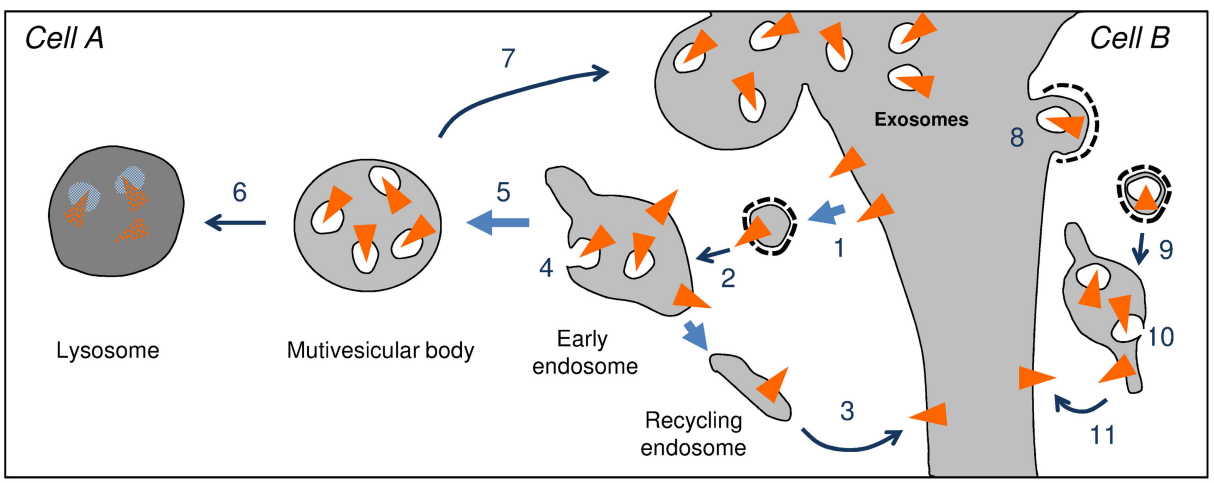

FIGURE 1 | Endosomal trafficking of transmembrane proteins (triangle). After endocytosis (1) the endocytic vesicle fuses to early endosomes (2). Proteins can be concentrated into recycling endosomes, which fuse to the plasma membrane and allow re-expression at the cell surface (3). Alternatively proteins can be entrapped in vesicles budding from the limiting membrane of the endosome (4). Maturation of the endosome leads to the individualization of a multivesicular body containing intraluminal vesicles (ILV) (5). The multivesicular body can fuse with lysosomes in which the ILVs and their cargoes are hydrolyzed (6). The multivesicular body can also fuse with the plasma membrane (7) thereby releasing ILVs. Once in the extracellular milieu ILVs are referred to as exosomes. Exosomes released by cell A, can bind to and be endocytosed by a receiving cell [cell B, 8]. The endocytic vesicle containing the exosome fuses with the early endosomes (9). Once inside the endosome, the exosome undergoes back-fusion with the endosomal membrane (10). Fusion of recycling endosomes to the plasma membrane allows expression of protein of the cell A at the surface of cell B. Back-fusion also allows the release of the intraluminal content of exosomes [proteins and RNAs of cell A] into the cytosol of cell B. It is important to note that steps 9, 10, and 11 remain speculative.

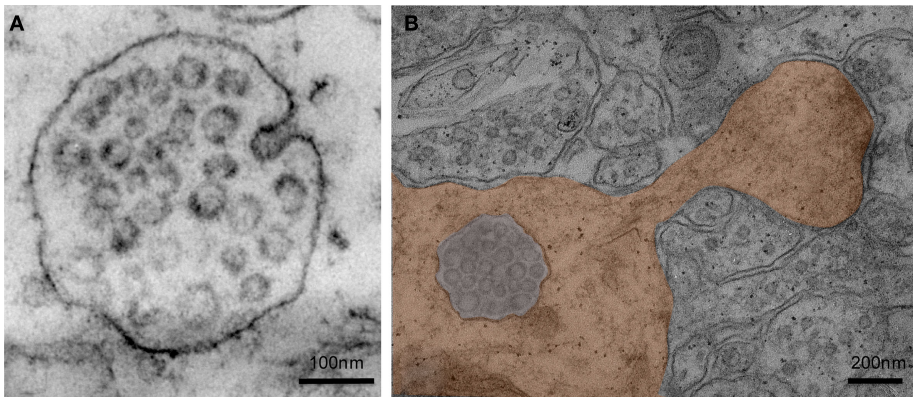

FIGURE 2 | (A) Electron micrograph of a multivesicular body present in a neuron of the CA1 region of the adult rat hippocampus. Note the budding of a vesicle from the limiting membrane of the MVB (upper right; Fiona Hemming, unpublished). **(B)** Electron micrograph of a multivesicular body in a dendrite (colored; CA1 region of an adult rat hippocampus). The protrusion of the dendrite, called dendritic spine, corresponds to the post-synaptic part of a glutamatergic synapse. Two post-synaptic densities, which anchor ionotropic glutamate receptors, are visible. In this case the multivesicular body is present within the dendritic shaft at the base of the spine neck (Fiona Hemming, unpublished).

of spines, which represent post-synaptic parts of glutamatergic synapses (**Figure 2B**; Cooney et al., 2002). Noteworthy, is that MVBs are about 50 times more represented in somatodendritic compartments than in axons (Von Bartheld and Altick, 2011). Endosome-containing spines are mostly mushroom-like spines i.e., with the most active synapses (Kasai et al., 2003). Enhancement of synaptic activity after injection of peptides known to improve cognitive functions or during kindling, significantly increased the proportion of MVBs inside spines in the dentate gyrus (Popov et al., 2008; Kraev et al., 2009). Similarly, water maze training of rats led to the migration of MVBs to the vicinity of PSDs in dendrites of CA3 pyramidal cells, while chronic restraint stress diminished the number of MVBs associated with PSDs (Stewart et al., 2005). Similarly neutrotrophic factors (BDNF, GDNF) induced a relocalization of dendritic MVBs very near PSDs of hypoglossal motoneurons (Rind et al., 2005). Thus, in the CNS, movements of MVBs to synapses are tightly linked to synaptic plasticity (Von Bartheld and Altick, 2011).

Studies of the trafficking of synaptic AMPA type-receptors, which represent the major mediators of fast synaptic transmission among glutamate receptors of the CNS, led to the demonstration that dendritic endosomes act as stores and sorting platforms for synaptic receptors (Kennedy and Ehlers, 2006). During long-term potentiation (LTP), a form of synaptic plasticity now widely accepted as a model of learning and memory processes (Ehlers, 2000), membrane insertion of new post-synaptic AMPA receptors increases excitatory post-synaptic currents thereby potentiating the synapses. Live cell imaging of dissociated hippocampal neurons demonstrated that glycine stimulation, a protocol used to induce chemical LTP through activation of synaptic NMDA-receptors, leads to the recruitment of endosomes into, or near spines and their fusion with the plasma membrane (Correia et al., 2008; Wang et al., 2008). Thereby, AMPA-Rs present in the limiting membrane of endosomes become inserted at the neuronal surface and diffuse laterally to synaptic sites where they accumulate through interaction with proteins of PSDs. Live imaging of the insertion at the plasma membrane of transferrin receptors (TfR) contained in endosomes, showed the requirement of Rab11 or syntaxin 13 for the endosomal fusion at the dendritic surface (Park et al., 2006). Accordingly, expression of a dominant negative form of Rab11 was found to inhibit LTP in slice cultures (Brown et al., 2007; Wang et al., 2008), demonstrating that endosomal fusion to the dendritic membrane is a necessary step for synaptic potentiation.

The compartments fusing at the plasma membrane of cultured neurons were identified as recycling endosomes because TfR is usually detected in recycling endosomes, and Rab11 or syntaxin 13 are known regulators of recycling endosomes. However, the strict separation between recycling endosomes and MVBs, considered as late endosomes, needs to be made with caution. Indeed, in reticulocytes, TfR is present in exosomes (Geminard et al., 2004) and Colombo and collaborators found that Rab11 is required for MVB fusion to the plasma membrane in an erythroleukemic cell line (Savina et al., 2005). Furthermore, NEEP21, known to regulate recycling of AMPA receptors at the synapse, has been localized by immunofluorescence inside TfR-containing endosomes of cultured hippocampal neurons (Steiner et al., 2005). However, EM observations of rat brain sections demonstrated that the protein is expressed at PSDs as well as in intralumenal, but not limiting, membranes of MVBs (Utvik et al., 2009). Thus, even if fluorescence data suggest that endosomes fusing to the plasma membrane during synaptic plasticity are recycling endosomes, one cannot yet exclude that some of these endosomes are MVBs. The final proof that MVBs fuse to the dendritic surface awaited the visualization of this process by EM and the demonstration that exosome release is modified by synaptic glutamate receptor activity.

REGULATED SECRETION OF EXOSOMES BY NEURONS

We made the first demonstration that cortical neurons in culture release exosomes (Faure et al., 2006). As in the case of other cells, exosomes isolated from neuron culture media floated on sucrose gradients at a density of 1.1–1.2 g/ml and contained

both Tsg101 and Alix. Tsg101 belongs to the *endosomal sorting complex required for transport* (ESCRT-0 to III), necessary for the making of ILVs accumulating inside MVBs (Babst, 2011). Alix is acytoplasmic protein binding to Tsg101 of ESCRT-I and CHMP4B of ESCRT-III (Missotten et al., 1999; Matsuo et al., 2004). Endophilin A, which also interacts with Alix (Chatellard-Causse et al., 2002), was not detected in exosomes, demonstrating that entry of cargoes into ILVs is regulated. Other cytoplasmic proteins and enzymes were present inside exosomes, including GADPH, ubiquitin, and Hsc70. This is in good agreement with the recent finding thatHsc70 binding to GADPH drives its ESCRT-dependent engulfment into MVB-ILVs (Sahu et al., 2011). Exosomes also contained AMPA-, but not NMDA-receptors and the cell adhesion molecule L1/NgCAM, which, in the central nervous system is expressed only by neurons (Maness and Schachner, 2007), thus demonstrating that exosomes are secreted by neurons. We also observed that electrical activity regulates exosomal secretion since long-term depolarization of neurons with 25 mM potassium strongly increased the release of AMPA-R containing exosomes.

Three other studies reported secretion of exosomes by neurons (Vingtdeux et al., 2007; Putz et al., 2008; Ghidoni et al., 2009). These studies, as well as our initial one, used embryonic neurons cultured for only 3–8 days. In short term cultures, neurons make only few synapses and neurite outgrowth is still on-going. Thus, exosome release could simply reflect the fusion of late endosomes/lysosomes at growth cones necessary for neurite elongation (Arantes and Andrews, 2006).

More recently, we have studied exosome release from fully differentiated cultures (15 DIV; Lachenal et al., 2010). Dissociated cortical cells contain both glutamatergic and GABAergic neurons, which make functional networks within the second week in culture. Thus, incubation with GABA receptor antagonists, such as picrotoxin or bicuculline, alleviates inhibitory activities within the networks and increases synaptic glutamatergic activity. Picrotoxin or bicucullin rapidly (10–15 min) and massively augmented the secretion of exosomes in a way dependent on AMPA- and NMDA-receptors (Lachenal et al., 2010). We also found that increasing cytosolic calcium, using the calcium ionophore ionomycin, drastically elevated exosome secretion. EM examination of cultures treated for 1 min with ionomycin revealed clusters of exosomes at the surface of dendrites visualizing the fusion of MVBs with the plasma membrane. Altogether, our data suggest that calcium entry through synaptic NMDA-receptors is a potent activator of MVB fusion to the plasma membrane and thereby of exosome secretion. The enhanced secretion of AMPA-R-containing exosomes following glutamatergic synaptic activation, underlines exosomal release as a way of local elimination of receptors at synapses undergoing plastic changes. The loss of AMPA receptors upon extensive synaptic activation could be a mechanism of homeostatic synaptic scaling, necessary for adjusting the strength of all of a neuron's excitatory synapses to stabilize firing (Turrigiano, 2008). Thus, while fusion of endosomes leads to an increase of receptors at synapses undergoing potentiation, sustained synaptic activation would lead to calcium increase within the dendritic shaft triggering fusion of MVBs at the base of nearby synapses to allow the local elimination of the intracellular pool of AMPA receptors and thereby synaptic down-scaling. In this scenario, regulation of the pool of surface synaptic receptors by exosome secretion would be a local event, avoiding retrograde transport of MVBs necessary to hydrolyze the receptors in lysosomes, which are only present in proximal dendrites and soma.

THE FATE OF EXOSOMES RELEASED BY NEURONS

Exosomes were first shown to be endocytosed by dendritic cells of the immune system (Skokos et al., 2003). Those released by neurons into the CNS parenchyma could potentially be endocytosed by nearby cells as shown for oligodendrocyte derived exosomes which are endocytosed by microglial cells (Fitzner et al., 2011). Astrocyte end feet, which enwrap a number of glutamatergic synapses, can also endocytose/phagocytose cellular debris (Haydon and Carmignoto, 2006) and could thus capture exosomes released at synapses. Transfer of exosomes could also occur between spines of the same neuron or across synapses to end up in afferent neurons. Indeed, the diameter of neuronal exosomes is compatible with possible endocytosis in neuronal clathrin coated pits occurring in presynaptic boutons, in spines, or dendritic shafts (Lu et al., 2007). We have recently obtained evidence that exosomes bind to and are endocytosed by hippocampal neurons (unpublished observations) and that they allow the inter-neuronal transfer of Tetanus Toxin, which is known to cross synapses *in vivo* (Lachenal et al., 2010). EM observations are now needed to characterize the site of entry of exosomes and their fate inside endosomes. In non-neuronal cells, the fact that exosomal RNAs can act on receiving cells demonstrates that exosome intralumenal cargoes are released into the cytosol i.e., that the membrane of exosomes fuses with the plasma membrane or with endosomal membranes after their endocytosis (**Figure 1**). Back-fusion of intralumenal vesicles has been demonstrated to occur in MVBs (Falguieres et al., 2009) and could thus concern exosomes, which have the same origin. Such a process would lead to the entry of exosomal membrane proteins into the endosomal protein pool and possibly re-expression at the cell surface (e.g., AMPA receptors). It would also allow the release into the cytosol of the exosome content, including signal transduction molecules and miRNAs.

RELEVANCE OF NEURONAL EXOSOMES FOR NEURODEGENERATIVE DISEASES

Exosomes can contain pathogenic proteins such as alpha-synuclein, PrPsc, amyloid precursor protein (APP), and phosphorylated Tau, which are involved in Parkinson's, Prion, and Alzheimer's diseases respectively. The scrapie form of the Prion protein (PrPsc) contained in exosomes is secreted via exosomes and remains infectious under this form (Fevrier et al., 2004). Thus, trans-synaptic exchange could be one way for the propagation of Prion diseases from the periphery to the CNS. Alpha-synuclein secreted together with exosomes released by neuroblastoma cells causes cell death of recipient neuronal cells suggesting that alpha-synuclein secretion via exosomes serves to amplify and propagate Parkinson's disease-related pathology (Emmanouilidou et al., 2010). The catabolism of APP giving rise to the amyloidogenic C-terminal APP fragment occurs in endosomes and this fragment as well as Aβ amyloid peptides, are released by way of exosomes (Rajendran et al., 2006; Vingtdeux et al., 2007; Sharples et al., 2008;

Ghidoni et al., 2011). Exosomes could therefore contribute to the spreading of the pathology throughout interconnected cortical areas. These puzzling hypotheses require *in vivo* work (i) to show that exosomal released from MVBs occurs *in situ* (ii) to find out the privileged site of this release (iii) to demonstrate transynaptic exchange of exosomes. Furthermore, even though the activity dependent-release of exosomes suggests a genuine function of exosomes in synaptic plasticity, molecular tools to specifically block MVB fusion with the plasma membrane must be developed to test this hypothesis. Nevertheless, studies on exosomes in the CNS are bound to shed new light on intercellular exchanges within the brain and to open new avenues toward understanding how neurodegenerative diseases spread over time throughout the nervous system.

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Emerging roles of exosomes in neuron–glia communication

Carsten Frühbeis, Dominik Fröhlich and Eva-Maria Krämer-Albers*

Department of Molecular Cell Biology, Johannes Gutenberg University Mainz, Mainz, Germany

Edited by:
Claudia Verderio, CNR Institute of Neuroscience, Italy

Reviewed by:
Felipe A. Court, Pontificia Universidad Catolica de Chile, Chile
Rémy Sadoul, Institut National de la Santé et de la Recherche Médicale, France

***Correspondence:**
Eva-Maria Krämer-Albers, Department of Molecular Cell Biology, Johannes Gutenberg University Mainz, Bentzelweg 3, 55128 Mainz, Germany.
e-mail: emkraemer@uni-mainz.de

Brain function depends on coordinated interactions between neurons and glial cells. Recent evidence indicates that these cells release endosome-derived microvesicles termed exosomes, which are 50–100 nm in size and carry specific protein and RNA cargo. Exosomes can interact with neighboring cells raising the concept that exosomes may mediate signaling between brain cells and facilitate the delivery of bioactive molecules. Oligodendrocytes myelinate axons and furthermore maintain axonal integrity by an yet uncharacterized pathway of trophic support. Here, we highlight the role of exosomes in nervous system cell communication with particular focus on exosomes released by oligodendrocytes and their potential implications in axon–glia interaction and myelin disease, such as multiple sclerosis. These secreted vesicles may contribute to eliminate overproduced myelin membrane or to transfer antigens facilitating immune surveillance of the brain. Furthermore, there is emerging evidence that exosomes participate in axon–glia communication.

Keywords: microvesicles, exosomes, neuron–glia communication, oligodendrocytes, axon–glia interaction, myelin disease

INTRODUCTION

Glial cells actively participate in brain development and function necessitating communication between neurons and glia (Allen and Barres, 2009). Their functions range from metabolic support to myelination, immune defense, and engagement in synapse formation and plasticity. Oligodendrocytes ensheath axons with an insulating myelin sheath facilitating electric impulse propagation. Myelination requires intense communication between oligodendrocytes and neurons, which is also essential for the maintenance of axonal integrity over the lifetime (Nave, 2010a).

Recent reports describe the horizontal transfer of biomolecules by secreted extracellular vesicles, which is increasingly becoming established as a general mode of intercellular communication (Simons and Raposo, 2009; Camussi et al., 2010). Neurons and the major types of glia release vesicles, raising the possibility that communication mediated via extracellular vesicles is a common mechanism in the CNS. Small vesicles, here referred to as microvesicles, shed directly from the plasma membrane or originate from the endosomal system (Lakkaraju and Rodriguez-Boulan, 2008; Cocucci et al., 2009). A mixed population of such vesicles has been detected in body fluids including cerebrospinal fluid (Vella et al., 2008). Exosomes are released by fusion of multivesicular bodies (MVBs) with the plasma membrane and secretion of the intraluminal vesicles (ILVs) into the extracellular space. They are 50–100 nm in diameter and carry specific protein and RNA cargo. Exosomal membranes are enriched in cholesterol and sphingomyelin. Proteins relating to their biogenesis (Alix and Tsg101), distinct cytosolic proteins such as heat-shock proteins, and certain membrane proteins (tetraspanins, integrins) are sorted into exosomes whereas others are excluded (for review, see Thery et al., 2009; Thery, 2011, and references therein).

Cells utilize exosomes to dispose of unwanted proteins or to exchange signals with neighboring cells. As an example, erythrocytes remove the transferrin receptor via exosomes during maturation, instead of eliminating it via internal degradation. Proteins implicated in cell interaction are strikingly abundant in exosomes, which thus suggests a role in cell–cell communication. In the immune system, antigen presenting cells (APCs) release exosomes containing MHC and costimulatory molecules to modulate T-cell activation (Thery et al., 2009). Furthermore, microvesicles (including exosomes) transport miRNAs and mRNAs from cell to cell. Translation of microvesicle-derived mRNAs is initiated and new proteins are synthesized. In turn, transferred miRNAs inhibit expression of resident proteins. Thus, shuttled RNAs can alter the proteome of recipient cells (Valadi et al., 2007; Skog et al., 2008; Pegtel et al., 2010; Zhang et al., 2010).

This review describes the characteristics and functions of microvesicles secreted by neurons and glia (collectively referred to as neural cells), with particular focus on exosomes released by oligodendrocytes. We propose a role of oligodendroglial exosomes in axon–glia interaction and hypothesize that they shuttle functional molecules to neurons, thus influencing neuronal properties.

CLASSIFICATION AND GENERAL COMPONENTS OF EXOSOMES

Exosomes and other microvesicles can be isolated from culture supernatants or body fluids by differential centrifugation and filtration. With current technology, the isolation of extracellular vesicles directly from tissues is impossible because membrane debris and internal vesicles contaminate the preparation. Furthermore, discrimination between different types of microvesicles is difficult. To distinguish exosomes from other secreted vesicles,

their characteristic size (diameter below 100 nm), protein composition, density, and endosomal origin are commonly used criteria. Exosomes are derived from MVBs and correspond to the ILVs, which bud from the limiting membrane into the lumen of late endosomes. ILV-budding involves the action of the ESCRT (endosomal sorting complex required for transport) machinery, though its exact role in exosome biogenesis is not clear and appears cell-type dependent (Simons and Raposo, 2009; Bobrie et al., 2011). Anyhow, ESCRT and associated proteins such as Tsg101 and Alix are integrated in exosomes and serve as markers of their identity. In addition, ESCRT independent mechanisms of ILV formation have been described involving sorting of exosome cargo to endosomal domains and ceramide-mediated budding from the limiting membrane (Trajkovic et al., 2008; Buschow et al., 2009). Possibly, distinct endosomal sorting mechanisms lead to the generation of subpopulations of exosomes. MVB trafficking and fusion is regulated by Rab-family GTPases. In oligodendroglial cells, Rab35 regulates docking of MVBs to the plasma membrane (Hsu et al., 2010). Moreover, Rab27a and Rab27b are involved in the docking step in Hela cells (Ostrowski et al., 2010).

Proteomic and microarray analysis on a range of exosome preparations yielded a reproducible compendium of exosome-associated proteins and RNAs, summarized in the database Exocarta (Mathivanan et al., 2012). Exosomes contain a distinct set of proteins conserved across different cell types and species. Typical proteins are cytoskeletal proteins such as tubulin and actin, heat-shock proteins (Hsp70, Hsp90), metabolic enzymes of the glucose metabolism, Flotillin-1, signal transduction proteins (kinases, heterotrimeric G proteins), MHC molecules, clathrin, proteins involved in transport and fusion (annexins, Rab proteins), and translation elongation factors. Strikingly abundant in exosomes are proteins of the tetraspanin family, e.g., CD9, CD63, CD81, and CD82 (van Niel et al., 2006; Simpson et al., 2008; Thery et al., 2009). Additionally, exosomes contain cell-type specific components reflecting the host cell identity and presumably also hinting at the biological function of the released exosomes.

CHARACTERISTICS OF CNS EXOSOMES

In the CNS, neurons, microglia, astrocytes, and oligodendrocytes have been reported to secrete microvesicles into the extracellular environment. In response to glutamatergic synaptic activity, cultured cortical and hippocampal neurons release microvesicles with the characteristics of exosomes. Neuronal exosomes carry the cell adhesion molecule L1, the GPI-anchored prion protein, as well as the GluR2/3 subunits of the AMPA receptor (Faure et al., 2006; Lachenal et al., 2011). The Parkinson disease related protein α-synuclein is secreted from a neuroblastoma cell line by exosomes and can influence the viability of neighboring cells (Emmanouilidou et al., 2010).

Oligodendrocytes release exosomes that include the lipids galactocerebroside, sulfatide, and cholesterol (Krämer-Albers et al., 2007), which are also prominent in oligodendroglial lipid rafts and represent characteristic myelin lipids (Krämer et al., 1997). The proteomic profile of oligodendroglial exosomes mirrors the exosome-pattern of marker proteins (Alix, Tsg101, Flotillin-1), ubiquitous tetraspanins (CD81, CD63), and chaperones (**Figure 1**). They are furthermore characterized by the presence of unique myelin proteins, such as PLP, CNP, MAG, and MOG. Remarkably, oligodendroglial exosomes carry a range of enzymes such as the NAD-dependent deacetylase sirtuin-2, oxidative stress alleviating peroxiredoxins and

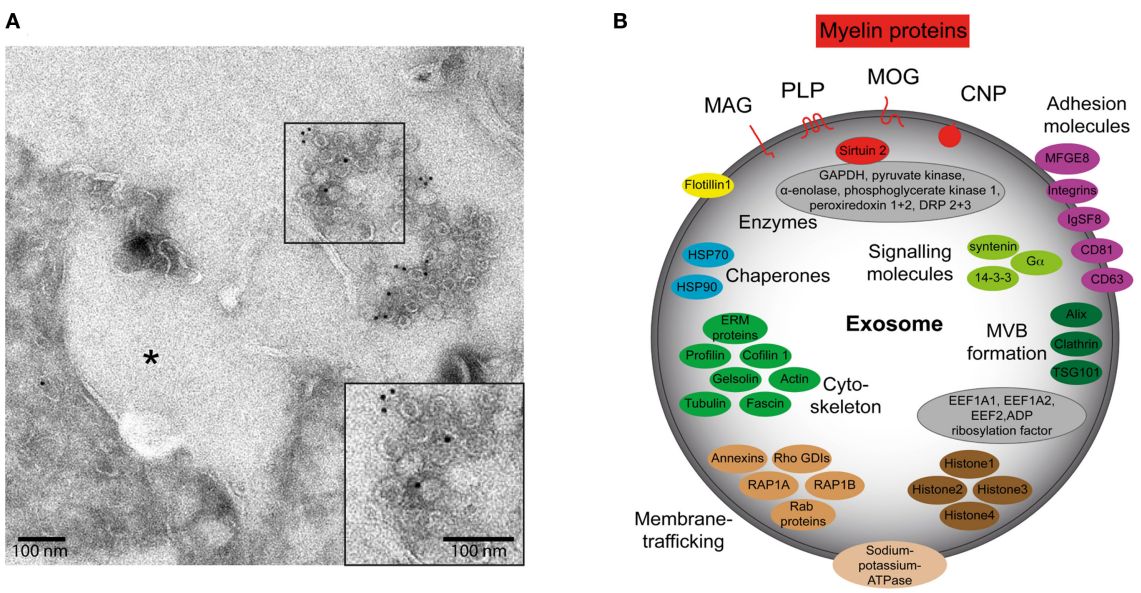

FIGURE 1 | Characteristics of oligodendroglial exosomes. (A) Electron micrograph of exosomes in the extracellular space released by primary oligodendrocytes. Immuno-gold labeling was performed with antibodies recognizing myelin-associated glycoprotein (MAG). Asterisk indicates the putative MVB fusion profile that resulted in exosome release. Scale bar, 100 nm. **(B)** Protein composition of a typical oligodendroglial exosome. The illustration is based on proteomic analyses of exosome preparations derived from oligodendroglial cells (Krämer-Albers et al., 2007).

dihydropyrimidinase-related proteins, and glycolytic enzymes (GAPDH, pyruvate kinase, α-enolase).

Microglia are the resident macrophages of the CNS. In pathological situations, they become activated and execute immune functions such as antigen presentation (Kettenmann et al., 2011). Microglia secrete exosomes containing the expected exosomal proteins as well as a set of proteins previously reported for B cell- and dendritic cell-derived exosomes. In addition, they carry the surface-bound aminopeptidase N (CD13) and the monocarboxylate transporter 1 (MCT1). CD13 cleaves N-terminal amino acids from polypeptides and exosome-associated CD13 degrades enkephalins, influencing the activity of ligands of the opioid receptor and thus neuronal cAMP levels. Since these exosomes contain the lactate transporter MCT1 together with glycolytic enzymes, they may serve to deliver energy substrates to neurons (Potolicchio et al., 2005). Moreover, microvesicles carrying the proinflammatory cytokine IL-1β shed from the plasma membrane of microglial cells and astrocytes in response to ATP stimulation and activation of acid sphingomyelinase (Bianco et al., 2009).

In response to oxidative and heat stress, cultured astrocytes release elevated amounts of the heat-shock protein 70 (Hsp/Hsc70) as well as synapsin 1 in association with exosomes (Taylor et al., 2007; Wang et al., 2011). Intriguingly, astrocyte-derived exosomes have been reported to contain mitochondrial DNA (Guescini et al., 2010). Secretion of microvesicles comprising a mixture of exosomes, shedding vesicles, and possibly also apoptotic bodies is a prominent feature of brain tumor cells, which often originate from astrocytes (van der Vos et al., 2011). Microvesicles derived from highly aggressive glioblastoma multiforme tumors (GBM), carry oncogenic EFGRvIII in addition to immunosuppressive and angiogenic factors (Al-Nedawi et al., 2008; Graner et al., 2009). They are moreover characterized by the presence of nucleic acids, including mRNAs, miRNAs, non-coding RNAs, retrotransposon elements, genomic DNA, and cDNA derived from oncogenic sequences (Skog et al., 2008; Balaj et al., 2011).

FUNCTIONS OF NERVOUS SYSTEM EXOSOMES

Two general functions have been ascribed to exosome secretion: disposal of unneeded cell components and signaling to neighboring cells involving the horizontal transfer of biomolecules (Lotvall and Valadi, 2007; Simons and Raposo, 2009). Both functional properties assigned to exosomes appear eligible to be utilized by neural cells, which operate as a long-term cellular network relying on finely tuned cell–cell interactions, mutual support, and the clearance of remnants. The physiological and pathological impact of exosomes in the nervous system has been a topic of discussion (Smalheiser, 2007; Aguzzi and Rajendran, 2009), however, we are only beginning to capture a true picture (**Figure 2**). Exosomes secreted by neurons have been implicated in synaptic plasticity. Enhanced glutamatergic activity within cultures of mature cortical neurons stimulates the release of exosomes carrying the AMPA receptor subunit GluR2 from the somatodendritic postsynaptic compartment (Lachenal et al., 2011). These findings suggest that activity-dependent exosome secretion may help to adapt the efficacy of synaptic transmission by depletion of neurotransmitter receptors from the postsynaptic compartment. What happens to the exosomes after the release, whether they become internalized by the presynaptic neuron or surrounding glial cells remains to be determined. A synaptic transfer of wnt-signaling molecules involving exosome-like vesicles has been shown to occur in drosophila larva at the neuromuscular junction (Koles et al., 2012).

In the context of CNS pathology, it appears that pathogenic proteins such as β-amyloid peptide, prion protein, α-synuclein, tau, and superoxide dismutase are released from cells in association with exosomes (Fevrier et al., 2004; Rajendran et al., 2006; Gomes et al., 2007; Emmanouilidou et al., 2010; Saman et al., 2011). These proteins have in common the activity to form aggregates (amyloids, prionoids) that escape the normal cellular degradation machinery. It is not clear yet whether exosome-associated secretion of amyloidogenic proteins helps to relieve cells of potentially detrimental components, or if the pathogen-filled exosomes engage in propagating neurodegenerative "seeds" within the tissue. Exosomes carrying prions have been shown to be infectious (Fevrier et al., 2004; Vella et al., 2007). However, the processing of pathogenic exosomes by recipient cells remains to be demonstrated.

More is known about the role of membrane vesicles released by glial cells. Microvesicles (including exosomes) are released by glioma cells, which carry the mRNA and protein of oncogenic EGFRvIII as well as angiogenic factors. They can be internalized by surrounding cells, promoting cell transformation or mediating tubular growth of endothelial cells (Al-Nedawi et al., 2008; Skog et al., 2008; Graner et al., 2009; Svensson et al., 2011; van der Vos et al., 2011). Thus, glioma-derived microvesicles appear to modulate the environment to favor tumor growth. Intriguingly, tumor-derived microvesicles can also be detected in the circulation of glioma-bearing mice or human glioblastoma patients demonstrating that they are produced *in vivo* and are capable of crossing the blood brain barrier. Microvesicle release from primary cortical astrocytes and microglial cells appears to be triggered by ATP-mediated activation of $P2X_7$ receptors and downstream stimulation of acid sphingomyelinase (Bianco et al., 2009). A recent study suggests that this special type of microvesicle signals to neurons resulting in modulation of synaptic activity (Antonucci et al., 2012). Application of microvesicles isolated from ATP-stimulated microglial cells to neurons *in vitro* and *in vivo* gave rise to increased neurotransmission, that appeared to be due to an enhanced release probability at the presynaptic site. However, the contribution of exosome release to these phenomena is unclear. Scanning electron micrograph pictures of glioma cells and fluorescence microscopy analysis of ATP-stimulated glial cells, as well as the heterogeneous size profile of the released microvesicles indicate that shedding from the cell surface is the dominant form of vesicle generation in these paradigms. Nevertheless, it is possible that exosomes account for some of the described functions since they are also present in the vesicular fractions analyzed. Astrocytes release exosomes carrying Hsp70 and synapsin 1 in response to heat or oxidative stress, which have been suggested to ship neuroprotective cargo to neurons facilitating their survival (Taylor et al., 2007). Intriguingly, synapsin 1 is first released from the exosomal cytosolic compartment under these conditions, before it acts on neurons in its soluble form (Wang et al., 2011).

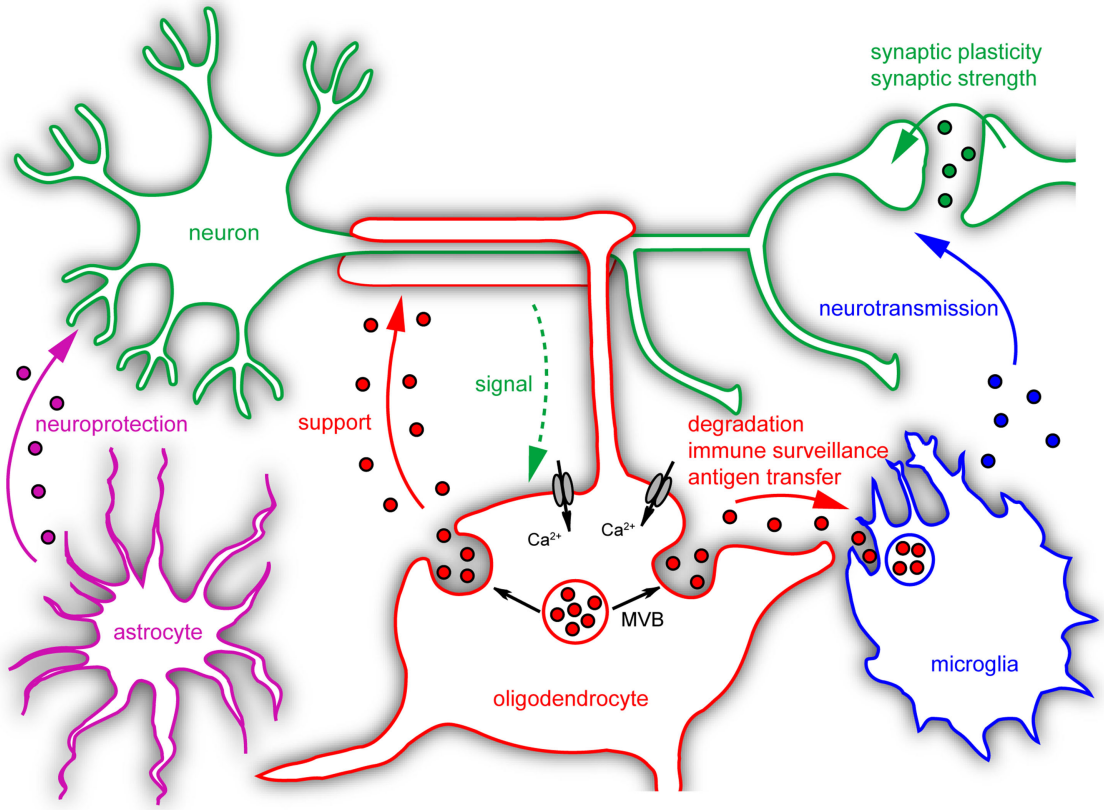

FIGURE 2 | Postulated roles of microvesicles in neural cell communication. Neural cells release different types of microvesicles with several known or suggested functions. Neurons secrete exosomes which may influence synaptic plasticity. Microglia modulate neurotransmission via shedding microvesicles. Astrocyte-derived exosomes carry neuroprotective cargo and could contribute to neuronal survival. Neuronal signals trigger exosome release from oligodendrocytes by raising intracellular Ca^{2+}-levels. Upon internalization by neurons these exosomes could provide support to axons. Microglia take up and degrade oligodendroglial exosomes without changing their inflammatory properties. Under specific pathological conditions these exosomes may transfer antigens to microglial cells or other APCs and induce inflammatory responses.

ROLE OF OLIGODENDROGLIAL EXOSOMES IN CELL COMMUNICATION

Oligodendrocytes secrete MVB-derived exosomes in a Ca^{2+}-dependent fashion, which also carry mRNA and miRNA in addition to myelin proteins and lipids (Krämer-Albers et al., 2007; Krämer-Albers et al., unpublished observations). These exosomes appear to convey autocrine signals that inhibit membrane expansion and myelin formation by oligodendrocytes in culture in response to neuron-derived trophic factors (Bakhti et al., 2011). This autocrine signaling pathway does not involve exosome reinternalization but is mediated by activation of second messenger cascades involving RhoA, Fyn, and FAK. Furthermore, it has been shown that microglia take up oligodendroglial exosomes by macropinocytosis, which subsequently become degraded and apparently fail to provoke an immune response (Fitzner et al., 2011). Internalization is observed preferentially in MHC class II negative microglia and does not promote microglial activation. The authors suggest that microglia are engaged in the degradation of excess myelin components secreted by the exosome pathway. It will be interesting to determine whether exosomes derived from oligodendrocytes exposed to pathogens or specific forms of stress will be able to induce an inflammatory response. This is particularly relevant to the still unresolved etiology of multiple sclerosis and the question of how CNS self-antigens are transferred to trigger a myelin-specific autoimmune response.

Our recent work indicates that oligodendroglial exosomes play an important role in mutual communication between oligodendrocytes and neurons. We found that neurotransmitter release stimulates oligodendroglial exosome secretion by activating Ca^{2+}-permeable ionotropic receptors on the surface of oligodendrocytes. Furthermore, neurons internalize oligodendroglial exosomes by endocytosis and utilize their cargo (submitted manuscript). These findings suggest that neuronal activity triggers the transfer of oligodendroglial exosomes and their cargo to neurons. Thus, neurons would regulate their supply of glia-derived exosomal proteins, mRNAs, and miRNAs. Myelinated axons exist as long protrusions at a distance from the neuronal cell body and are shielded from the CNS environment by the myelin membrane (Nave, 2010b). Hence, external support by exosome-mediated transfer of glial substrates could be contributing to and be critical for long-term axonal maintenance. A lack of glial support is modeled by PLP and CNP knockout mice, which develop a

progressive axonal degeneration (Edgar and Nave, 2009). PLP and CNP are both components of oligodendroglial exosomes and thus, it is tempting to speculate about the potential contribution of exosomes to glial support. Oligodendroglial exosomes additionally include substances that can contribute to neuroprotection such as Hsp/Hsc70. There is evidence that the squid giant axon locally picks up Hsps from periaxonal glial cells (Tytell et al., 1986) and it was indeed suggested that the transfer is mediated by exosomes (Tytell, 2005). Moreover, newly synthesized glial RNAs are delivered to the giant axon in response to axonal depolarization (Eyman et al., 2007). In the PNS, it has been described that axons receive ribosomal subunits in association with vesicles from myelinating Schwann cells (Court et al., 2008). It will be interesting to determine, whether oligodendroglial exosomes exhibit neuroprotective or neurotrophic functions.

RELEVANCE FOR MYELIN DISEASE

Myelin diseases are characterized by a developmental hypo/dysmyelination or a secondary demyelination. They can be inherited (leukodystrophies) or acquired such as multiple sclerosis. The pathology of multiple sclerosis involves an immune-mediated degeneration of the myelin sheath. Which factors direct the specificity of the immune response toward myelin and whether myelin antigens in multiple sclerosis are processed by APCs is unknown. Exosomes are known to deliver antigens to APCs (Bobrie et al., 2011). In spite of reports that oligodendroglial exosomes appear not to activate microglia, it is feasible that other APCs such as dendritic cells, which invade the CNS under certain conditions, process oligodendroglial exosomes and present the antigens on their surface to initiate an immune response. Future studies will determine whether oligodendroglial exosomes can mediate myelin antigen transfer to dendritic cells. Furthermore, exosome composition may be altered in certain pathological conditions, causing a switch of immunologically inert into immunologically active exosomes. Oligodendroglial exosome composition indeed may be altered in Pelizaeus–Merzbacher disease (PMD) resulting from duplications in the *PLP1* gene. Overexpression of PLP leads to its accumulation in late endosomes (Simons et al., 2002) and may yield an increased release of PLP in association with exosomes. Increased exosome release or an altered exosome protein/lipid stoichiometry may thus trigger inflammatory reactions in the CNS contributing to PMD pathology.

Common to most myelin diseases is the phenomenon of progressive axonal degeneration due to lack of glial support. This secondary neuronal damage is the major cause of irreversible disability and death of the patients. Thus, it is of clinical importance to decipher the potential implications of oligodendroglial exosomes in neuroprotection and to identify the beneficial components, with the ultimate goal to develop therapeutic strategies mitigating axonal degeneration. Furthermore, there is accumulating evidence that exosomes qualify as promising vehicles for the delivery of therapeutic agents into the brain, as they can be delivered across biological barriers such as the blood brain barrier (Lakhal and Wood, 2011).

CONCLUSION

The hypothesized role of secreted microvesicles/exosomes in neural cell communication is materializing into a picture substantiated by experimental evidence (**Figure 2**). Microvesicles may execute their functions by distinct modes of action: (1) internalization by target cells and cargo retrieval, (2) binding to the cell surface and triggering second messenger pathways, and (3) release of components into the extracellular matrix. However, their interaction with target cells is not well understood at a mechanistic level. To date, most theories of exosome function have arisen from *in vitro* data. The field awaits genetic mouse models that interfere with neural exosome secretion to demonstrate the *in vivo* relevance of exosome-mediated processes. Nonetheless, the versatile role of CNS microvesicles opens up new perspectives for the understanding and treatment of neurodegenerative diseases including diseases of myelin.

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Transfer of vesicles from Schwann cells to axons: a novel mechanism of communication in the peripheral nervous system

M. Alejandra Lopez-Verrilli*[1]* and Felipe A. Court[1,2]

[1] *Millennium Nucleus for Regenerative Biology, P. Catholic University of Chile, Santiago, Chile*
[2] *NeuroUnion Biomedical Foundation, Santiago, Chile*

Edited by:
Claudia Verderio, Institute of Neuroscience, Italy

Reviewed by:
Dandan Sun, University of Pittsburgh, USA
Carla Taveggia, San Raffaele Scientific Institute, Italy

****Correspondence:***
M. Alejandra Lopez-Verrilli and Felipe A. Court, Department of Physiology, Faculty of Biology, Pontificia Universidad Católica de Chile, Av. B. O'Higgins 340/Casilla 114-D, Santiago 8331150, Chile.
e-mail: fcourt@bio.puc.cl; alejanddra@gmail.com

Schwann cells (SCs) are the glial component of the peripheral nervous system, with essential roles during development and maintenance of axons, as well as during regenerative processes after nerve injury. SCs increase conduction velocities by myelinating axons, regulate synaptic activity at presynaptic nerve terminals and are a source of trophic factors to neurons. Thus, development and maintenance of peripheral nerves are crucially dependent on local signaling between SCs and axons. In addition to the classic mechanisms of intercellular signaling, the possibility of communication through secreted vesicles has been poorly explored to date. Interesting recent findings suggest the occurrence of lateral transfer mediated by vesicles from glial cells to axons that could have important roles in axonal growth and axonal regeneration. Here, we review the role of vesicular transfer from SCs to axons and propose the advantages of this means in supporting neuronal and axonal maintenance and regeneration after nerve damage.

Keywords: Schwann cell, axon, vesicular transfer, exosomes, microvesicles, axonal regeneration

INTRODUCTION

Originally, glial cells were considered as a sort of glue that filled the space between neurons, which is largely a passive role. In time, this view changed substantially. In the peripheral nervous system (PNS), Schwann cells (SCs) were recognized to regulate a wide variety of ongoing functions of axons (Mirsky and Jessen, 1999). It is well known that the myelin sheath, by increasing the operational resistance of the axolemma, greatly increases the velocity of the nerve impulse, which is a passive effect (Hartline and Colman, 2007).

A number of observations indicate that the cellular biology of axons is regulated by SCs. In the axolemma of unmyelinated fibers, sodium and potassium channels exist side by side (Garrido et al., 2003) but in axons surrounded by myelin, the axolemma under the sheath is poor in sodium and rich in potassium channels while the converse occurs at the nodal axolemma, where sodium channels accumulate (Salzer et al., 2008; Feinberg et al., 2010). This indicates that SCs regulate at a molecular scale the local organization of axons, thus regulating the axonal phenotype.

Nerve injury and its ensuing repair illustrate the mutual regulation of axons and SCs. Waller (1850) established that nerve section is followed by degeneration of the distal domain while SCs evolve to a dedifferentiated state. Nerve repair re-establishes the original condition (Jessen and Mirsky, 2008). After nerve injury, elongation of axons was shown to be prevented by SCs as long as they remained differentiated distal to injury (Tapia et al., 1995; Court and Alvarez, 2000), which strongly suggests that nerve repair proceeds in close interaction with the SC and not commanded by the cell body (Bray and Aguayo, 1974; Court and Alvarez, 2005). When SCs in a segment of an intact nerve are treated with a protease inhibitor, which has been shown to induce SC dedifferentiation (Alvarez et al., 1992, 2000; Tapia et al., 1995), the associated axon extends sprouts in that segment in spite of being surrounded by SCs, i.e., branches arise in an uninterrupted axon. This indicates that the axon has a growth program repressed by the differentiated SC (Court and Alvarez, 2005). Together, these phenomena illustrate that the SC locally affects the underlying axon, from its passive electrical properties, to organization of the axolemma, and even complex cellular programs embodied in the axoplasm.

We will consider now the first step of regulatory mechanisms between cells that operate on a local basis. Adhesion molecules are an important and well characterized mechanism that allows contact-mediated signaling between cells. Another mechanism involves extracellular free ligands that are produced by a cell and operate on a very short range, from its site of release to its receptor in the target cell. These two mechanisms share an important feature, namely, the machinery that produces the response belongs entirely to the target cell. A third regulatory mechanism has emerged in which a cell produces vesicles that are taken up by the target cell and the cargo is incorporated into the recipient cytoplasm (Simons and Raposo, 2009). This mechanism opens a new dimension to the intercellular interaction in that the recipient cytoplasm may contain an incomplete machinery that is completed by molecules of the donor cell upon their release from the vesicle. Our review focuses on the regulation of axons by SCs mediated by secreted vesicles and proposes the advantages of this means of communication in supporting neuronal and axonal maintenance and regeneration after nerve damage.

EVIDENCES FOR VESICULAR TRANSFER BETWEEN SCs AND AXONS

That proteins may enter the cytoplasm from the outside is an old notion. About fifty years ago, it was established that some proteins of the oocyte yolk of the mosquito *Aedes aegypti* were synthesized in the gut, moved to the ovary, and were taken up from the extracellular space via pinocytic vesicles to be stored essentially as a reservoir of amino acids for the embryo (Roth and Porter, 1964). In the nervous system, glia-to-axon transfer of protein was proposed about forty years ago. The giant axon of the squid was incubated with radiolabeled amino acids and labeled proteins were recovered from its axoplasm (Lasek et al., 1974). However, the notion of transcellular transfer emerged under the assumption that axons were unable to synthesize proteins, but since this assumption was wrong as axons do synthesize protein (Koenig and Giuditta, 1999; Alvarez et al., 2000; Donnelly et al., 2010; Gumy et al., 2010), the notion of glia-to-axon transfer of protein awaited further experimental support.

Around the 1980s, the groups of Stahl and Johnstone provided evidence to support that vesicles can mediate the release of proteins during reticulocytes maturation (Harding et al., 1983; Pan et al., 1985). These vesicles named exosomes were contained within multivesicular endosomes whose fusion with the plasma membrane was followed by exosome secretion (Johnstone et al., 1987; Simons and Raposo, 2009; Thery et al., 2009). In turn, vesicles originated after the evagination of the plasma membrane were named microvesicles (Cocucci et al., 2009; Thery et al., 2009). That was the beginning of a new era in cell communication, the release of membrane vesicles.

Based on these antecedents, transfer of macromolecules from SC to axons was reconsidered, this time mediated by vesicles. Thus, Buchheit and Tytell (1992) described transfer of fluorescently labeled vesicles from SCs to squid giant axons. They proposed that these vesicles carried the proteins previously thought to be transferred directly from the SC to the axon, such as heat shock protein (Hsp) 70 (Tytell et al., 1986) – a protein also carried in exosomes secreted by reticulocytes (Davis et al., 1986) – but they did neither confirmed these possibilities nor their functional significance. Nowadays, exosomes and microvesicles have been described in glial cells from the central nervous system (CNS, see **Table 1**), although in the PNS the evidence is scarce. Hsp70 is present in exosomes secreted from a SC cell line (Fevrier et al., 2004), SC primary cultures (Lopez-Verrilli M. A. and Court F. A., unpublished results) and in exosomes secreted by glial cells from the CNS, including astrocytes, oligodendrocytes, and microglia (Potolicchio et al., 2005; Krämer-Albers et al., 2007; Taylor et al., 2007). It remains to be investigated whether vesicular transfer of Hsp70 to axons confers neuroprotection to stress stimuli and neurodegenerative disorders.

Schwann cells are essential during regenerative processes after nerve injury, not only by secreting growth factors (Madduri and Gander, 2010; Quintes et al., 2010) but also by supplying components of the protein synthesis machinery to axons. Court et al. (2008, 2011) demonstrated *in vivo* the transfer of ribosomes from SCs to axons after axonal damage as well as during axonal regeneration. Electron microscope images showed ribosomes in the axoplasm but also within vesicles surrounded by two or multiple membranes. Interestingly, even multimembrane vesicles open to the axoplasm were still partially loaded with ribosomes and abundant free ribosomes seemingly discharged in the vicinity, suggesting that SC-derived vesicles were secreted and internalized in axons by endocytosis. Nevertheless, the molecular mechanism for ribosomal transfer after axonal damage and during axonal regeneration has not been disclosed yet.

Considering that SC exosomes diameter varies between 50 and 120 nm (Lopez-Verrilli M. A., and Court F. A., unpublished results), only a small amount of ribosomes could be transported within each exosome. On the other hand, microvesicles are larger vesicles (up to 1 μm; Cocucci et al., 2009) and might even transport polyribosomes. Since mRNAs can be stored in a dormant state in the distal axon until needed (Yoo et al., 2010), the transfer of mRNA-containing ribosomes from SC to axon could supply transcript for storage and translation in response to acute stimuli (e.g., nerve damage) or the transfer be triggered by the stimuli itself. In addition, vesicular transfer from SCs would accelerate the arrival of ribosomes to the axon, compared to ribosomes derived from the neuronal cell body (Twiss and Fainzilber, 2009).

In the dark side of vesicular transfer, SCs have been shown to secrete exosomes containing pathogenic prions upon cell infection *in vitro*, therefore prion secretion via SC-derived exosomes may spread these pathogenic proteins from the PNS to the CNS (Fevrier et al., 2004). Prions are misfolded proteins that act as infectious agents and cause neurodegenerative diseases (Weissmann et al., 2011). Furthermore, pathological cell–cell communication by endogenous vesicular vectors could be one of the mechanistic explanations for non-cell autonomous processes playing critical roles in neurodegenerative diseases (Garden and La Spada, 2012).

Summing up, SCs might provide by means of secreted vesicles, an efficient, specific, and highly localized support for axons. Vesicles interact specifically with the target cell (Rana and Zoller, 2011) supplying many copies and many kinds of macromolecules, which might allows SCs to locally regulate axonal functions without direct involvement of the neuronal cell body. Together, the evidence suggests that machineries of a given cytoplasm may be incomplete requiring the contribution of a neighboring cell to become operative (Court et al., 2008, 2011). From another point of view, the phenotype of a cell may require the contribution of an external genome to supply the missing messenger RNAs. SCs contain mRNAs coding for neurofilament proteins, which they barely translate (Roberson et al., 1992), and its transfer within vesicles could be instrumental for protein homeostasis in axons. In brief, the transfer of vesicles and their cargo of protein and RNAs open a novel mode for intercellular interaction, and a broad avenue of research.

POTENTIAL ROLES AND FUNCTIONS OF SECRETED VESICLES IN THE PNS

Vesicle secretion as a means to supply components to a target cell offers a number of advantages considering the SC and axon dynamics, e.g., during myelination and regeneration conditions.

In the myelinated nerve fiber (**Figure 1A**), vesicles could be released from microvilli domains to the node of Ranvier and transfer scaffolding proteins required for the proper node formation, such as actin, tubulin, cofilin, and ankyrin-G (Krämer-Albers et al., 2007; Valadi et al., 2007). Vesicles could be secreted along

Table 1 | Vesicles secreted by glial cells: content and effects upon target cells.

Cell of origin	Vesicle type	Recipient cell	Relevant vesicle content	Effect over recipient cell or secretion details	Reference
Schwann cells	Exosomes	ND	PLP, CNP, MBP, PrPc, PrPsc	Prions-infected SCs secrete infectious exosomes containing PrPsc	Fevrier et al. (2004)
	Unknown	Neuron (axonal compartment)	Ribosomes	Transfer of ribosomes stimulated by axonal damage and during regeneration	Court et al. (2008), Court et al. (2011)
Oligodendrocytes	Exosomes	ND	PLP, MOG, MBP, CNP	Oligodendrocytes secrete exosomes upon Ca^{+2} influx to the cytoplasm. Characterization of protein and lipid composition of oligodendrocyte derived exosomes	Krämer-Albers et al. (2007)
		ND	PLP	Exosome formation into multivesicular bodies is dependent of ceramide synthesis and independent of the ESCRT machinery.	Trajkovic et al. (2008)
		ND	PLP	Exosome secretion is regulated by Rab35 GTPase and its GAPs TBC1D10A–C	Hsu et al. (2010)
		ND	Flotillin-2, cholesterol	Exosomes containing flotillin-2 allow the discharge of cholesterol from oligodendroglial cells.	Strauss et al. (2010)
		Oligodendrocyte	PLP, MOG, MAG, CNP	Exosomes inhibit oligodendrocyte differentiation and myelin formation	Bakhti et al. (2011)
		Microglia	PLP, MOG, CNP	Oligodendrocyte derived exosomes are selectively internalized by microglial cells via macropinocytosis	Fitzner et al. (2011)
		Astrocytes	Hsp70, TRAIL	Oligodendroglial exosomes induce apoptosis in astrocytes	Lo Cicero et al. (2011)
	Exosomes and microvesicles	Cortical neurons	FasL, Nogo protein B	Vesicles induces apoptosis of cortical neurons	D'Agostino et al. (2006)
Astrocytes	Exosomes	ND	Hsp70	Astrocytes secrete exosomes containing Hsp70 upon heat shock stress	Taylor et al. (2007)
		ND	mtDNA	Exosomes from astrocytes and glioblastoma tumors secrete exosomes carrying mitocondrial DNA	Guescini et al. (2010)
		HUVECs	RNA and DNA, mutated and amplified oncogene sequences and transposable elements	Glioblastoma tumors secrete vesicles with functional RNAs and DNAs to potentially promote tumor progression	Balaj et al. (2011)
		ND	Synapsin	Extracellular synapsin stimulates neurite outgrowth	Wang et al. (2011)
	Microvesicles	ND	IL-1β	ATP acting on P_2X_7 increases microvesicle secretion from astrocytes, a mechanism dependent on sphingomyelinase activation	Bianco et al. (2009)
		ND	MMP2, MMP9 and TIMP2	MMP 2 and 9, and tissue inhibitors of MMP are released in microvesicles from astrocytes.	Sbai et al. (2010)
		ND	Ectonucleotidase NTPDase	Microvesicles containing NTPDase degrades extracellular ATP in an *in vitro* model of the blood brain barrier.	Ceruti et al. (2011)
	Exosomes and microvesicles	ND	β1-integrin, FGF-2, VEGF	Astrocytes secrete vesicles containing β1-integrin, FGF-2 and VEGF	Proia et al. (2008)

(Continued)

Table 1 | Continued

Cell of origin	Vesicle type	Recipient cell	Relevant vesicle content	Effect over recipient cell or secretion details	Reference
Microglial cells	Exosomes	ND	Aminopeptidase CD13	Characterization of exosome release and measurement of exosomal aminopeptidase CD13 activity.	Potolicchio et al. (2005)
		ND	IDE	Exosomes carrying IDE degrade extracellular β-amyloid peptide	Tamboli et al. (2010)
	Microvesicles	ND	IL-1β	ATP-derived astrocytes promotes microvesicle secretion by microglial cells	Bianco et al. (2005)
		Hippocampal neurons	ND	Microvesicles influence synaptic activity by increasing spontaneous and evoked excitatory transmission in neurons	Antonucci et al. (2012)
Glioblastoma cells	Exosomes	Endothelial and glioma cells	Angiogenic proteins, mRNAs, microRNAs and the tumor-specific EGFRvIII mRNA	Tumor vesicles mRNA content can be translated in endothelial and glioma cells, promoting angiogenesis, and tumor proliferation	Skog et al. (2008)
		Cancer cells lacking EGFRvIII	EGFRvIII	Exosomes carrying EGFRvIII transfer oncogenic activity to cancer cells lacking EGFRvIII and promote the expression of EGFRvIII-regulated genes	Al-Nedawi et al. (2008)
	Exosomes and microvesicles	ND	RNA	Vesicle-derived RNA analysis purified from serum of control and glioblastoma multiforme patients	Noerholm et al. (2012)

ND, not determined; CNP, 2′3′-cyclic-nucleotide-phosphodiesterase; EGFRvIII, truncated isoform of endothelial growth factor receptor; ESCRT, endosomal sorting complex required for transport; FasL, Fas ligand; FGF-2, fibroblast growth factor 2; Hsp70, heat shock protein 70; HUVECs, human umbilical vein endothelial cells; IDE, insulin-degrading enzyme; IL-1β, interleukin-1β; MBP, myelin basic protein; MMP, matrix metalloproteases; MOG, myelin oligodendrocytes glycoprotein; mtDNA, mitochondrial DNA; NTPDase, Nucleoside triphosphate diphosphohydrolase; PLP, proteolipid protein; PrP, prion protein; PrPsc; prion protein scrapie; TIMP2, metallopeptidase inhibitor 2; SCs, Schwann cells; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; VEGF, vascular endothelial growth factor.

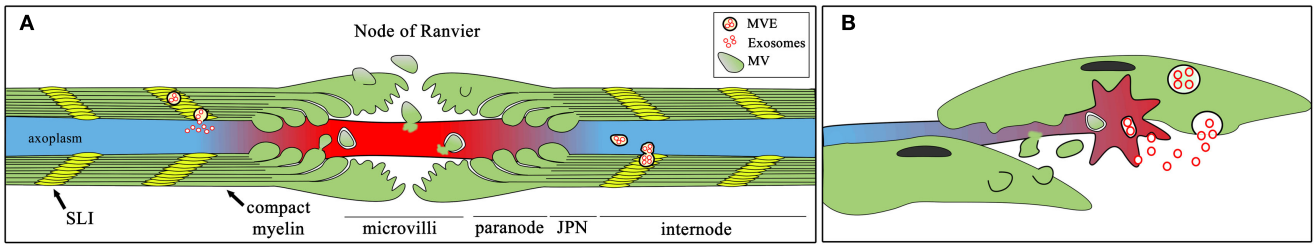

FIGURE 1 | Possible routes and conditions for SC to axons transfer of vesicles. Schematic representation of possible routes of exosomal (red circles) and microvesicle (larger green ovoids) transfer between SCs and axons in a myelinated fiber **(A)** and in an axonal growth cones during axonal regeneration **(B)**. Exosomes are contained within multivesicular endosomes (MVE) in the secreting cell, and then can move to the axon through cytoplasmic-rich region in SCs, including Schmidt-Lantermann incisures [SLI, yellow regions in **(A)**] or paranodal domains of myelinating fibers **(A)** or can be released close to the growth cone by dedifferentiated SC **(B)**. Microvesicles, in turn, are generated from the evagination of SC plasma membrane and they can fuse or be internalized by axons.

the Schmidt-Lantermann incisures cytoplasm channels across the myelin sheath or paranodal domains directly to the axoplasm, providing macromolecules in a temporally and spatially regulated fashion. Both exosomes and microvesicles deliver not only proteins but also microRNAs and mRNAs that can be translated into recipient cells (Ratajczak et al., 2006; Valadi et al., 2007). In fact, elongation factors needed for mRNA translation have been found in exosomes from oligodendrocytes and microglial cells (Potolicchio et al., 2005; Krämer-Albers et al., 2007).

Exosomes and microvesicles may actively participate in processes activated after nerve damage, when SCs dedifferentiate and begin to proliferate (**Figure 1B**). This scenario is quite complex since axons degenerate distal to the injury, while in the proximal stump, regeneration takes place (Coleman, 2005; Twiss and van Minnen, 2006). In these conditions, SCs could secrete vesicles containing mRNAs and microRNAs to negatively regulate myelination and stimulate proliferation, as observed for glial tumor cells (Skog et al., 2008). In addition, SCs-secreted vesicles could sustain protein synthesis in regenerating axons (Court et al., 2011), independently from axonal mRNA synthesized in the neuronal nuclei, which needs to be transported a long way before translation, or even provide guiding clues to regenerating axons, such as the axonal guidance protein Wnt, which has been detected in exosomes from motor neurons (Zou, 2004; Korkut et al., 2009).

If SC-derived vesicles are demonstrated to have functional roles in axonal regeneration, SC differentiation, or other processes crucial for neural tissue regeneration, these vesicles can be used for therapeutic purposes by using their endogenous potentials or by loading them with specific transcript or proteins by modifying glial cells, which can be easily manipulated *in vitro* (Schmitte et al., 2010; Zhang et al., 2010). It has been demonstrated that neuronal-targeted exosomes obtained from genetically modified dendritic cells *in vitro* can be electroporated with specific siRNA, and after intravenous injection, they specifically knock-down their target gene in brain neurons (Alvarez-Erviti et al., 2011). Vesicle-mediated drug delivery promises to overcome important challenges, such as delivery of drugs across impermeable biological barriers and using patient-derived cells to obtain tolerogenic vesicles.

CONCLUDING REMARKS

In this review we presented evidence for SC to axon communication via secreted vesicles and highlighted the functional role this process may have in the maintenance of peripheral axons and during regeneration. Increasing evidence is suggesting that axons have the ability to respond to a challenge autonomously from the cell body albeit under SCs regulation (Alvarez et al., 2000; Court and Alvarez, 2005). Since axons are of any length up to several meters, this anatomy clearly poses logistic problems. The evolutionary solution may be that SC packs the requisite components in a vesicle to convey its cargo to the axoplasm. We propose that vesicles secreted by SCs and transferred to axons are a major mechanism by which SCs locally support axonal maintenance and regeneration after nerve damage. We hope that the study of the processes will enrich our understanding of the cellular biology of the nervous system.

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Extracellular membrane vesicles and immune regulation in the brain

Chiara Cossetti, Jayden A. Smith, Nunzio Iraci, Tommaso Leonardi, Clara Alfaro-Cervello and Stefano Pluchino*

Cambridge Centre for Brain Repair, Department of Clinical Neurosciences, Stem Cell Institute, University of Cambridge, Cambridge, UK

Edited by:
Claudia Verderio, Università di Milano, Italy

Reviewed by:
Roberto Furlan, San Raffaele Scientific Institute, Italy
Giovanni Camussi, University of Torino, Italy

****Correspondence:***
Stefano Pluchino, Cambridge Centre for Brain Repair, Department of Clinical Neurosciences, Stem Cell Institute, University of Cambridge, ED Adrian Building, Forvie Site, Robinson Way, Cambridge CB2 0PY, UK.
e-mail: spp24@cam.ac.uk

The brain is characterized by a complex and integrated network of interacting cells in which cell-to-cell communication is critical for proper development and function. Initially considered as an *immune privileged* site, the brain is now regarded as an *immune specialized* system. Accumulating evidence reveals the presence of immune components in the brain, as well as extensive bidirectional communication that takes place between the nervous and the immune system both under homeostatic and pathological conditions. In recent years the secretion of extracellular membrane vesicles (EMVs) has been described as a new and evolutionary well-conserved mechanism of cell-to-cell communication, with EMVs influencing the microenvironment through the traffic of bioactive molecules that include proteins and nucleic acids, such as DNA, protein coding, and non-coding RNAs. Increasing evidence suggests that EMVs are a promising candidate to study cross-boundary cell-to-cell communication pathways. Herein we review the role of EMVs secreted by neural cells in modulating the immune response(s) within the brain under physiological and pathological circumstances.

Keywords: extracellular membrane vesicles, exosomes, immune regulation, central nervous system, neural stem cells, microglia, endothelial cells, brain tumors

INTRODUCTION

The central nervous system (CNS) is built upon complex cellular networks consisting of neurons – embodying about half the volume of the CNS – and glial cells that make up the rest of the volume and provide support and protection for neurons. CNS cells are structurally in close contact with vascular cells that control their microenvironment through blood flow and the formation of the blood–brain barrier (BBB), whose presence renders the CNS a site that is in principle immunologically distinct. As such, until a few decades ago the brain was generally considered a functionally *immune privileged* site devoid of immune cells, thus implying its likely "*invisibility*" to some aspects of immune surveillance (Wilson et al., 2010).

However, since the late 1980s accumulating evidence has been provided that immune cells gain access to (and eventually persist into) the healthy CNS and that immune responses can also be mounted within the CNS (Wekerle et al., 1986). Currently, the brain is more precisely regarded as an immune "specialized" site where a close association between resident CNS cells and, importantly, a bidirectional cell trafficking between the brain and the blood stream is needed primarily for brain integrity and homeostasis, but also for immune functions and repair upon injury (Bradl, 1996). This relevant neuro-immune interplay takes place mainly at the level of the neurovascular unit (NU) that represents a sort of "master checkpoint" of the brain microenvironment. The NU is the dynamic assembly of endothelial cells within a capillary vessel, the surrounding extracellular matrix (ECM), and a number of accessory cells, including pericytes, capillary-bound astrocytes, perivascular dendritic cells (DCs), macrophages, and neurons (Hawkins and Davis, 2005). On the basis of the evidence to date, it appears that the brain and the immune system have the capacity to establish a very sophisticated biochemical intercourse, which is made of a plethora of paracrine molecules that used to be naively considered solely immune-modulating, while later showing remarkable additional effects on the CNS (Boulanger et al., 2001).

The immune status of the CNS at the level of the NU becomes therefore dichotomous when comparing homeostatic (healthy) to reactive (pathological) conditions.

In the healthy CNS (i) the intact BBB is a gate to the influx of immune cells and macromolecules from the blood stream; (ii) chemokines and cytokines are only produced to a basal level which is not enough to chemoattract immune cells; (iii) CNS endothelial cells have a basal (low) expression of adhesion molecules that are not capable of guiding the migration of immune cells; and (iv) the antigen presentation capacity of brain immune cells is very limited, due to negligible expression of molecules of the major histocompatibility complex (MHC; Carrithers et al., 2000). This situation very rapidly changes under certain pathological conditions in which the brain microenvironment reacts to infections or injuries, or when disease-induced stresses result in secondary effects in the CNS, ultimately leading to neuro-inflammation (Carrithers et al., 2000).

As a consequence of the above, the injured CNS instantly becomes locally immune competent and immune reactive, as suggested by the different organized immune responses that are observed both in and outside the injured area(s). These immune responses can be either tightly adapted against a specific (self vs.

Table 1 | Extracellular membrane vesicles in the brain.

Cell type	Type of EMVs	Function	Reference
Microglia	Shedding vesicles	Non-classical release of IL-1β	Bianco et al. (2005)
	Exosomes	Neuropeptide degradation, glucose catabolism, and lactate production	Potolicchio et al. (2005)
	Exosomes	Secretion of insulin-degrading enzyme	Tamboli et al. (2010)
Endothelial Cells	Endothelial microparticles (EMPs)	Cerebrovascular diseases (e.g., stroke)	Simak et al. (2006), Jung et al. (2009)
		Contribution to MS pathophysiology (enhanced inflammation and increasing migration), functional interactions between EMP and leukocytes	Minagar et al. (2001), Jy et al. (2004), Sheremata et al. (2006)
		Contribution to cerebral malaria pathogenesis	Combes et al. (2005, 2006, 2010), Coltel et al. (2006)
NPCs	Membrane particles	CD133/prominin-1 release	Marzesco et al. (2005), Huttner et al. (2008)
	Exosomes	None	Kang et al. (2008)
Brain tumors	Microvesicles	miRNA and protein transfer; oncogenesis	Skog et al. (2008)
	Microvesicles	Tumor progression	Skog et al. (2008), Al-Nedawi et al. (2009)
	Exosomes	mtDNA release	Guescini et al. (2010)
	Exosomes	Retrotransposon and oncogene transfer	Balaj et al. (2011)
	Microvesicles	Angiogenesis	Svensson et al. (2011)
	Microvesicles	Transformation	Antonyak et al. (2011)
	Microvesicles	None	Graner et al. (2009)
	Microvesicles	None	van der Vos et al. (2011)

non-self) antigens or involve cellular and molecular pathways specific of innate responses (Griffiths et al., 2007) that involve different types of immune cells (Doring and Yong, 2011; Graeber et al., 2011; Miljkovic et al., 2011) and encompass different cell-to-cell communication programs.

Focusing at cell non-autonomous mechanisms of neuroinflammation in auto/dysimmune inflammatory CNS disorders – multiple sclerosis (MS) being among the most didactical examples – cytokines, chemokines, and other known secreted paracrine factors of intercellular communication are first released by myelin-reactive peripheral T lymphocytes, then contribute to the bystander activation of other circulating immune cells (e.g., macrophages, neutrophils, and monocytes), and finally are secreted within the CNS also by endothelial cells and neurons/glia alongside the synthesis of classical neurotransmitters (Doring and Yong, 2011; Graeber et al., 2011; Miljkovic et al., 2011). Following these and other mechanisms of information spread, the BBB is disrupted and the CNS acquires signals that allow cells from the hematopoietic system (e.g., T lymphocytes and monocytes) to leave the blood stream and accumulate at the level of the NU. There, they come in contact with local CNS cells, including microglia, leading to a second wave of immune activation, and damage within the CNS (Mae et al., 2011).

While there is general agreement on the ubiquitous and redundant nature of some of the mechanisms regulating the neuroimmune cross talk, still there is very little knowledge of the modalities (as well as the messages) that can be used to convey immune signals into (and around) the CNS (Blalock, 1994).

The recent description of new specialized structures for intercellular communication, such as extracellular membrane vesicles (EMVs; Cocucci et al., 2009; Thery et al., 2009; Thery, 2011) and tunneling nanotubes (Gerdes and Carvalho, 2008; Gousset et al., 2009), has significantly broadened the range of modalities of intercellular communication. This has led to the challenging hypothesis that some of these extracellular organelles might work as long-distance signaling structures acting into either the extracellular space or biological fluids prior (or as an alternative) establishing direct cell-to-cell contacts between cells in the CNS (Antonucci et al., 2012; Huttner et al., 2012; Saman et al., 2012; Street et al., 2012).

Here we will review the most recent evidence on the role of (secreted) EMVs in regulating the immune response(s) in the brain.

EXTRACELLULAR MEMBRANE VESICLES, EXOSOMES, MICROVESICLES, AND BEYOND

Cells produce many vesicles regulating the transfer of components between intracellular compartments, but it is now clear that eukaryotic cells also generate membrane vesicles that are secreted into the extracellular space, and are therefore potential carriers for intercellular communication. EMVs are spherical structures that are formed by a lipid bilayer and that contain hydrophilic soluble components. EMVs can form at the plasma membrane (membrane particles) by direct budding or shedding into the extracellular space, giving rise to large-size (>100 nm) membrane particles, also defined as microvesicles, ectosomes, microparticles, or exovesicles (Thery et al., 2009). Alternatively, EMVs can form inside internal (late endocytic) compartments from where they are subsequently secreted by fusion with the plasma membrane. The recycling pathways of endocytosed components from the cell surface involve several sorting events, which are regulated by

molecular motors and take place at different steps of the pathways (Maxfield and McGraw, 2004). The EMVs generated in multivesicular endosomes (exosomes) are small in size (40–100 nm). The exact nature of the intracellular compartments from which exosomes derive is still unclear. Recently, another class of microvesicles known as gesicles have been identified (Mangeot et al., 2011). These particles, approximately 100 nm in diameter and slightly less dense than exosomes, are highly fusogenic and originate from cells that have been induced to overexpress the spike glycoprotein of the vesicular stomatitis virus (VSV-G). Finally, also exosome-like vesicles (20–50 nm) that express the full-length 55-kDa tumor necrosis factor (TNF) receptor 1 may originate from multivesicular internal compartments (not necessarily being part of the endosomal system), though their nature is not completely clear (Hawari et al., 2004). Irrespective of their origin, all these extracellular vesicles contain cytosol and expose on their outer surface the extracellular side of the membrane from which they are formed (flip–flop mechanism). Large membrane particles and ectosomes have high levels of phosphatidylserine exposed on the outer membrane – usually depending on cell type and stimuli – and express the complement receptor (CR)-1, whereas exosomes are enriched in tetraspanins (e.g., CD63, CD9). EMVs highly enriched in histones are also released by dying and apoptotic cells (Thery et al., 2009).

Despite the lack of definitive evidence for their physiological function *in vivo*, EMVs appear to constitute a newly recognized means of communication found to be shared by an increasing number of cell types. In 2009 one of the first hypotheses postulating that this sort of mechanism of communication might not only exist, but also be relevant for neural cells, challenged the field (Smalheiser, 2007, 2009). Concomitantly, the capacity to secrete vesicles other than synaptic vesicles has been demonstrated for almost every cell type that constitutes the brain (Von Bartheld and Altick, 2011), namely neurons (Faure et al., 2006; Schiera et al., 2007; Smalheiser, 2007; Putz et al., 2008; Lachenal et al., 2011), astrocytes (Taylor et al., 2007; Guescini et al., 2010), oligodendrocytes (Trajkovic et al., 2008; Hsu et al., 2010; Fitzner et al., 2011), and microglia (Bianco et al., 2005, 2009; Potolicchio et al., 2005; Tamboli et al., 2010). Importantly, EMVs are also secreted by neural stem/precursor cells (Marzesco et al., 2005; Huttner et al., 2008; Kang et al., 2008; Pluchino et al., 2009b). Elevated levels of EMVs expressing the neural stem cell marker prominin-1/CD133 in the cerebrospinal fluid (CSF) are observed in glioblastoma and partial epilepsy, two disease states that are described to be associated to significant changes in adult neurogenesis (Ming and Song, 2011). This suggests the potential value of circulating EMVs as biomarkers of either disease statuses or specific micro-environmental cues in monitoring the behavior of neural progenitors (Huttner et al., 2008, 2012).

Increasing evidence also suggests that EMVs may control fundamental cellular responses, such as intercellular signaling and immune reactions (Simons and Raposo, 2009; Thery et al., 2009). Several types of interactions have been proposed as being mediated by secreted EMVs, mostly based on indirect *in vitro* evidence. These include the adhesion of EMVs to the recipient cell surface (e.g., through lipids or ligand–receptor interactions; Segura et al., 2007), the internalization of whole EMVs into endocytic compartments (e.g., mediated by receptors; Miyanishi et al., 2007), or the direct fusion of VSV-G expressing gesicles (Mangeot et al., 2011). However, whether EMV fusion occurs on the surface of the recipient cell or after endocytosis via internal compartments (or both) is still unclear. The functional consequences of this sophisticated mode of intercellular communication include the amplification and/or modulation of cellular (e.g., immune) responses (Thery et al., 2009), as well as the acquisition of new functional properties by recipient cells, such as migratory, adhesive, or metastatic abilities (Al-Nedawi et al., 2008).

Extracellular membrane vesicles in the brain have been linked to a number of different processes, such as regulation of myelin membrane biogenesis (Bakhti et al., 2011), transfer of proteins or mRNAs locally in highly polarized structures like neurons (Twiss and Fainzilber, 2009), or trafficking of Nedd4 family interacting protein 1 (Ndfip1) and associated Nedd4 family proteins for the exosomal sequestration of unwanted metal cation-transporting proteins during times of stress (Putz et al., 2008). Besides, EMVs participate in the processing of misfolding/aggregation-prone proteins associated with neurological diseases into their pathological conformations, as well as their subsequent intercellular trafficking (Vella et al., 2008). In particular, exosomes containing α-synuclein have been demonstrated to cause cell death in neurons *in vitro*, thus leading to an amplification and propagation of Parkinson's disease-related pathology, *in vitro* (Emmanouilidou et al., 2010). In Alzheimer's disease (AD), it has also been reported that β cleavage occurs in early endosomes followed by routing of β-amyloid to multivesicular bodies (MVBs). Subsequently, a minute fraction of Aβ peptides can be secreted from the cells in association with exosomes. Also, exosomal proteins were found to accumulate in the plaques of AD patient brains, suggesting a role in the pathogenesis (Rajendran et al., 2006; Sharples et al., 2008; Bulloj et al., 2010; Saman et al., 2012).

Moreover, exosomes are involved in the formation/transfer of pathogenic proteins such as prions (Fevrier et al., 2004; Vella et al., 2007; Alais et al., 2008), and may play a role in the spread of hyperphosphorylated tau, the misfolded protein most commonly associated with human neurodegenerative diseases (Goedert et al., 2010).

In addition to proteins, some recent evidence shows that secreted EMVs also contain nucleic acids, including DNA and RNAs, some of which are specifically packaged into EMVs and shuttled to neighboring recipient cells. The RNA has indeed several advantages as an extracellular signaling molecule, and a number of recent reports have envisaged a significant role for both coding and non-coding RNAs carried within EMVs as biologically relevant extracellular signals (Dinger et al., 2008).

Micro RNAs (miRs) are small (21–23 nt) non-coding RNAs that post-transcriptionally regulate gene expression by translational inhibition or destabilization of mRNAs (Bartel, 2009). As such, a specific miR transferred within EMVs may simultaneously regulate multiple target genes, thereby enabling complex changes in multiple protein expression profiles. Recently, miRs have been found in the extracellular space and fluids such as blood plasma, urine, saliva, and sperm. Extracellular miR profiles have been considered as putative biomarkers of pathological states (De Smaele et al., 2010; Ciesla et al., 2011), and the relatively high stability of the

cell-free, circulatory miRs has been attributed to their associations with RNA-binding proteins or their encapsulation within vesicles (Hunter et al., 2008; Wang et al., 2010; Chen et al., 2012). Intriguingly, a reminiscence of this evidence dates back to 1977 when small particles possessing the characteristic of polysomal tumor virus-specific RNA were first detected in the CSF of patients with various types of CNS solid tumors (Cuatico et al., 1977). The miR profiles of tumor-secreted exosomes commonly mirror those of the parent cells (Taylor and Gercel-Taylor, 2008; Rabinowits et al., 2009), while viral nucleic acids may hijack the host's exosomes to affect immune regulation in recipient cells (Gourzones et al., 2010; Meckes et al., 2010; Pegtel et al., 2010).

More recent perspectives have expanded beyond the role of extracellular miRs as passively released indicators of disease. Indeed, the extracellular secretion of miRs has been found to be both active and energy-dependent, with sorting of specific miRs into EMVs occurring prior to release (Gibbings et al., 2009; Kosaka et al., 2010; Zhang et al., 2010). Some of these studies have in fact highlighted the fact that some of the identified RNAs [e.g., over 120 mature miRs within exosomes from mast cells and recently also pre-miRs in EMVs from mesenchymal stem cell (MSC)] are expressed at higher levels or even uniquely within EMVs, as compared to donor cells (Valadi et al., 2007; Chen et al., 2010). Moreover EMVs derived from MSC not only contain pre-miRs but also Ago2, a component of the machinery for their maturation, as well as mature, functional miR (Collino et al., 2010). This would suggest the existence of dedicated (and still not clarified) cellular trafficking control mechanisms for recycling, collecting, and packaging specific nucleic acids into EMVs.

EXTRACELLULAR VESICLES AS CONVEYORS OF IMMUNE RESPONSES

Recently, several studies have focused on the role of EMVs as conveyors of immune response (Clayton and Mason, 2009; Anand, 2010; Bobrie et al., 2011; Chaput and Thery, 2011). The impact of EMV-mediated immune modulation remains an on-going controversy, with the net functional effect – that is promotion or suppression of the immune response – being very much dependent on the nature of the parent cell (Thery et al., 2009).

Exosomes secreted by antigen presenting cells (APCs), such as DCs and B lymphocytes, carry a range of immune-stimulatory molecules including MHC-I, MHC-II, as well as co-stimulatory molecules such as CD80/B7.1 and CD86/B7.2. DC exosomes activate T cells, and participate to the development of antigen-specific immune responses (Raposo et al., 1996; Zitvogel et al., 1998; Thery et al., 1999; Clayton et al., 2001; Segura et al., 2005a,b; Bhatnagar et al., 2007).

Similarly, exosomes derived from B lymphocytes are enriched in proteins that facilitate antigen presentation and can stimulate T cells *in vitro*, implying a role in the maintenance of T cell memory or T cell tolerance (Raposo et al., 1996; Escola et al., 1998; Muntasell et al., 2007). Furthermore, B cell-derived exosomes have been found to be specifically delivered to follicular DCs (FDCs) *in vitro*, thus suggesting a potential route by which FDCs might passively acquire peptide-loaded MHC class II molecules for further stimulation of $CD4^+$ T cells (Denzer et al., 2000). Preclinical studies have also demonstrated that antigens increase their immunogenicity when trafficked by exosomes (Chaput and Thery, 2011).

While it is firmly established that miRs play an important role in immune regulation (O'Connell et al., 2010), only recently has evidence been presented supporting the exosomal transfer as a route by which miRs can affect such activity. Mittelbrunn et al. (2011) have demonstrated *in vitro* an antigen-driven, immune synapse (IS)-dependent, unidirectional transfer of exosomes between T cells and APCs. Antigen-induced IS formation was found to result in a polarization of exosome-generating MVBs toward the IS and a concomitant enhancement in exosome secretion. Furthermore, J77 T cells transduced to overexpress miR-335 were observed to knockdown the miR-335 target gene Sox4 in recipient Raji B cells in an antigen-specific manner correlating with the transfer of the exosomal marker CD63. Inhibition of IS and exosome formation were found to impair T cell to APC exosome and miR transfer, respectively. While not directly affirming the role of exosomal miR delivery in immune regulation, these results do imply the feasibility of such a mechanism (Mittelbrunn et al., 2011). Nevertheless, the ultimate contribution of exosomes to immune regulation, and particularly the role of miRs in this process, remains controversial due to the inherent complexities of the immune response mechanism.

Given that almost all cell types secrete EMVs it should be taken into account that, in contrast to the *in vitro* conditions in which often only one cell type is analyzed, the *in vivo* interplay is much more complex and the vesicle exchange may very likely be bidirectional. The mechanism of vesicle transfer (as well as the signals conveyed with vesicles) might also be different between various cell types, thus leading to cell- or context-specific vesicle effects (Koppler et al., 2006). When trying to translate the immune properties of a certain subset of vesicles, the perspective from which the system is evaluated should be carefully taken into account (Brown et al., 2008). As such, depending on the donor cell type, EMVs either activate or suppress the immune response (Valenti et al., 2006, 2007; Wieckowski and Whiteside, 2006; Zhuang et al., 2011). While the delivery of exogenous miRs to target cells appears to be facilitated by a vesicle-mediated specificity, a firm understanding of the recipient uptake mechanisms remains elusive (Chen et al., 2012).

In the brain, the regulation of immune functions by EMVs has been convincingly reported for microglia/macrophages, endothelial cells, and brain tumor cells, while only indirectly ascribed to stem cells, so far (**Table 1**).

EXTRACELLULAR VESICLES AND MICROGLIA/MACROPHAGES

Microglia, the resident macrophages of CNS parenchyma, and macrophages, are two related classes of cells (Raivich and Banati, 2004) that are now recognized as the prime components of the intrinsic brain immune response, alternatively defined as the vanguard in host defense and tissue repair (Streit and Kincaid-Colton, 1995; Rock and Peterson, 2006).

Historically, the function of microglia has been somewhat controversial. This is in part due to the extremely plastic phenotype and broad activity of this cell type (Graeber, 2010). However, our understanding of the role of microglia is now evolving. In addition to their well-established housekeeping properties (Kettenmann et al., 2011; Tremblay et al., 2011), microglia are increasingly being

attributed the function of coordinators of the trafficking of other immune cells into the nervous system (Aloisi et al., 2001). The current concept of microglia is as dynamic sensors of brain trauma, disease, and degeneration (Kettenmann, 2007), yet there is a general agreement that they exhibit both a bright and a dark side in this role.

Microglia function in normal brain physiology is still poorly defined, but resident non-activated microglial cells act as poor APCs due to their constitutive low levels of MHC-I/II molecules. However, upon activation they rapidly express MHC-I/II proteins and quickly become efficient "non-professional" antigen presenters. The non-professional nature and CNS-localization of microglia result in a notably different antigen presentation mechanism than that generally established for DCs. Upon internalizing antigens, DCs exit the tissue in which they reside (typically thought to be restricted to the spleen, lymph nodes, the skin, and mucosal surfaces) and enter the draining lymph nodes where they stimulate naïve T cells (Ransohoff and Cardona, 2010). In contrast, microglia encounter T cells during inflammation when T cells cross the BBB thanks to specialized surface antigens and then directly bind to microglia in order to receive antigens. Once they have been presented with antigens, T cells fulfill a variety of effector functions including pro-inflammatory recruitment, formation of immunological memories, and secretion of cytotoxic molecules (Yang et al., 2010).

Microglial cells secrete EMVs, and proteomic studies have identified several microglial vesicle proteins that were already reported in EMVs from B cells and DCs (Potolicchio et al., 2005). Microglial EMVs (mirroring their parent cells) also express MHC class II molecules, the levels of which are up regulated in response to stimulation with interferon (IFN)-γ (Potolicchio et al., 2005). The release of EMVs from microglial cells is also enhanced in critical conditions where immune activation is required. Upon activation, microglia release both soluble pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6, and TNF-α, as well as shed membrane vesicles (Bianco et al., 2009). Activated microglial cells also rapidly release IL-1β by a non-classical pathway of secretion leading to vesicle shedding (MacKenzie et al., 2001; Bianco et al., 2005) (**Table 1**).

There is also evidence of increased number of microglia-derived EMVs secreted by microglial cells into the CSF circulation in rodents with CNS inflammation, thus suggesting that circulating EMVs can be considered as surrogate markers of local (compartmentalized) vs. systemic inflammation ultimately affecting the CNS (Antonucci et al., 2012).

EXTRACELLULAR VESICLES AND ENDOTHELIAL CELLS

Endothelial cells release small membrane vesicles, known as endothelial microparticles (EMPs), that have been regarded as useful indicators of the functional state of the diseased endothelium; they may also potentially play key roles in the disease pathogenesis (Chironi et al., 2009; Morel et al., 2011). EMPs are found in the circulation of healthy subjects but their numbers increase in various pathological conditions such as thrombotic or infectious diseases, suggesting that these vesicles can act as pro-inflammatory and pro-coagulant regulators (Rabelink et al., 2010; Morel et al., 2011). EMPs released from the injured endothelium after cerebrovascular diseases such as cerebral stroke are found associated with microcirculatory injury, capillary blocking, acute and chronic inflammatory processes, and disruption of the BBB. The level of circulating EMPs has also been correlated with stroke severity, brain lesion volume, and outcome (Simak et al., 2006; Jung et al., 2009).

During CNS inflammation, the increased permeability of the BBB largely results from interactions among activated monocytes and T cells with cerebral endothelial cells, which – coupled with lymphokine and chemokine production – leads to cell adhesion to the cerebrovascular endothelium and trans-endothelial migration across the BBB. Upon activation by inflammatory cytokines such as IFN-γ and TNF-α, endothelial cells secrete EMPs. This has been reported on MS studies in which a correlation between plasma $CD31^+$ EMP levels and clinical exacerbations, as well as brain magnetic resonance imaging (MRI) disease activity, has been reported (Minagar et al., 2001). Interestingly, the plasma levels of $CD31^+$ EMP decrease upon disease modifying drug therapy with IFN-β1a (Sheremata et al., 2006) and this decrease correlates well with decrease in the number and volume of contrast enhancing T1-weighed lesions in MRIs (Lowery-Nordberg et al., 2011).

The role of EMPs in the pathogenesis of MS has been further explored and functional interactions between EMP and leukocytes have been assessed *in vitro*. These studies revealed that EMPs are captured preferentially by monocytes, less so by neutrophils, and have little affinity for lymphocytes (Jy et al., 2004). Bound EMPs activated monocytes leading to an increased expression of CD11b and migration through the cerebral endothelial cell layer. In an *in vitro* model for trans-endothelial migration, EMP–monocyte complexes showed a higher rate of migration of monocytes through monolayers of human cerebral microvascular endothelial cells (ECs) vs. monocytes alone.

Endothelial microparticles may then contribute to MS pathophysiology, enhancing inflammation, and increasing trans-endothelial migration of monocytes by binding to and activating monocytes, likely through CD54 (Jy et al., 2004).

Circulating EMPs are also increased in patients with severe cerebral malaria (CM; Coltel et al., 2006; Combes et al., 2006, 2010), the major fatal complication of plasmodium infection, where they appear to have a pathogenic role. This is strongly supported by the finding that ABCA1 transporter deletion associated with an impaired EMP production confers a complete protection against CM (Combes et al., 2005).

Hergenreider et al. have recently investigated a putative role for EMPs in mediating the atheroprotective properties of the parent cell. The shear-responsive transcription factor Krüppel-like factor 2 (KLF2), was recently found to up-regulate the miR-143/145 cluster in KLF2-transduced (or simply shear-stress-stimulated) ECs. Given the role of miR-143/145 in controlling the phenotype of smooth muscle cells (SMCs), Hergenreider et al. have proposed a miR-143/145-based – EMV-mediated – communication between parent ECs and SMCs. Real-time PCR analysis of EMVs isolated from KLF2-activated ECs showed enrichment in miR-143/145. *In vitro* EC-to-SMC transfer of miR-143/145 via EMVs was evidenced in co-cultures by restoration of SMC miR-143/145 content to levels near those measured pre-knockdown and a concomitant knockdown of mRNAs known to be targeted by the miR-143/145 cluster (e.g., ELK1, KLF4, CAMK2d, and SSH2). *In vivo*, the delivery of isolated atheroprotective EC EMVs into

atherosclerosis-prone ($ApoE^{-/-}$) mice resulted in a reduction of aortic atherosclerotic lesion formation (Hergenreider et al., 2012) (**Table 1**).

EXTRACELLULAR VESICLES AND BRAIN TUMORS

The literature describes contradictory functions in immune responses of vesicles secreted by tumor cells *in vitro*: on one hand transferring antigens to DCs to allow cross-presentation (Wolfers et al., 2001), and subsequent stimulation of cytotoxic lymphocytes (Zitvogel et al., 1998; Gastpar et al., 2005); on the other inhibiting several players of the effector step of anti-tumor immune responses that include the inhibition of natural killer (NK) cell proliferation and cytolytic function (Liu et al., 2006; Clayton et al., 2007).

In this context the glioma model system (gliomas are classified according to the cells that give rise to the tumor, e.g., astrocytoma, oligodendroglioma, glioblastoma (GBM), and oligo/astrocytoma) has been extensively examined for vesicle release and function (Trams et al., 1981; Skog et al., 2008; Al-Nedawi et al., 2009; Graner et al., 2009; Guescini et al., 2010; Balaj et al., 2011; Graner, 2011; Svensson et al., 2011; van der Vos et al., 2011).

The original idea was that tumor-derived EMVs would behave like cancer vaccines because of the presence of tumor-specific antigens and heat shock proteins known to favor APC activation. Evidence of induction of anti-tumor immune responses *in vivo* (Graner et al., 2009) involving both specific $CD8^+$ cytotoxic T-lymphocyte (CTL) response against autologous tumor cells (Bu et al., 2011) and an effective antibody production (Graner et al., 2009) have been reported.

A significant proportion of human gliomas express a truncated, constitutively active mutant of the epidermal growth factor receptor (EGFR) known as EGFR variant III (EGFRvIII). EGFRvIII expression is specific to some tumors and defines clinically distinct glioblastoma subtypes (Pelloski et al., 2007). Thus, a patient's EGFRvIII status (positive or negative) determined by analysis of EMVs carried in the serum can be diagnostic of their tumor type. EGFRvIII mRNA is found in EMVs from many patients tested, but in none of the controls. This finding might have diagnostic implications for EMV as a biomarker (Skog et al., 2008).

Recently it has been demonstrated that EMVs shed by glioma cells promote oncogenic transformation of neighboring cells thought the transfer of the above described EGFRvIII (Al-Nedawi et al., 2008) as well as the protein cross-linking enzyme tissue transglutaminase (tTG) that conferred certain characteristics of cancer cells (e.g., anchorage-independent growth and enhanced survival capability) to non-transformed fibroblasts and epithelial cells (Antonyak et al., 2011).

Much of the current knowledge regarding the immunology of tumor-derived EMVs is dominated by the opinion that such vesicles mediate immune suppression by increasing the activity of regulatory T cells (Treg) and myeloid-derived suppressor cells, suppressing activated T cells and NK cells, and by inhibiting DC maturation (Iero et al., 2008). In the same population of vesicles therefore seems to be a range of immune-stimulatory and potentially immune-suppressive functions. To date, these findings have been robustly demonstrated for EMVs released by tumor types other than brain tumors, but initial evidence of immune suppression has also been reported for GBM, thus correlating with the abnormal cellular immune response observed in patients.

In particular, peripheral blood mononuclear cells from healthy donors exposed *in vitro* to GBM exosomes displayed a suppressed phenotype with higher CD14 expression and lower HLA-DR (vs. non-exposed cells; de Vrij et al., 2011). Moreover, exosomes isolated from U87 and U138 human glioblastoma cell lines significantly inhibited the proliferation of T cells *in vitro* an effect likely resulting from FasL expression by tumor cells inducing apoptosis of activated T cells (de Vrij et al., 2011; Sabin et al., 2011).

The stem cell theory of carcinogenesis (Trosko and Chang, 1989) might suggest that a common cell signaling system operates in normal and malignant neural stem cells (Gilbertson and Rich, 2007). It has been in fact suggested that intrinsic brain tumors originate from a population of neural stem/precursor cells (NPCs) within prototypical germinal niches of the post-natal brain, including the sub-ventricular zone of the lateral ventricles (Hemmati et al., 2003; Singh et al., 2004; Uchida et al., 2004; Vescovi et al., 2006). NPCs, as self-renewing precursors capable of producing progeny along neuronal or glial lineages, commonly possess features associated with CNS tumors, including a robust proliferative potential and a diversified progeny (Nguyen et al., 2012). Cancer stem cells also express the stem cell marker prominin/CD133 (Nguyen et al., 2012). Brain tumor cells are described as "abnormal deranged cells" of the CNS and may recapitulate many features, albeit exaggerated, of normal cells, thus in turn helping in the understanding of normal cell biology and cell differentiation (Sanai et al., 2005; Jacques et al., 2010) (**Table 1**).

It is therefore plausible that the similarities between normal stem cells and cancer stem cells could extend to the unique relationship that stem cells have with their immediate microenvironments.

EXTRACELLULAR VESICLES AND STEM CELLS

Stem cells are the leading candidates as a source for transplantation in patients with neurological diseases. A variety of stem cells – including hematopoietic stem cells, MSCs and NPCs – display the potential to promote immune regulation, thus giving rise to the speculation that these properties are likely due to a common functional signature that in turn widens the possible source for cell therapy (Uccelli et al., 2008; Martino et al., 2011). Many studies focusing at the understanding of the possible crosstalk between NPCs and immune cells have been conducted, based on the observation that mouse and human NPCs share the expression of an array of functional immune-like receptors (e.g., cell adhesion molecules and pro-inflammatory chemokine receptors; Butovsky et al., 2006). The immune regulatory actions of transplanted NPCs have been described in different experimental models of neurological diseases, such as acute and chronic experimental autoimmune encephalomyelitis (EAE), spinal cord injury (SCI), stroke, and neurometabolic diseases. In all these models transplanted cells improved the clinical outcome mostly by immune modulation, neurotrophic, and neuroprotective effects, rather than substantial replacement of endogenous cells.

This has been generally attributed to an *in vivo* NPC capability to modulate the infiltration of blood-borne encephalitogenic T cells at the level of the CNS, through (i) the down-regulation

of intercellular adhesion molecule (ICAM)-1 and leukocyte function associated (LFA)-1, two cell adhesion molecules involved in T cell migration, at the level of the BBB; (ii) the induction of T cell-specific apoptosis or; (iii) the increase of the number of regulatory T cells (Einstein et al., 2003; Pluchino et al., 2005). The very same findings are consistent with *in vitro* observations documenting the capacity of NPCs to inhibit both non-antigen-specific as well as antigen-specific T cell activation and proliferation (Einstein et al., 2003; Fainstein et al., 2008), and apoptosis induction via a FasL-dependent mechanism (Knight et al., 2010). The suppression of T cell proliferation has been attributed at least in part to the production of soluble mediators, such as nitric oxide (NO) and prostaglandin E2 (PGE2; Wang et al., 2009). An enhanced efficacy has been obtained with genetically engineered NPCs expressing IL-10, an anti-inflammatory cytokine that efficiently suppresses EAE (Croxford et al., 2001), as compared to control (non-engineered) NPCs (Yang et al., 2009). Similar results to those achieved in rodent EAE have been obtained in non-human primates with human NPCs (Kim et al., 2009a; Pluchino et al., 2009a). Further work on rodent models of stroke documented that, thanks to their reactivity to CCL2/CCR2 and CXCL12/CXCR4 axes, NPCs migrate in the perilesional area and persist there in an undifferentiated phenotype (Imitola et al., 2004; Darsalia et al., 2007; Bacigaluppi et al., 2009; Sun et al., 2010; Andres et al., 2011). Transplanted NPCs, engaged in a complex interplay with the inflammatory environment, are able to reduce the number of infiltrating cells (neutrophils) in the brain as well as the numbers of activated macrophages in lymphoid organs (Lee et al., 2008). NPCs also vary the bioavailability of immune mediators (Kilic et al., 2008), i.e., increasing gene expression levels of vascular-endothelial growth factor (VEGF) and down-regulating in the ischemic region multiple RNA species involved in inflammation, including IFN-γ, TNF-α, IL-1β, IL-6, and leptin receptor (Bacigaluppi et al., 2009). In models of stroke, NPCs demonstrated the ability to modify the ischemic environment via induction of neurotrophic factors [such as stromal-derived factor (SDF)-1/CXCL12, insulin-like growth factor (IGF)-1, VEGF, transforming growth factor (TGF)-β, and brain-derived growth factor (BDNF)] and activation of selected aspects of the inflammatory response, particularly $CD11b^+$ microglia/macrophages (Capone et al., 2007).

Several studies have also highlighted the crucial role of the interaction between transplanted NPCs and microglia/macrophages, although with controversial results. On one side there is the idea that microglia activation might be required for transplanted NPCs to exert their neuroprotective action, given the indirect evidence of its increased number after NPC-transplantation in models of stroke (Capone et al., 2007; Daadi et al., 2010). This is also more directly suggested by the increased ischemic volume in mice affected by experimental middle cerebral artery occlusion (MCAo) after selective ablation of $CD11b^+$ microglia in CD11b-thymidine kinase mutant-30 mice (Lalancette-Hebert et al., 2007). On the other hand, significant reduction of microglia/macrophages is observed after the intravenous NPC-transplantation in wild type MCAo mice that leads to increased neuronal survival and recovery of locomotor functions (Lee et al., 2008; Bacigaluppi et al., 2009).

In experimental SCI, NPCs injected into the CSF synergize with myelin-specific T cells used as a vaccination therapy that stimulated transplanted NPCs to specifically migrate to the site of injury, while also instructing the local macrophages/microglial cells toward a tissue-protective phenotype (Ziv et al., 2006). Recently, NPCs that were implanted focally at the level of the severely contused mouse spinal cord, survived at the boundaries of the injured spinal cord, always in very close contiguity with blood vessels, while retaining undifferentiated morphology and ultrastructure and intimately interacting with phagocytic cells and astrocytes via cellular junctional coupling. This was associated to increased levels of inflammatory mRNAs and significant reduction of the proportion of "classically activated" (M1) infiltrating macrophages and, in turn, remarkable promotion of the healing of the injured cord (Cusimano et al., 2012).

Immune regulation mediated by transplanted NPCs may take place in the CNS (Pluchino et al., 2005), as well as in secondary lymphoid organs such as the lymph nodes or the spleen (Einstein et al., 2007; Pluchino et al., 2009b). Einstein et al. have shown that in the production of pro-inflammatory cytokines in response to myelin oligodendrocyte glycoprotein (MOG) 33–35 peptide EAE-derived lymph node cells were strongly inhibited by NPCs. Furthermore, primed T cells from mice treated with NPCs were also deficient in their ability to adoptively transfer EAE to a naïve host (Einstein et al., 2007). We have shown striking peripheral (i.e., at the level of the secondary lymphoid organs) accumulation, survival, and long-term persistence of NPCs injected sub-cutaneously into mice with EAE. In this experimental context, NPCs showed negligible propensity to accumulate into the brain, but rather were consistently capable of modifying the perivascular lymph node microenvironment by hindering the activation of myeloid DCs via a bone morphogenetic protein (BMP)-4 dependent mechanism, which in turn limited the expansion of antigen-specific encephalitogenic T cells at the sites of antigen presentation (Pluchino et al., 2009b). The survival of NPCs outside the CNS was likely promoted by the *in situ* increased levels of major stem cell-fate determinants, including the BMP-4 and -7, sonic hedgehog (Shh), and the BMP antagonist Noggin, which were released both by transplanted NPCs and immune cells (Pluchino et al., 2009b). Also human NPCs have been shown to interfere with a number of major DC functions, such as the differentiation of myeloid precursor cells (MPCs) into immature DCs (iDCs), and the maturation of iDCs into functional (antigen presenting) mature DCs. (Pluchino et al., 2009a).

The general consensus from these and other studies is now that transplanted non-hematopoietic stem cells promote remarkable clinical and pathological amelioration from inflammatory-driven CNS damage and that this is due to mechanisms alternative to the initially expected cell replacement (Martino and Pluchino, 2006).

It is therefore possible to speculate that in addition to paracrine and endocrine factors that transplanted stem cells will undoubtedly release at the level of the extracellular space, EMVs are also likely to play a role in the mediation of some of the parental cell's functions in shaping the host microenvironment.

One of the first reports on stem cell-derived EMVs identified two classes of membrane particles (named P2 and P4 by the authors), with diameters of 600 and 50–80 nm, respectively, which carry the stem cell marker prominin-1/CD133, a pentaspan

membrane protein found on the membrane protrusions of the apical surface of neuroepithelial cells, in the lumen of the neural tube in the developing embryonic mouse brain. The P2 and P4 classes of particles were observed in the ventricular fluid during the onset and early stages of neurogenesis, respectively, and their presence correlated with a change in the nature of the neuroepithelial membrane protrusions. It has been hypothesized that these particles may exert a signaling role or they may be a mean of discharging membrane microdomains that endow these cells with stem/progenitor cell properties contributing to their differentiation (Marzesco et al., 2005).

Embryonic stem (ES) cells have also been shown to be a rich source of EMVs. Mouse and human ES cell–EMVs traffic various stem cell-specific molecules that regulate self-renewal of pluripotent cells in embryoid bodies and may affect the growth of recipient cells, e.g., contributing to cell-fate decision.

Embryonic stem cell–EMVs are in fact highly enriched in Wnt-3 protein and mRNAs for transcription factors such as Oct-4, Nanog, and Rex-1, which are markers of pluripotency that are typically implicated in self-renewal. Furthermore, they are capable of reprogramming hematopoietic progenitors cells (HPCs) not only by stimulating them with surface-expressed ligands but also by delivering ES-derived Oct-4 mRNA which is subsequently translated into Oct-4 protein within the recipient cell (Ratajczak et al., 2006).

Recently, ES cell–EMVs have been demonstrated to be capable of transferring a subset of miRs to mouse embryonic fibroblasts (MEFs); the most efficiently transferred miRs (as determined by real-time quantitative RT-PCR analysis of the recipient cells) were those abundant in the parent ES cells but relatively deficient in recipient fibroblasts (i.e., miR-290, miR-291-3p, miR-292-3p, miR-294, and miR-295), suggesting a tightly regulated transfer process (Yuan et al., 2009).

It has been also recently proposed that the interaction of stem cells with the microenvironment has a critical role in defining stem cell phenotype. This concept acquires relevance especially in the context of tissue/cellular injury, as the continuous genetic modulation through EMV transfer between neighboring cells can be a key determinant of stem cell phenotype variation (Quesenberry and Aliotta, 2008; Aliotta et al., 2010). This hypothesis was first proposed for marrow cells and their capacity to assume the phenotype of other hematopoietic cells or non-hematopoietic cells (conversion). The "continuum model" of stem cell regulation states that the potential of marrow stem cells continually changes with cell cycle transit and that marrow stem cell are indeed cycling cells (Quesenberry and Aliotta, 2008; Aliotta et al., 2010). Studies with mouse lung-derived EMVs and mouse bone marrow cells have shown that the capacity to take up EMVs varies with cycle phase. Thus, phenotype modulation at the stem cell level involves both cell cycle and EMV phenotype change (Quesenberry and Aliotta, 2008).

Extracellular membrane vesicles derived from human liver stem cells (HLSC) induce proliferation and apoptosis resistance in cultured human hepatocytes and favor liver regeneration in hepatectomized rats through the transfer of a defined pattern of mRNAs associated with cell functions related to the control of transcription (e.g., DMRT2, HOXC12, NFIX, and HOXA3), translation (AGO2), and proliferation (e.g., MATK, MRE11A, CHECK2, and CDK2; Herrera et al., 2010).

Interestingly, the pattern of genes present in HLSC-derived EMVs is substantially different from that of EPCs and MSCs (Deregibus et al., 2007; Bruno et al., 2009) indicating a parental cell-specific signature.

Exosomes from human ES cell-derived MSCs have been recently shown to reduce infarct size in a mouse model of myocardial ischemia/reperfusion injury and in this setting exosomes have been identified as the cardioprotective component in the MSC paracrine secretion (Lai et al., 2010). These very same MSC-derived exosomes contained the hsa-let-7b and hsa-let-7g predominantly in the precursor form (Chen et al., 2010).

Systemically injected EMVs from human bone marrow-derived MSCs have been shown to accelerate kidney repair in a mouse model of acute kidney injury (AKI) by inhibiting apoptosis and stimulating tubular epithelial cell proliferation. EMVs also significantly reduced the impairment of renal function. Pretreatment of EMVs with RNase to inactivate their RNA cargo abrogated these protective effects. Moreover, EMVs capable of reducing the acute injury also protected from later chronic kidney disease (Gatti et al., 2011).

All these studies suggest the existence of a bidirectional exchange of genetic information between stem and neighboring cells, or reciprocally from injured cells to bone marrow-derived or resident stem cells that in turn lead to tissue repair (Camussi et al., 2010). In this context, embryonic and adult stem cell-derived EMVs shuttle defined patterns of mRNAs and miRs that are internalized by a receptor-mediated mechanism in target cells, and may induce de-differentiation of cells surviving injury with cell cycle re-entry and tissue self-repair; conversely, it might be envisaged that transcripts delivered by EMVs from injured cells may reprogram the phenotype of stem cells to acquire specific features of the inflamed/damaged microenvironment.

The first tentative evidence of immune modulation by NPC-derived exosomes emerged from experiments in which the culture supernatant of hNPC (HB1.F3) suppressed the activation and proliferation of human T cells by apoptosis and cell cycle arrest. Exosomes isolated from hNPCs and added to the supernatant of cultured T cells resulted in a similar suppression by G0/G1 cell cycle arrest. This reinforces the possibility that (at least part of) the immune modulatory effects of hNPCs might be mediated by secreted EMVs/exosomes (Kim et al., 2009b).

The hypothesis of EMV secretion by NPCs introduces a completely different dimension to the therapeutic applications of NPCs in regenerative medicine. By replacing transplantation of NPCs with administration of their secreted products (including EMVs), many of the limitations and safety concerns associated with the transplantation of viable replicating cells, such as tumors arising from transplanted NPCs, could be mitigated (Amariglio et al., 2009).

As naturally occurring "nanoparticles," EMVs may benefit from the expression of specific membrane molecules that might confer them a potential mechanism for the homing to a specific tissue or microenvironment.

The future challenge is the discovery of the molecules (i.e., proteins, mRNAs, or miRs) that might recapitulate the therapeutic

efficacy of transplanted NPCs, the engineering or modification of the exosome surface antigen and internal content, and their *in vivo* delivery to the target site.

Very recent studies report on the use of exosomes as a fast and selective brain-targeted delivery system of therapeutic molecules able to overcome the major hurdle imposed by the BBB. Systemically injected immature DC-derived exosomes, engineered to express Lamp2b fused to the CNS-specific rabies viral glycoprotein (RVG) peptide, have been shown to deliver GAPDH short interfering RNA (siRNA) to the mouse brain (Alvarez-Erviti et al., 2011). The siRNA was efficiently delivered to neurons, microglia, oligodendrocytes, and oligodendrocyte precursors. The same report details similar achievements with siRNA that interfere with the enzyme β-secretase 1 (BACE-1), a foremost target for the treatment of AD, where the amyloid-β (Aβ) peptide is believed to play a key role in the pathogenesis of AD (Alvarez-Erviti et al., 2011).

Zhuang et al. have recently explored the use of T cell-derived exosomes to deliver anti-inflammatory drugs to the mouse brain through a non-invasive intranasal route. The authors challenged three different models of brain inflammation that included lipopolysaccharide (LPS)-induced inflammation, MOG-induced EAE and the orthotopic glioblastoma (GL-26) model. Intranasally administered exosome-encapsulated anti-inflammatory drugs (curcumin or the signal transducer and activator of transcription 3 (Stat3) inhibitor JSI124) were selectively taken up by microglia, both "resting" and "activated." The administration of exo-curcumin led to a significant reduction in the number of microglial cells, while exo-JSI124 resulted in the enhancement of tumor apoptosis and a concomitant reduction in disease progression in all the tested models. The immune reaction toward the parental cell exosomal antigens, in terms of immune tolerance or immune responsiveness, needs further evaluation. The authors speculate that exosomes taken up by naïve microglial cells may lead to the induction of an immune tolerance to antigens released from the cells producing the exosomes, whereas the exosomes taken up by activated microglial cells may lead to activation of immune cells (Zhuang et al., 2011) (**Table 1**).

The feasibility of scaling up production and purification of clinical grade exosomes using a Good Laboratory Practice process using DC-derived exosomes has been partially addressed (Escudier et al., 2005), and various clinical trials are about to start (Chaput and Thery, 2011).

CONCLUSION

Depending on their origin, EMVs are able to either stimulate or repress functions of the immune system and drive regenerative processes. Vesicles secreted by stem cell sources other than NPCs have begun to prove that vesicles are endowed with immune modulatory properties that might make them promising agents to be exploited for therapeutic purposes.

The future challenge for exosomal research is to continue looking into innate (physiological) mechanisms with the focus of translating the knowledge of basal (vs. reactive) cell functions into innovative highly clinical impact therapeutics.

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Microglial microvesicle secretion and intercellular signaling

Elena Turola[1,2], Roberto Furlan[3], Fabio Bianco[1,2], Michela Matteoli[1,2,4] and Claudia Verderio[1,2]*

[1] *CNR Institute of Neuroscience, Milano, Italy*
[2] *Department of Medical Pharmacology, Università di Milano, Milano, Italy*
[3] *INSPE, Division of Neuroscience, Fondazione San Raffaele del Monte Tabor, Milano, Italy*
[4] *Istituto Clinico IRCCS Humanitas, Milan, Italy*

Edited by:
Mauro Maccarrone, University of Teramo, Italy

Reviewed by:
Giuseppe Astarita, University California Irvine, USA
Maria Marino, University Rma Tre, Italy

****Correspondence:***
Claudia Verderio, CNR Institute of Neuroscience, Via Vanvitelli 32, 20129 Milano, Italy.
e-mail: c.verderio@in.cnr.it

Microvesicles (MVs) are released from almost all cell brain types into the microenvironment and are emerging as a novel way of cell-to-cell communication. This review focuses on MVs discharged by microglial cells, the brain resident myeloid cells, which comprise ∼10–12% of brain population. We summarize first evidence indicating that MV shedding is a process activated by the ATP receptor $P2X_7$ and that shed MVs represent a secretory pathway for the inflammatory cytokine IL-β. We then discuss subsequent findings which clarify how IL-1 β can be locally processed and released from MVs into the extracellular environment. In addition, we describe the current understanding about the mechanism of $P2X_7$-dependent MV formation and membrane abscission, which, by involving sphingomyelinase activity and ceramide formation, may share similarities with exosome biogenesis. Finally we report our recent results which show that microglia-derived MVs can stimulate neuronal activity and participate to the propagation of inflammatory signals, and suggest new areas for future investigation.

Keywords: microvesicles, microglial cells, IL-beta, neuronal activity, brain inflammation

SUBCELLULAR ORIGIN AND COMPOSITION OF MVs SHED FROM THE CELL SURFACE

Microvesicles (MVs), also referred to as shed vesicles or ectosomes (Sadallah et al., 2011), are small (0.1–1 μm) vesicles which bud directly from the plasma membrane and are released into the extracellular environment upon cell activation. Shedding of MVs typically involves a budding process, in which surface blebs selectively accumulate cellular constituents that are packaged into MVs. MVs contain a variety of cell surface receptors, intracellular signaling proteins and genetic materials derived from the cell of origin. In terms of composition, MVs originating from distinct cell types are molecularly different from each other, reflecting the differential expression of proteins of various donor cells. Composition and biological activity of MVs also vary depending on the state (e.g., resting, stimulated) of donor cells and on the agent employed for stimulation (Bernimoulin et al., 2009). The mechanisms involved in MV budding and discharge are beginning to emerge and suggest the involvement of ESCRT and/or ARF6 (Cocucci et al., 2009; Muralidharan-Chari et al., 2009; Gan and Gould, 2011).

Besides MVs, most cells secrete into the environment a markedly distinct type of small extracellular vesicles by an alternative two-step process. This mechanism includes the bud of intraluminal vesicles at endosomes during multivesicular bodies (MVBs) maturation and subsequent vesicle secretion upon fusion of MVBs with the plasma membrane (Cocucci et al., 2009; Simons and Raposo, 2009). Small (40–80 nm) extracellular vesicles released by this process are called exosomes and represent a more homogeneous type of vesicles, enriched in specific components (tetraspanning proteins, CD63 and CD9, and alix).

BIOLOGICAL ACTIVITY AND MECHANISM OF MV INTERACTION WITH TARGET CELLS

Until a decade ago, MVs were considered as *in vitro* artifact, or alternatively regarded as a way of eliminating unwanted material from cells. MVs were also often confused with apoptotic bodies generating during cell death or with exosomes, discharged upon fusion of MVBs with the plasma membrane. Several reasons may explain why for many years MVs have been largely overlooked. First, discriminating between different types of extracellular vesicles is difficult and requires a combination of electron microscopy and biochemical techniques. In addition, while the presence of markers of endosomal origin (alix, Tg110, CD63, and CD9) is an accepted criterion to identify exosomes, no universal markers have been identified yet for MVs discharged outside the cells from the cell surface, which are highly heterogeneous in composition. Also, studies of MVs have been constrained by the limitations of current methodology employed for their isolation and quantification. MVs are usually isolated from culture medium or body fluids by differential centrifugation or affinity capture. However, immunosorbent or bead capture assays do not allow isolating all the vesicles present in the samples and differences in centrifuge speeds used to eliminate whole cells may discard materials which in different laboratories are measured as MVs (Horstman et al., 2007). Furthermore the most widely used

Abbreviations: A-SMase, acid sphingomyelinase; CNS, central nervous system; ESCRT, endosomal sorting complex required for transport; MVs, microvesicles; MVBs, multi vesicular bodies; PS, phosphatidylserine; SM, sphingomyelin.

methods to quantify MVs, i.e., flow cytometry and dynamic light scattering, are biased toward the detection or larger MVs (Dragovic et al., 2011). Although interesting advances in MV quantification and isolation have been recently achieved with introduction of new methodology such as a nanoparticle tracking or micro- and nano fluidics (Chen et al., 2010), these new technologies for sizing and quantifying MVs still need to be standardized in order to provide reliable and reproducible methods. Despite these technical limitations, now-a-days MVs attract great interest as their shedding is recognized as a widespread mode of intercellular communication in different body compartments. Indeed shed MVs, similarly to exosomes, may serve as information packets to guide the phenotype of surrounding cells by transferring lipids, proteins, and genetic material from donor to target cells (Thery et al., 2009). Furthermore shed MVs, being enriched in various bioactive molecules, play pleiotropic roles in many physiological processes, including development (Liegeois et al., 2006; Kolotuev et al., 2009), coagulation, and immune reaction (Thery et al., 2009), as well as in diseases, such as cancer progression (Yu et al., 2006, 2009; Keller et al., 2009; Gan and Gould, 2011), viral infection (Dukers et al., 2000; Gould et al., 2003; Fang et al., 2007; Logozzi et al., 2009; Nazarenko et al., 2010), and amyloidopathies (Fevrier et al., 2004; Leblanc et al., 2006; Alais et al., 2008). Released MVs may remain in the extracellular space in close proximity to the place of origin or move by diffusion and enter biological fluids, such as blood, urine, and synovial fluid, where they are emerging as clinically valuable markers of disease states (Doeuvre et al., 2009). MVs have the same topology as the cell of origin but loose membrane asymmetry and are characterized by the presence of the phospholipid phosphatidylserine (PS) externalized at their surface (Zwaal and Schroit, 1997; Sims and Wiedmer, 2001). PS exposed on shed MVs represents a determinant for recognition on recipient cells, through binding to the corresponding cellular PS receptors (Al-Nedawi et al., 2009). The interaction of MVs with recipient cells can be followed by fusion or endocytosis. Alternatively, MVs can undergo rupture and release their luminal active components, thus modulating, by protein secretion, the activity of target cells.

BLEBBING AND MV FORMATION INDUCED BY $P2X_7$ RECEPTOR ACTIVATION

A specialized type of MV release exists for cells that express the ATP receptor $P2X_7$, and which shed MVs from the cell surface when this receptor is activated by ATP. $P2X_7$ receptor is an ATP-gated ion channel highly expressed in immune cells, particularly macrophages (Steinberg et al., 1987) mast cells (Cockcroft and Gomperts, 1979), and microglia (Visentin and Levi, 1997) where it controls the release of inflammatory cytokines, such as IL-1β and IL-18 (Ferrari et al., 2006). Activation of $P2X_7$ receptor induces efficient assembly of inflammosome, the protein complex which activates the IL-1β processing enzyme caspase-1. This process is followed by rapid cytokine secretion (Qu et al., 2007). $P2X_7$ receptor differs from other members of the P2X family in its relatively low affinity for ATP and the presence of a long cytoplasmic C-terminus that contains several protein–protein interaction motifs. Depending on the ATP concentration and time of exposure, $P2X_7$ receptor functions as either an ion channel or a non-selective pore, the latter generally leading to cytotoxicity and apoptotic cell death. Many studies have shown that dramatic morphological changes occur in cells endogenously or heterologously expressing $P2X_7$ receptors during and subsequent to receptor activation (Hogquist et al., 1991; Ferrari et al., 1997). These changes consist in rapid formation of cell membrane blebs and are associated to cell death upon sustained $P2X_7$ receptor activation. Membrane blebbing results from several intracellular signaling events, which are induced by occupancy of the receptor, such as the activation of protein kinases and other effector enzymes (Duan and Neary, 2006). In particular, several lines of evidence indicate that $P2X_7$ – induced blebbing is dependent upon P38 and requires ROCK activation, which causes local disassembly of the cytoskeletal elements, associated to the $P2X_7$ C-terminus (Budagian et al., 2003; Morelli et al., 2003; Verhoef et al., 2003). Notably, surface blebbing is preceded by loss of plasma membrane asymmetry and exposure of phosphatidylserine (PS) at the outer leaflet of the plasma membrane, a process controlled by specific enzymes, named flippase, floppase, and lipid scramblase, which control PS segregation in the inner leaflet of the plasma membrane (Hugel et al., 2005). Externalized PS is a commonly accepted marker for cell apoptosis. However, a pioneer study by Surprenant and colleagues (MacKenzie et al., 2001) dissociated the $P2X_7$-induced bleb formation from cell apoptosis, by showing in monocytes that $P2X_7$-induced PS externalization and bleb formation occur within the first few minutes of receptor activation and is reversible after brief stimulation. MacKenzye and colleagues also showed that, during blebbing, MVs with externalized PS can be formed and released into the extracellular space as a result of bleb detachment from the cell surface. Notably the pro-inflammatory cytokine IL-1β is packaged into plasma membrane blebs, which are subsequently shed, as MVs, into the extracellular space from reactive monocytes (**Figure 1A**). Almost ten years ago these results provided the first evidence that $P2X_7$-induced MV shedding acts as a secretory pathway for rapid release of IL-1β and may represent a general mechanism for secretion of leaderless secretory proteins from $P2X_7$-expressing myeloid cells.

EMERGING ROLE OF MVs DERIVED FROM BRAIN CELLS: $P2X_7$-DEPENDENT MV SHEDDING AND IL-1β RELEASE IN MICROGLIA

In the recent years, a series of studies has indicated relevant physiological and pathological functions for extracellular vesicles within the brain. These functions include fundamental processes occurring in brain, such as axonal growth and regeneration, axon-glia communication, inter-neuronal transfer of information across synapses, modulation of neuro-immune interactions, as well as disease-associated events, including tumor progression, and spreading of pathogenic agents or misfolded proteins. The majority of these studies focused, however, on exosomes rather than MVs shed from the cell surface of brain cells (**Table 1**). Indeed only one report indicates the existence of MVs of neuronal origin (Schiera et al., 2007) and there is no evidence for shedding of MVs from oligodendrocytes. Nevertheless, a mixed population of MVs and exosomes has been detected *in vivo* in the cerebrospinal fluid isolated from sheep (Vella et al., 2008), and our recent evidence indicate that a fraction of large MVs (mean size = 420 nm) pelleted from rat CSF by differential centrifugation

displays neuronal or oligodendroglial markers (Verderio et al., 2012), suggesting that even neurons, and oligodendrocytes produce MVs *in vivo*. Larger interest has been raised so far by MVs shed from the cell surface of microglia, the immune cells of the nervous system, which play key role in inflammatory and degenerative brain pathologies. Microglial cells are brain resident myeloid cells, which migrate into the CNS during early embryogenesis and comprise ∼10–12% of total brain population (Ransohoff and Cardona, 2010). Although they are traditionally distinguished from infiltrating peripheral macrophages, which can migrate to the brain from blood upon CNS damage or inflammation and do not differentiate into microglia, in the injured CNS, activated microglia and infiltrating macrophages cannot be distinguished by their morphology or by specific antigenic markers.

As immune cells, the primary function of microglia is to maintain brain tissue homeostasis, to provide the first line of defense during infection or brain injury and to promote tissue repair. In normal brain "surveillant" microglia display a ramified morphology, characterized by long and thin processes, which communicate with surrounding neurons and other glial cells and continuously scan the microenvironment to exert a guard function for incoming pathogens and brain alterations. In response to many types of alarm signals (cytokines, material from apoptotic cells, and exogenous viral factors) "surveillant" microglia undergo several levels of activation and migrate to the site of infection or injury to eliminate pathogens or to phagocyte dead cells and protein aggregates. Depending upon the nature and duration of environmental signals, microglia can undergo a "classical" pro-inflammatory activation, transforming into fully activated inflammatory effector cells (cytokines-secreting cells), or an "alternative" activation, generally associated with tuning of inflammatory response, protection from disease, and tissue repair. These two extremes along multiple states of microglia activation are commonly indicated as M1 (pro-inflammatory) and M2 (pro-regenerative) phenotypes, in analogy to the distinction originally made between M1 and M2 macrophages (Mantovani and Locati, 2009; David and Kroner, 2011; Saijo and Glass, 2011). Experimentally, these states are commonly achieved by treating cells *in vitro* with polarizing agents, such as anti inflammatory cytokines (Saijo and Glass, 2011).

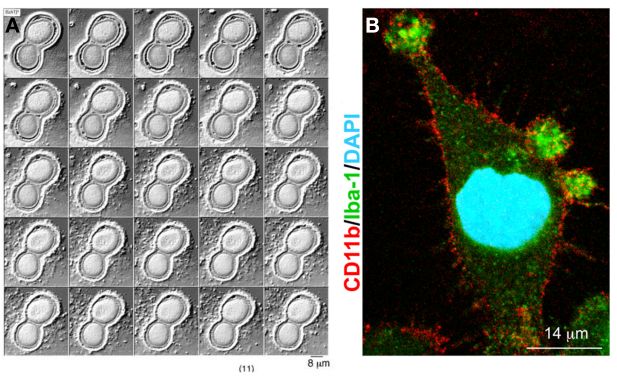

FIGURE 1 | P2X$_7$ receptor-induced MV shedding from monocytes and microglial cells. (A) Fluorescent images of two THP-1 monocytes labeled with NBD membrane, NBD-labeled particle shedding, and membrane bleb during exposure to BzATP. From MacKenzie et al. (2001). **(B)** Fluorescent image of a cultured microglial cell exposed for 48 h to a cocktail of inflammatory cytokines, stained for Iba-1 (green), CD11b (red), and DAPI (blue). Note the presence of many blebs at the cell surface double positive for Iba-1 and Cd11b. From Verderio et al. (2012).

A few years ago we reported that a MV-mediated mechanism for IL-1β release occurs in microglial cells (Bianco et al., 2005), very similar to that first described in monocytes (MacKenzie et al., 2001). By video microscopy experiments, we showed that cultured microglia form membrane blebs (**Figure 1B**) and shed MVs from the cell surface upon P2X$_7$ receptor activation. Isolation of MVs produced from reactive microglia, followed by IL-1β evaluation by ELISA or western blotting revealed that MVs produced by LPS-treated microglia store and subsequently release IL-1β into the environment in an ATP- and P2X$_7$-dependent manner. IL-1β efflux from shed MVs is enhanced by ATP stimulation and inhibited by pretreatment with the P2X$_7$ receptor antagonist oxidized ATP, thus indicating a crucial involvement of the pore-forming P2X$_7$ receptor in the release of the cytokine. Notably, we found that shedding of MVs from microglial cells is not only promoted

Table 1 | Microvesicles of different brain cell origin.

Cell of origin	Exosomes	Ectosomes/shed MVs	Mixed population
Astrocytes	Taylor et al. (2007), Bianco et al. (2009), Guescini et al. (2010), Sbai et al. (2010), Wang et al. (2011)	Bianco et al. (2005)	Proia et al. (2008), Ceruti et al. (2011), Verderio et al., 2012; *in vivo*)
Microglia	Potolicchio et al. (2005), Bianco et al. (2009), Tamboli et al. (2010)	Bianco et al. (2005, 2009), Chahed et al. (2010), Tamboli et al. (2010), Antonucci et al. (2012), Verderio et al. (2012)	Verderio et al., 2012; *in vivo*)
Oligodendrocytes	Kramer-Albers et al. (2007), Trajkovic et al. (2008), Strauss et al. (2010), Fitzner et al. (2011), Bakhti et al. (2011)		Scolding et al., 1989; *in vivo*), Verderio et al., 2012; *in vivo*)
Neurons	Faure et al. (2006), Korkut et al. (2009), Lachenal et al. (2011), Ghidoni et al. (2011), Yuyama et al. (2012)		Schiera et al. (2007), Verderio et al., 2012; (*in vivo*)

by $P2X_7$ receptor activation through exogenous ATP, but also by ATP endogenously released from healthy astrocytes in astrocyte-microglia co-cultures. Although ATP is typically considered a danger signal, this observation represented a first indication that MVs can be released from microglia even in the absence of cellular damage.

HOW DOES IL-1β GET THROUGH THE MEMBRANE OF MVs?

The MV-mediated mechanism for IL-1β release originally proposed by Surprenant's laboratory left a question unsolved: how does mature IL-1β get through the membrane of MVs and reach the extracellular space? Later observations from our laboratory (Bianco et al., 2005) and from Di Virgilio's group (Ferrari et al., 2006; Pizzirani et al., 2007) provided a possible answer: MVs shed from microglia and dendritic cells, bear $P2X_7$ receptors in their membranes and are loaded with caspase-1. This enzyme becomes activated upon $P2X_7$ receptor stimulation and is responsible for intravesicular processing of the biological inactive precursor of IL-1β (pro-IL-1β) into the active form of the cytokine. Recent evidence obtained in macrophages confirmed these findings by showing that extracellular vesicles, both exosomes and shed MVs, carry components of the inflammosome, in addition to the IL-1β converting enzyme (Qu et al., 2009; Sarkar et al., 2009).

Notably, activation of $P2X_7$ receptors, followed by opening of large pores and MV lysis may represent the mechanism by which IL-1β gets through membrane of MVs. $P2X_7$-dependent disruption of MVs was indeed reported to mediate the efflux of IL-1β from dendritic cells (Ferrari et al., 2006). However, differently from what described in dendritic cells, we found that IL-1β release from microglia is not the consequence of MV lysis. This was indicated by the observation that IL-1β release is not paralleled by GFP efflux from GFP-labeled MVs, produced by N9 microglial cells (Balcaitis et al., 2005), which stably express the fluorescent protein (Bianco et al., 2005). GFP is a 40-kDa cytosolic protein, that can be released extracellularly upon MV disruption but not through the $P2X_7$ pore, which is permeable to molecules up to 1 kDa.

Although further studies are necessary to better define how IL-1β gets through membrane of MVs, it can be hypothesized that, once microglia-derived MVs approach the plasma membrane of target cells, where the ATP concentration is higher than in the bulk solution, the $P2X_7$ receptor is activated, IL-1β is processed by caspase-1, and released from MVs.

BIOGENESIS OF MVs INDUCED BY $P2X_7$ RECEPTOR ACTIVATION

Microvesicles emanate from viable cells through the outward blebbing of their plasma membrane. Budding of MVs shares many features with budding of viral particles and intraluminal vesicle budding inside endosomes, during MVBs biogenesis. The latter process occurs through the invagination of small intraluminal vesicles of about 50 nm in diameter which then pinch off from the endosomal membrane and are released extracellularly as exosomes, upon fusion of MVBs with the plasma membrane. Exosome formation, as well as the egress of a few enveloped viruses, is generally dependent on the ESCRT (endosomal sorting complex required for transport) machinery, which regulates membrane scission. Recent studies indicated that fission of exosome membrane is catalyzed, in particular, by components of the ESCRT-III complex, called charged multivesicular body proteins (CHMPs; Hanson et al., 2009; Wollert et al., 2009; Wollert and Hurley, 2010) and by the AAA-ATPase vacuolar protein sorting-associated 4 (VPS4; Babst, 2005). Other pathways that promotes exosome biogenesis are emerging, which depend on lipids raft composition, the phospholipid LBPA (Matsuo et al., 2004), the sphingolipid ceramide, and activity of neutral sphingomyelinase (Trajkovic et al., 2008). However it is still unclear how these factors combine to promote exosome secretion (Gan and Gould, 2011). Trajkovic and coworker, for example, clearly showed that enrichment in ceramide is sufficient to trigger spontaneous vesiculation by an invagination mechanism which is independent of the ESCRT machinery, thus suggesting that distinct mechanisms may control the biogenesis of specific subsets of exosomes.

A few years ago we gained some insights into the molecular mechanism which mediates bleb formations and (exo)vesiculation upon activation of $P2X_7$ receptor in glial cells, both microglia and astrocytes (Bianco et al., 2009). It was known that $P2X_7$-dependent blebbing is preceded by alteration of the transbilayer lipid distribution and requires ROCK and P38 MAP kinase activation, similarly to apoptotic blebbing (Piccin et al., 2007; Al-Nedawi et al., 2009; Pap et al., 2009; Cocucci and Meldolesi, 2011). However an unsolved question was how signaling by $P2X_7$ receptor leads to alterations of the biophysical properties of the plasma membrane, which together with actin-cytoskeleton re-organization are a prerequisite for membrane blebbing and vesiculation at the surface of healthy cells.

We found that biogenesis of MVs storing IL-1β is controlled by acid sphingomyelinase (A-SMase), the enzyme which hydrolyzes sphingomyelin (SM) to the sphingolipid ceramide. Following $P2X_7$ receptor activation, a src-protein tyrosine kinase interacts with the C-terminus of the receptor (Denlinger et al., 2001) and promptly phosphorylates P38 MAP kinase. P38 phosphorylation, in turn, induces translocation of A-SMase to the plasma membrane outer leaflet, where it generates ceramide, thereby inducing budding of MVs (Bianco et al., 2009; **Figure 2**). Although A-SMase has been historically associated to lysosomes, our data are consistent with evidence indicating that the enzyme is activated rapidly upon stimulation of various receptors, and is recruited to the plasma membrane to mediate receptor-dependent signaling (Grassme et al., 2001; Gulbins and Kolesnick, 2003; Marchesini and Hannun, 2004; Perrotta et al., 2010). Formation of blebs is likely caused by redistribution of extracellularly synthesized ceramide within the bilayer and by local enrichment of the cone-shape sphingolipid into the inner leaflet of the membrane. Indeed due to its spontaneous negative curvature, ceramide may induce membrane subdomains with curvature different from the adjacent planar membrane (Subra et al., 2007). In addition, hydrolysis of SM, which has a high affinity for cholesterol, may result in increased efflux of cholesterol. As this lipid is a major determinant of membrane fluidity and structural integrity of the plasma membrane (Simons and Ikonen, 1997) cholesterol efflux may cause an increase in membrane fluidity (Slotte et al., 1989; Neufeld et al., 1996) thus contributing to membrane destabilization and facilitating blebbing and MV shedding (Van Blitterswijk et al., 1982; Chang et al., 1993; Tepper et al., 2000). Bleb formation probably

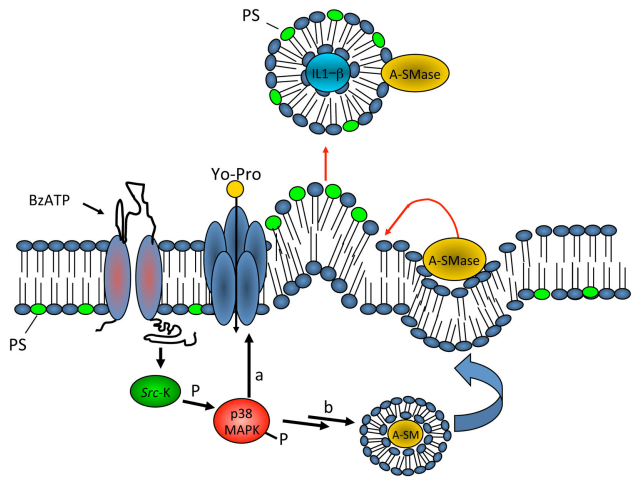

FIGURE 2 | Model for P2X$_7$ receptor-induced signaling pathway involved in MV shedding. On stimulation with ATP or the selective agonist BzATP, P2X7 receptor activates P38 cascade through src-kinase-mediated phosphorylation. In turn, P38 triggers different pathways, among which PM pore formation (a), and mobilization of A-SMase from luminal lysosomal compartment to plasma membrane outer leaflet (b) where the enzyme alters membrane structure/fluidity leading to plasma membrane blebbing and shedding. Shed MVs carry IL-1β, present A-SMase and high levels of PS on their membrane outer leaflet. From Bianco et al. (2009)

occurs from surface lipid rafts (Del Conde et al., 2005), where the P2X$_7$ receptor localizes. In such domains, cytoskeleton/membrane proteins, directly interacting with the P2X$_7$ receptor or P2X$_7$ receptor-dependent signaling components, can be recruited. We demonstrated the key role of A-SMase in MV formation using the pharmacological inhibitor imipramine, and genetic inactivation of the enzyme. Both approaches strongly abolished release of MVs and of IL-1β from reactive glial cells (Bianco et al., 2009).

Other pathways besides shed MVs have been proposed to mediate IL-1β release from myeloid cells, including exosomes and exocytosis of secretory lysosomes (Andrei et al., 1999; Qu et al., 2007). However, enrichment of IL-1β in larger MVs, derived from the plasma membrane, and complete blockade of MV shedding and IL-1β release from A-SMase KO cells indicates that MV shedding represents the major mechanism mediating secretion of the inflammatory cytokine from reactive microglial cells.

Our results are consistent with the involvement of neutral sphingomyelinase and ceramide formation in the budding of exosomes in oligodendrocytes (Trajkovic et al., 2008) and represent further evidence that MV budding may share features with exosome biogenesis. The role of acid-rather than neutral-sphigomyelinase in MV formation suggests that different members of the SMase family may control the release of distinct types of extracellular vesicles from brain cells, independently of the ESCRT complex.

What remains to be clarified is whether acid SMase activity may play a general role in shedding of MVs that occurs independently of P2X$_7$ activation, such as discharge of MVs from the plasma membrane of highly proliferating cells, which is involved in tumor growth and invasion. Similarly, it remains to be determined if sphingomyelinase activity and ceramide production may be also involved in nanotube formation, by promoting the protrusion of filopodia – like structures which then extend as tubes toward distant cells.

WHY DO MICROGLIA USE MVs TO RELEASE IL-1β?

Which is the main advantage for a cell to release IL-1β through MVs rather than exporting the cytokine directly? Shed MVs, containing packages of IL-1β, can deliver the cytokine at significant distance from the donor cell, in possible proximity to IL-1β receptors present on target cells, thus preventing dispersal and degradation and avoiding dilution of the cytokine in the extracellular environment. Furthermore, TNFα (Hide et al., 2000) and proteases, such as caspase-1 and cathepsin D (Qu et al., 2009; Sarkar et al., 2009), which are synthesized and released upon P2X$_7$ receptor activation, could be released via MVs together with IL-1β. Biogenesis of MVs may indeed serve as a mechanism of regulated assembly of multiple factors (Al-Nedawi et al., 2009) and reactive microglial cells may use MVs as complex "units" of information to mediate an integrated biological response. The presence of inflammatory cytokines and proteolytic agents might be important for the onset of detrimental effects of MVs toward degenerating cells, which are known to release large amounts of ATP, thereby promoting MV shedding. In this regard, monocytes-derived MVs, containing functional caspase-1, have been described to deliver a cell death message to vascular smooth muscle cells (Sarkar et al., 2009).

ROLE OF MICROGLIA-DERIVED MVs IN BRAIN INFLAMMATION

Notably, besides inflammatory mediators and proteases, MVs shed upon P2X$_7$-activation from monocytes and dendritic cells contains MHCII proteins (Qu et al., 2009). This suggests that MVs produced from reactive myeloid cells may provide an efficient route for rapid dissemination and presentation of antigens, as part of an adaptive immune response. Indeed it has been recently shown that MVs shed by macrophages upon P2X$_7$ receptor activation propagate an inflammatory signal among peripheral immune cells (Thomas and Salter, 2010). In the same study, Thomas and Salter identified membrane phospholipids as the active components of MVs, responsible for upregulation of co-stimulatory receptors and cytokine secretion in non-primed macrophages, through a TLR4-dependent process (Thomas and Salter, 2010).

Consistent with a pro-inflammatory role of MVs shed upon P2X$_7$ receptor activation our recent data indicate that microglia-derived MVs induce an immuno-stimulatory activity in recipient microglia, which upregulate the co-stimulatory molecule CD86 and express inflammatory genes in a dose dependent manner upon MV exposure (Verderio et al., 2012). The inflammatory reaction occurring in recipient microglia is associated to MV internalization and MVs-mediated transfer of mRNA codifying for IL-1β. However, it still unclear whether transfer of genetic information from MVs to target microglia contributes to the inflammatory response induced by MVs and further studies are required to identify the inflammatory component/s of MVs. Interestingly, we validated *in vivo* these results by demonstrating that MVs

of microglial origin are detectable in the cerebrospinal fluids of rodents and that their concentration increases in the course of Experimental Autoimmune Encephalomyelitis (EAE), a model of the prototypic neuroinflammatory disease multiple sclerosis (Verderio et al., 2012). We also found that injection of MVs into the brain of mice with subclinical EAE induces recruitment of inflammatory cells at the site of delivery, while A-SMase knock out mice, genetically impaired in MV production, are largely protected from EAE (Verderio et al., 2012). All together these data indicate that microglia-derived MVs act as amplifying agents of inflammation and identify MVs as a marker and therapeutic target of brain inflammation.

It should be noted, however, that MV shedding can also serve functions other than antigen dissemination and propagation of inflammation. Evidence from Di Virgilio's group and our laboratory (Bianco et al., 2005; Pizzirani et al., 2007), by demonstrating the presence of $P2X_7$ receptors on isolated vesicles, suggested that MV shedding could represent a defense strategy against apoptotic insults, produced by excessive or repetitive ATP stimulation. Removal of functional $P2X_7$ receptors from the cell surface could facilitate cell survival and avoid $P2X_7$-mediated apoptosis (Verderio and Matteoli, 2001).

MICROGLIA-DERIVED MVs ENHANCE EXCITATORY NEUROTRANSMISSION

Microvesicles however may play a functional role also in different scenarios. In a recent study we explored the potential of MVs produced by microglia to interact with neurons and to modulate neurotransmission (Antonucci et al., 2012). We found that MVs shed from the surface of microglia interact with the plasma membrane of neurons and enhance spontaneous and evoked excitatory transmission. Indeed analysis of miniature excitatory postsynaptic currents (mEPSCs) in neurons acutely exposed to MVs revealed an increase in mEPSC frequency without changes in mEPSC amplitude and paired recording analysis showed an increase the amplitude of EPSCs (**Figure 3**). MVs mainly act on the presynaptic site of the excitatory synapse, by increasing the ready releasable pool of synaptic vesicles and enhancing release probability at hippocampal synapses. This was indicated by increased sucrose-evoked exocytosis and reduction of paired pulse ratio in synaptically connected neurons. Notably, we found that MVs influence neurotransmission by inducing sphingolipid metabolism in neurons (**Figure 4**). Direct measurements of sphingolipid metabolism revealed an increase in ceramide and sphingosine production from sphingomyelin in cultured neurons exposed to MVs while pharmacological or genetic inhibition of sphingosine synthesis strongly prevented the stimulatory activity of MVs. Interestingly, the use of empty MVs, depleted of their luminal content, indicated that the presynaptic effect of MVs depends on surface components. Consistent with previous evidence by Thomas and Salter (2010) which identified phospholipids of MV membrane as the active pro-inflammatory agent of the MVs, we found that the lipid fraction of MVs shed from microglia is responsible for the enhancement of excitatory neurotransmission. However, additional studies are required to identify the active lipid/s of MVs and to fully define the receptors involved in MV recognition and coupled to sphingolipid metabolism in neurons.

Is MVs-DEPENDENT STIMULATION OF EXOCYTOSIS GOOD OR BAD FOR NEURONS?

This question remains still largely unsolved and we can only make some speculative considerations. Previous evidence indicated that

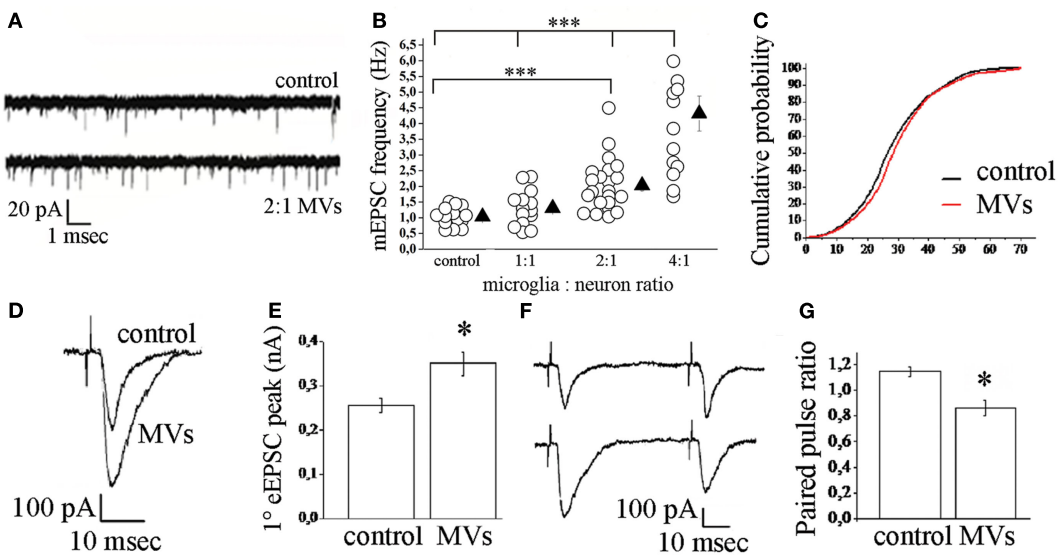

FIGURE 3 | Effect of microglia-derived MVs on neurotransmission in hippocampal cultures. (A) Representative traces of mEPSCs from control neurons and neurons exposed to MVs. **(B)** Changes of mEPSC frequency evoked by MVs in a microglia to neuron-ratio of 1:1 (MVs concentration = 1.2 mg/ml), 2:1 (MVs concentration = 2.38 mg/ml), and 4:1 (MVs concentration = 4.76 mg/ml). **(C)** Cumulative distribution of mEPSC amplitude from control and MVs-treated neurons. **(D–E)** Examples of stimulus-evoked EPSCs in control and MVs-treated paired mouse neurons **(D)** and corresponding mean amplitude **(E)**. **(F–G)** Representative traces of short-term plasticity in paired mouse neurons **(F)** (upper trace, control neurons; lower trace, MVs-treated neurons) and quantitative analysis of paired pulse ratio **(G)**. From Antonucci et al. (2012).

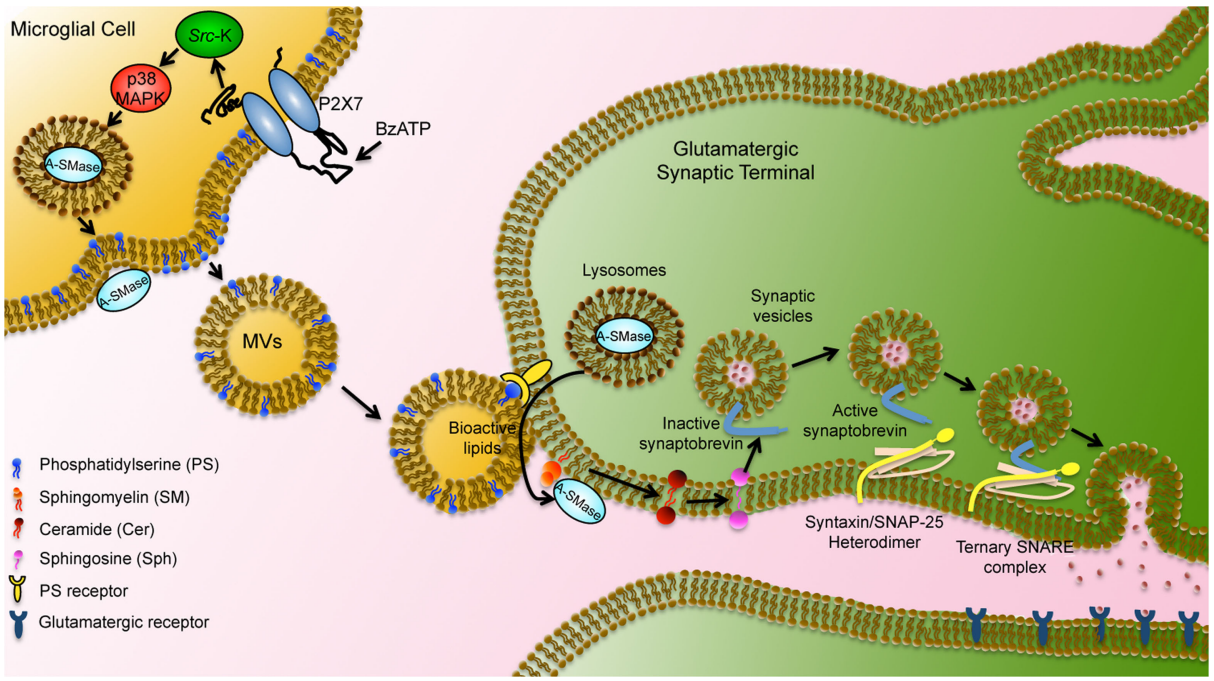

FIGURE 4 | Schematic representation of microglial MV-mediated activity in neurons. MVs shed from the microglial surface have externalized PS and bind via PS receptors to the surface of target neurons. MV lipids stimulate A-SMase activity and promote sphingomyelin metabolism to sphingosine in neurons. Sphingosine, in turn, mediates relief of the cytoplasmic part of synaptobrevin from inhibition by the vesicular membrane and facilitates further interaction with syntaxin/SNAP-25 heterodimer. Ternary SNARE complex formation leads to synaptic vesicle fusion with the plasma membrane modified by Darios et al. (2009).

ATP, the stimulus which triggers MV shedding, is a physiological gliotransmitter but also a typical danger signal, which accumulates in the extracellular microenvironment upon cell damage. The shedding process occurs more efficiently in reactive as compared to resting microglia (Bianco et al., 2009; Qu et al., 2009; Sarkar et al., 2009) and uncontrolled microglial activation is linked to neurotoxicity in a wide range of brain diseases. However there has been considerable debate as to whether the microglia response is good or bad for tissue protection and repair. Accumulating evidence indicates that microglial reaction may indeed support neurons by providing trophic factors, eliminating damaged cells (Olah et al., 2012), controlling neurogenesis (Butovsky et al., 2006) and synaptogenesis (Roumier et al., 2004), and monitoring the functional state of synapses (Wake et al., 2009).

Facilitation of exocytosis induced by microglia-derived MVs may represent a protective response of microglia, aimed at restoring neuronal activity upon functional deficit of synaptic transmission. Neurons constitutively release a number of "Off" signals capable of inhibiting microglia activation (Neumann et al., 1998; Mott et al., 2004; Biber et al., 2007). Thus reduction in secretion of "Off" signals at damaged synapses may favor the acquisition of reactive phenotype in surrounding microglia, and facilitate MV shedding to restore exocytosis. Alternatively, and more probably, MVs may impact neurotransmission in case of microglia overshooting. By causing overproduction of sphingolipids, MVs shed from reactive microglia may contribute to the excessive potentiation of excitatory transmission, which indeed occurs in neuroinflammatory and degenerative diseases (DeFelice et al., 2007; Busche et al., 2008; Centonze et al., 2009). Characterization of MV content in relation to the activation state of donor microglia may help deciphering the complex biological activity MVs may exert toward neurons either upon acute or chronic delivery in both physiological and pathological conditions.

CONCLUSION AND FUTURE PERSPECTIVE

Similar to membrane vesicles released by most cells, MVs shed from the surface of microglia contain various bioactive molecules which modulate neuron functionality and also influence the activity of surrounding non-neuronal cells. MVs contain a pro-inflammatory signals, i.e., IL-1β together with proteases and MHCII protein, act as amplifiers of inflammatory signals between glial cells and stimulate excitatory neurotransmission. This evidence suggests that MVs may play a pathogenic role not only in neuroinflammatory diseases, such as multiple sclerosis, but also in degenerative brain diseases, like Alzheimer's disease, where microglia is activated and IL-1β is implicated (Giulian et al., 1996; Lue et al., 2005). Hence, a better understanding of the molecular mechanisms involved in MV shedding and in the transfer of the inflammatory signals may help identifying a strategy to inhibit MV activity, which may be of therapeutic relevance for the treatment of inflammatory brain diseases. Few, important pieces of information are already available: we know the stimulus (ATP), the receptor ($P2X_7$ receptor), and the key enzyme (acid sphingomyelinase)

involved in regulated shedding of MVs from microglia. We can inhibit production of MVs with pharmacological and genetic tools and we can envisage a sorting mechanism for constituents of MVs which interact directly with the $P2X_7$ receptor or indirectly through its signaling components. However, the molecular composition of microglia-derived vesicles remains largely to be defined and little information are available about possible changes of MV cargo in relation to the activation state of donor microglia. A detailed characterization of proteins, lipids, and genetic components sorted inside MVs may greatly help deciphering the message stored inside MVs and sent by resting or reactive microglia toward surrounding cells, including non-neuronal cells such as astrocytes, oligodendrocytes, and other microglia. Also, the elucidation of intercellular trafficking of MVs and identification of ligand-receptor recognition events which mediate the specific interactions between MVs and target cells might facilitate the comprehension of the biological activity exerted by microglia-derived MVs toward distinct brain cells.

In vivo studies will greatly benefit from the creation of a mouse model, in which MV shedding can be inducible impaired, such as conditional A-SMase knock out mice. A further improvement would derive from selective and inducible inactivation of A-SMase in microglia as this would avoid the complex phenotype of currently available models, in which constitutive inactivation of the gene in all cells produces a phenotype similar to Niemann-Pick type A human disorder (Horinouchi et al., 1995; Otterbach and Stoffel, 1995). Furthermore the development of highly specific A-SMase inhibitors could reveal a therapeutic potential of MV shedding inhibitors in brain inflammation.

ACKNOWLEDGMENTS

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Oncogenic extracellular vesicles in brain tumor progression

Esterina D'Asti, Delphine Garnier, Tae H. Lee, Laura Montermini, Brian Meehan and Janusz Rak*

Pediatrics, Cancer and Angiogenesis Laboratory, RI MUHC, Montreal Children's Hospital, McGill University, Montreal, QC, Canada

Edited by:
Claudia Verderio, University of Milano, Italy

Reviewed by:
Carmelo Carlo-Stella, University of Milano, Italy
Xandra Breakefield, Mass General Hospital/Harvard Medical School, USA
Dolores Di Vizio, Children's Hospital, Harvard Medical School, USA

***Correspondence:**
Janusz Rak, Pediatrics, Cancer and Angiogenesis Laboratory, RI MUHC, Montreal Children's Hospital, Place Toulon, 4060 Ste Catherine West, PT-232 Montreal, QC, H3Z 2Z3, Canada.
e-mail: janusz.rak@mcgill.ca

The brain is a frequent site of neoplastic growth, including both primary and metastatic tumors. The clinical intractability of many brain tumors and their distinct biology are implicitly linked to the unique microenvironment of the central nervous system (CNS) and cellular interactions within. Among the most intriguing forms of cellular interactions is that mediated by membrane-derived extracellular vesicles (EVs). Their biogenesis (vesiculation) and uptake by recipient cells serves as a unique mechanism of intercellular trafficking of complex biological messages including the exchange of molecules that cannot be released through classical secretory pathways, or that are prone to extracellular degradation. Tumor cells produce EVs containing molecular effectors of several cancer-related processes such as growth, invasion, drug resistance, angiogenesis, and coagulopathy. Notably, tumor-derived EVs (oncosomes) also contain oncogenic proteins, transcripts, DNA, and microRNA (miR). Uptake of this material may change properties of the recipient cells and impact the tumor microenvironment. Examples of transformation-related molecules found in the cargo of tumor-derived EVs include the oncogenic epidermal growth factor receptor (EGFRvIII), tumor suppressors (PTEN), and oncomirs (miR-520g). It is postulated that EVs circulating in blood or cerebrospinal fluid (CSF) of brain tumor patients may be used to decipher molecular features (mutations) of the underlying malignancy, reflect responses to therapy, or molecular subtypes of primary brain tumors [e.g., glioma or medulloblastoma (MB)]. It is possible that metastases to the brain may also emit EVs with clinically relevant oncogenic signatures. Thus, EVs emerge as a novel and functionally important vehicle of intercellular communication that can mediate multiple biological effects. In addition, they provide a unique platform to develop molecular biomarkers in brain malignancies.

Keywords: extracellular vesicles, exosomes, oncogenes, cancer, brain

INTRODUCTION – INTERCELLULAR COMMUNICATION IN COMPLEX BIOLOGICAL SYSTEMS

The fascination with biological identity tends to overshadow the inherent interconnectedness of complex biological systems. The human brain epitomizes a biological context in which function and dysfunction is defined by patterns of information flow, which is reflected by the intercellular exchange of defined molecular signals.

Cellular interactions are mostly thought of as being organized into molecular pathways of autocrine, juxtacrine, paracrine, or endocrine nature (depending on the intercellular distances). According to this paradigm a target cell is subjected to iterations of individual receptor-ligand recognition events, and their networks, many of which are now well-characterized (e.g., in the case of hormones, neurotransmitters, growth factors, and membrane molecules and their respective receptors) (Avraham and Yarden, 2011).

This compelling model, however, has long eclipsed some other "non-conventional" forms of cellular communication (Mittelbrunn and Sanchez-Madrid, 2012). Indeed, it is increasingly understood that cells also produce combinatorial messages contained in cellular and membrane fragments including entities usually regarded as confined to insoluble, intracellular compartments (cytoplasm, nucleus, transport vesicles). Such bursts of multimolecular information may be received by other cells and lead to a change in their functional state along with elements of their molecular identity.

Indeed, this complex form of intercellular communication may have an ancient ancestry. This is exemplified by the phenomenon of horizontal gene transfer (HGT), which is implicated in certain forms of speciation and organismal symbiosis (Choi and Kim, 2007; Court et al., 2008). Intriguing remnants of such relationships include the insertion (and expression) of the entire genome (DNA) of the intracellular prokaryote *Wolbachia* in its carrier insect cell. In this sense cell fusion, phagocytosis, and formation of viral particles by higher organisms could be regarded as relics of intercellular integration developed during early evolution, a process "rediscovered" in the course of various physiological and pathological processes in higher species (Sinkovics, 2011).

The horizontal transfer of molecules is also known to occur between human cells, including those in the brain. This process may be executed through several different mechanisms involving rearrangements within specialized plasma membrane domains, and formation of direct cell–cell contact sites. Examples of such processes include formation of intercellular junctions, membrane swapping (trogocytosis), cellular synapses, extension of tunneling nanotubes or cytonemes, and other mechanisms acting mostly

between adjacent cells (Belting and Wittrup, 2008). However, cells also posses the capacity to exchange membrane fragments and associated complex molecular signals over longer distances (often systemically) subsequent to the formation and release of organelle-like structures often referred to as extracellular vesicles (EVs), which are the main focus of this article.

BIOGENESIS AND PROPERTIES OF EXTRACELLULAR VESICLES

EVs shed from individual cells are molecularly complex and often highly heterogeneous. Although there is no consensus as to the exact mechanisms that govern EV formation and their nomenclature, the most common descriptions point to at least four distinct vesiculation pathways. Thus, apoptotic cellular breakdown leads to the release of large EVs (>1000 nm in diameter) known as apoptotic bodies (AB) that contain cytoplasmic and membrane material, genomic DNA, and organelles. Even larger particles (large oncosomes, 1000–10,000 nm) are generated from plasma membrane blebs, as a by-product of the amoeboid motility exhibited by certain types of cancer cells (Di Vizio et al., 2009). Through a similar membrane blebbing mechanism various phagocytes, microglia, platelets, and cancer cells emit smaller EVs referred to as microvesicles (MVs), microparticles, shed vesicles, or ectosomes (usually 100–1000 nm in diameter) (Thery et al., 2009). In this case, the stimulation with biological agonists triggers calcium fluxes, regional loss of phospholipid asymmetry in the plasma membrane, exposure of phosphatidylserine (PS), followed by changes in membrane-cytoskeleton contacts, formation of membrane curvature, and vesicle scission (Piccin et al., 2007). In microgial cells, this process involves acidic sphingomyelinase (Asmase) and activation of intracellular kinase cascades (Bianco et al., 2009). A similar mechanism is also responsible for the extracellular release of certain integral membrane receptors such as tissue factor (TF), the main trigger of blood coagulation expressed by phagocytes and cancer cells, including glioma (Yu and Rak, 2004; Del Conde et al., 2005). Depending on their source, MVs may also contain cellular lineage markers, high levels of surface PS, integrins, cannabinoid receptor (CB1), matrix metalloproteinases (MMPs), TF, and other membrane-related entities defining their unique biological features along with lipids and possibly nucleic acids (Dolo et al., 2005; Bianco et al., 2009; Camussi et al., 2010; Lee et al., 2011a).

A relatively well-studied and distinct form of vesiculation involves the formation of exosomes. These EVs are believed to be generated intracellularly, as the so-called intraluminal vesicles (ILVs). These secondary vesicular structures emerge within larger endosomal vesicles described as multivesicular bodies (MVBs) (Simons and Raposo, 2009; Thery et al., 2009; Mathivanan et al., 2010). Formation of MVBs represents a step in membrane receptor signaling and processing cascade, which involves receptor internalization controlled by the endosomal sorting complex required for transport (ESCRT). This multimolecular apparatus controls the intracellular trafficking of membrane receptors between cell surfaces, endosomes and pathways of lysosomal destruction, or recycling (Williams and Urbe, 2007). It is believed that in some instances MVBs take an alternative path and are instead redirected to the plasma membrane in such a way as to allow the extracellular release of ILVs (as exosomes) (Trajkovic et al., 2008). Exosomes are relatively small (30–100 nm), rich in tetraspanins (CD63, CD9), Rab proteins, and other cargo including nucleic acids (Valadi et al., 2007; Thery et al., 2009).

EXTRACELLULAR VESICLES AS VEHICLES OF INTERCELLULAR COMMUNICATION AND MOLECULAR EXCHANGE

Emission of EVs constitutes a natural multiplexing mechanism whereby several molecules may be assembled, protected, and released from cells regardless of their compatibility with the classical secretory pathways. Indeed, vesiculation represents the key mechanism whereby proteins lacking a signal peptide (e.g., interleukin 1 beta–IL1β), or located in non-secretory cellular compartments (e.g., nuclear proteins) may reach the extracellular space (Bianco et al., 2009). Consequently, various proteins and nucleic acids are incorporated into EVs, often in concentrations higher than those found in parental cells. While the astonishing scope of this "packaging and shipment" process has been reviewed in the recent literature and cataloged in specialized databases (Exocarta) (Ratajczak et al., 2006b; Valadi et al., 2007; Thery et al., 2009; Mathivanan et al., 2012), the related mechanisms remain elusive, with only limited but intriguing insights (Bolukbasi et al., 2012).

The functional implications of cellular vesiculation can, at least to some extent, be inferred from the repertoire of EV-associated bioactive molecules. While EVs may contain high concentrations of soluble mediators (interleukins, growth factors, chemokines), their unique role in cell–cell interaction is thought to stem largely from their content of transmembrane, cytoplasmic and nuclear proteins, lipids, mRNA, miRs, genomic DNA sequences (Ratajczak et al., 2006b; Valadi et al., 2007; Mause and Weber, 2010; van der Vos et al., 2011).

EVs interact with various target cells through several mechanisms (**Figure 1**). The fate of EVs involved in such interactions may entail either a simple surface contact with the target cell, e.g., via receptor-ligand bridges, or several other processes. Those include rupture of the EV membrane leading to pericellular release of their cargo, and a burst of paracrine activity (Taraboletti et al., 2006). However, EVs may also reach the interior of their target cells by fusion with their plasma membranes, or through an endocytosis-like engulfment of the entire vesicle. In these instances, the bioactive cargo of EVs becomes released inside the target cell, and thereby may interact with their regulatory apparatus including adapter proteins and signaling circuitry (Al-Nedawi et al., 2009b). The efficiency and consequences of these cell-EV-cell interactions may depend on the nature of the cells involved and on the surrounding microenvironment (hypoxia, inflammation, acidity), all of which may control the emission, cargo, and uptake of EVs. In this regard, the brain represents a unique site for EV-mediated interactions.

EXTRACELLULAR VESICLES IN THE BRAIN MICROENVIRONMENT

There are several cellular sources of EVs that may enter the interstitium, fluid spaces, and other compartments of the brain microenvironment. For example, EVs are normally present within

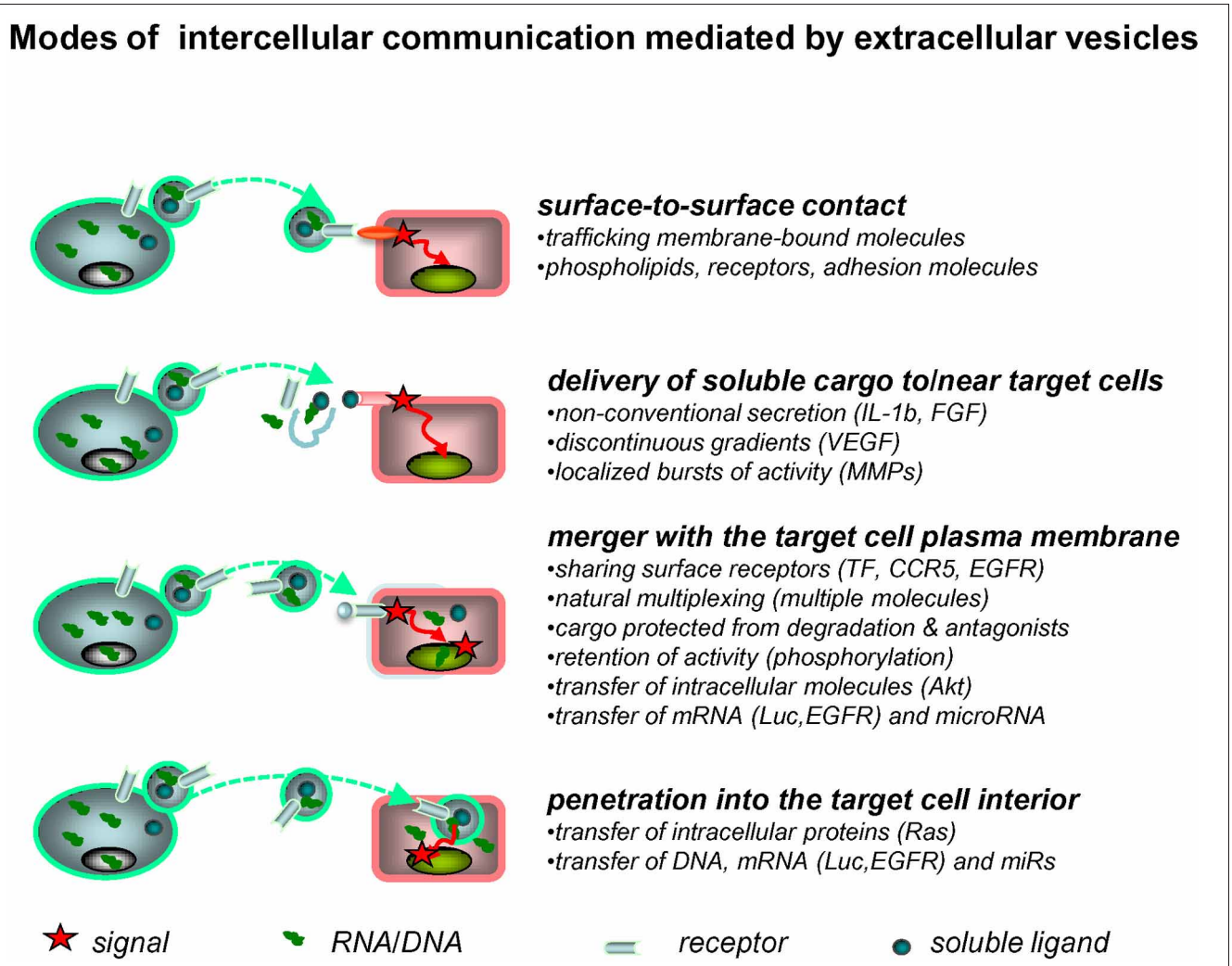

FIGURE 1 | Extracellular vesicles as mediators of intercellular communication. Exchange of molecular information between cells may be mediated by EVs in several ways. Thus, surface receptors of EVs may interact directly with counter-receptors on the surface of a target cell. The latter may also come into contact with the bioactive inner cargo of EVs upon their pericellular rupture. EVs may also merge with the plasma membrane of the target cell, or penetrate into its interior via endocytosis, or other processes, to release their content of proteins and nucleic acids into the intracellular compartments (see text for details).

the vascular system and may readily enter the brain microcirculation. In the absence of disease, those are mainly EVs (microparticles) released from activated blood platelets (Key et al., 2010) or inflammatory cells. In addition, other extracranial sources may contribute to the EV pool in the brain vasculature, including EVs generated by peripheral inflammatory cells, endothelium, or distant cancer cells (Smalheiser, 2009; Lee et al., 2011a). While certain formulations of dendritic cell exosomes have been shown to penetrate the blood-brain barrier (BBB) (Alvarez-Erviti et al., 2011), there is no conclusive evidence for a free and consequential exchange of naturally occurring EVs between brain parenchyma and peripheral tissues. This could likely take place, however, at sites of injury, or in hyperpermeable vessels associated with tumor growth. Circulating EVs may also freely interact with brain endothelial cells, and thereby potentially affect their state and function, or participate in thrombosis and other forms of vascular pathology (Chen et al., 2011). While many of these possibilities are poorly studied, EVs and their associated ectonucleotidases have been implicated in cytoprotective and repair events once BBB has been disrupted (Ceruti et al., 2011).

EVs have also been implicated in various processes involving brain parenchymal cells. For instance, neuronal stem cells (NSCs) produce EVs containing the CD133 progenitor marker (Marzesco et al., 2005). Exocytosis is also well-described in differentiated neurons and may impact their communication with non-neuronal cells (Smalheiser, 2009). Indeed, neurons contain MVBs, the structural precursor of exosomes (von Bartheld and Altick, 2011), and these EVs are also found in supernatants of corresponding cell cultures (Faure et al., 2006). Similarly, normal glial cells, such as astrocytes release EVs into their surroundings. In this manner glial-derived glutamate may reach and act on its receptors associated with adjacent neurons (Bergersen and

Gundersen, 2009). Astrocytes also shed EVs containing mitochondrial DNA, but the significance of this process is presently unclear (Guescini et al., 2010). Oligodendrocytes were found to produce exosomes (Kramer-Albers et al., 2007), a process that relies on a specific pathway involving neutral sphingomyelinase (Nsmase) (Trajkovic et al., 2008). These EVs are then selectively taken up by brain microglial cells, which are postulated to provide a constitutive mechanism for exosome clearance within the milieu of the brain (Fitzner et al., 2011). Microglial cells themselves emit EVs containing cytokines (Potolicchio et al., 2005), a process recently implicated in neuroinflammation (Bianco et al., 2009). In this regard, Verderio and colleagues described a regulatory pathway involving Asmase, which controls ATP stimulated release of EVs from microglial cells. In this fashion EV-associated IL-1β, which lacks secretory signal peptide, can be liberated from microglia and act as stimulator of phagocytosis, which is required for clearance of ATP emitting damaged cells (Bianco et al., 2009). Microglial EVs also play a previously unsuspected role in neuronal synaptic activity (Antonucci et al., 2012). Indeed, due to this emerging network of EV-mediated interactions in the brain the emission and content of various vesicles was recently proposed to serve as a putative biomarker for neurological disorders (Colombo et al., 2012). It remains to be established to what extent EV production, trafficking, and uptake contribute to the pathogenesis of these conditions, and whether their release also has notable systemic consequences.

BIOLOGICAL EFFECTS OF EXTRACELLULAR VESICLES IN CANCER

The process of cellular vesiculation is hijacked and distorted during malignant transformation and contributes to the phenotype of cancer cells and their associated stroma. This has been documented in several different disease settings, and reviewed extensively in recent literature (Ratajczak et al., 2006b; Thery et al., 2009; Camussi et al., 2010; Rak and Guha, 2012). The role of EVs in cancer is often a subject of generalizations, which will likely evolve to more disease-specific considerations as the underlying processes become better understood. It is reasonable to predict that EVs may differ in their type and relative role in the pathogenesis of different cancer types and disease subtypes, also as a function of such variables as host genetic background, in a similar manner as this applies to other effector mechanisms associated with malignancy (e.g., angiogenesis or metastasis) (Rohan et al., 2000; Hunter, 2006; Phillips et al., 2006). Moreover, the contribution of EV release is difficult to formally demonstrate due to the scarcity of suitable loss-of-function models *in vivo*, where tumor progression could be rigorously examined in the presence and absence of vesiculation. Nonetheless, correlative studies provide compelling evidence for the involvement of EV generation and exchange in several aspects of neoplasia.

Amongst the more extensively studied aspects of vesiculation is the involvement of EVs in cancer coagulopathy. Indeed, one of the first description of EVs was related to procoagulant microparticles emanating from activated platelets ("platelet dust") (Wolf, 1967). This "shedding" mechanism has since been implicated in prothrombotic, proangiogenic, and prometastatic events in cancer (Baj-Krzyworzeka et al., 2002; Janowska-Wieczorek et al., 2006). Seminal studies of Dvorak and colleagues revealed extensive shedding of procoagulant TF-containing microvesicles from cancer cells (Dvorak et al., 1983). Numerous subsequent analyses interrogated the relevance of this process in cancer biology (Yu et al., 2005), progression (Tesselaar et al., 2007), and paraneoplastic (prothrombotic) syndromes (Burnier et al., 2009; Aharon and Brenner, 2010; Khorana, 2010; Zwicker, 2010).

Production of exosomes by cancer cells has been frequently implicated in anticancer immunity (Wolfers et al., 2001). In this regard, both positive and negative effects of circulating exosomes were proposed to regulate antitumor responses (Wolfers et al., 2001; Taylor and Gercel-Taylor, 2005; Liu et al., 2006; Valenti et al., 2007). Among the most interesting examples is the discovery of exosomes containing Fas ligand, which could effectively destroy Fas receptor—expressing cytotoxic effector T cells before they could reach cancer cells (Abusamra et al., 2005). There are also indications that exosomes derived from glioblastoma (GBM) cells may exert immunomodulatory effects on monocytes (de Vrij et al., 2012).

EVs may harbor molecular mediators of drug resistance and transfer them between cells. This may lead to the exchange of pro-survival proteins (Al-Nedawi et al., 2008), molecular drug efflux pumps (e.g., P-glycoprotein/MDR1) (Jaiswal et al., 2012) or other cargo. A similar exchange of plasma membrane fragments containing drug resistance molecules may also occur upon cell–cell contact, through a mechanism known as trogocytosis (Rafii et al., 2008).

Cancer-derived EVs have also been implicated in metastasis. For example, recent experimental data suggests that exosomes cooperate with other pathways in the formation of pre-metastatic niches and promote hematogenous metastases at distant sites (Jung et al., 2009; Grange et al., 2011; Peinado et al., 2011). Similarly, the influence of exosomes has been observed in the context of lymphatic dissemination (Hood et al., 2011) and local invasion (Hendrix et al., 2010).

EVs may also influence disease dissemination through their impact on the vascular system including angiogenesis. In this regard, both host and tumor-derived EVs appear to possess an array of proangiogenic activities attributed to several elements of their cargo. Thus, EVs emanating from platelets (Janowska-Wieczorek et al., 2005) and endothelial progenitor cells (Deregibus et al., 2007) have the ability to stimulate the angiogenic program in resident endothelial cells. In another study, tetraspanin (Tspan8)-containing exosomes emanating from certain experimental cancer cells were found to elicit a systemic proangiogenic state in mice harboring the corresponding tumors (Gesierich et al., 2006). It is noteworthy that EVs may contain high concentrations of soluble angiogenic molecules such as IL-8, VEGF, FGF (Taraboletti et al., 2006; Skog et al., 2008) as well as proangiogenic matrix metalloproteinases (MMP9) and their regulators (CD147). In this manner, EVs may deliver bursts of activity to sites of blood vessel formation, in and around the tumor, or at distant sites (Taraboletti et al., 2006). EVs may also carry normally insoluble angiogenesis regulators such as delta like 4 (Dll4), the cellular ligand of Notch. Presentation of Dll4 to Notch in the EV-associated form alters the biological activity of this angiogenic pathway (Sheldon et al., 2010). Moreover,

interaction of EVs with target cells may modulate their angiogenic phenotype, either through EV-cell contact, or by horizontal transfer of signaling molecules (Ratajczak et al., 2006b; Al-Nedawi et al., 2008; Skog et al., 2008; Al-Nedawi et al., 2009a). While the requirement for such EV-mediated communication for the onset and regulation of angiogenesis is not fully explored, a multitude of angiogenic effectors are already known to be released via cellular vesiculation pathways, which likely influences their activity (e.g., by changing their spatial distribution and gradients) (Mause and Weber, 2010).

Several additional effects of EVs in cancer are also of considerable interest. This includes communication and reprogramming events that may occur through contact between cancer cells and EVs emanating from stem cells, as originally observed by Ratajczak and colleagues (Ratajczak et al., 2006a). Other types of progenitor-like cells are also known to shed EVs (Milsom et al., 2008; Collino et al., 2010), and this may include tumor initiating (cancer stem) cells (TICs) identified in several malignancies including brain tumors (Stiles and Rowitch, 2008). It is conceivable that TICs may possess the capacity to reprogram activities of other cells via the exchange of EVs.

NEOVESICULATION AND ONCOSOMES

Vesiculation of cancer cells may take several aberrant forms including quantitative increases in EV emission, changes in their size, structure, and molecular composition, as well as altered biological activity. Some of these anomalies may be a function of disease-related aberration in the EV biogenesis pathways, changes we collectively refer to as *neovesiculation*.

Several mechanisms have been described that effectively differentiate the EV emission by cancer cells from that of their corresponding non-transformed counterparts. For instance, in prostate cancer cells, deregulation of the Akt pathway, growth factor stimulation (EGF), and loss of the diaphanous related formin 3 (DRF3) leads to the acquisition of a cellular phenotype associated with invasiveness, amoeboid motility, and unique form of neovesiculation. The latter is characterized by formation of very large membrane blebs on the cell surface, and their subsequent scission as the aforementioned unusually large EVs (*large oncosomes*). Large oncosomes exhibit biological activities consistent with their content of signaling molecules, and their formation may be viewed as a hallmark of increased prostate cancer aggressiveness linked to a loss of a putative tumor suppressor (DRF3) (Di Vizio et al., 2009).

The term *oncosomes* was originally coined to reflect another distinct feature of tumor cell-derived EVs, namely their ability to carry cancer-specific mutant proteins and nucleic acids, the very drivers of oncogenic transformation and hitherto regarded as confined to cancer cells. Although oncogenic mutations are normally thought of as propagating along vertical clonal hierarchies, the release of their containing molecules (oncoproteins and nucleic acids) as cargo of EVs suggests that mutant gene products may traffic horizontally between cells. In this manner, transforming signals could be shared amongst wider cellular populations including indolent, normal, and unrelated (heterotypic) cells (Al-Nedawi et al., 2008). Notably, oncogene-containing EVs were found in the interstitium, body fluids, and circulating blood in tumor bearing animals and cancer patients (Al-Nedawi et al., 2008; Skog et al., 2008). Through this mechanism distant organ sites may become exposed to transforming activities, including cells within putative metastatic niches, stem cell reservoirs, and regulatory cell populations within the vascular system and bone marrow (Rak and Guha, 2012). Although several long and short range biological effects of EVs have already been described in various cancer settings (Al-Nedawi et al., 2008; Ghosh et al., 2010; Antonyak et al., 2011), the specific role of oncogenic molecules in these events is still to be formally demonstrated *in vivo*. Several types of EVs may contribute to the extracellular release of oncogenic cargo from cancer cells, including large and small oncosomes and exosome-like vesicles (Al-Nedawi et al., 2008; Skog et al., 2008; Al-Nedawi et al., 2009a; Graner et al., 2009). In this regard cancer cell apoptosis represents a distinct mechanism, whereby cellular remnants (AB) may serve as unique vehicles for vesicular trafficking of mutant DNA sequences in the pericellular milieu (Holmgren et al., 1999).

THE TRANSFORMING CARGO OF ONCOSOMES

Oncosomes may harbor several types of cancer-related molecules including active oncoproteins, oncogenic transcripts, transforming miR species, and genomic sequences containing mutant oncogenes. Likewise, wild type or mutant tumor suppressors (proteins and nucleic acids), and molecules affecting genetic stability (e.g., retrotransposons) have also been identified in the cargo of cancer-derived EVs, as reviewed in the recent literature (Ratajczak et al., 2006b; Muralidharan-Chari et al., 2010; van der Vos et al., 2011; Rak and Guha, 2012).

Amongst the best described examples of oncoproteins found in the cargo of cancer-derived oncosomes are members of the ErbB/HER family of receptor tyrosine kinases (RTKs), such as activated (phosphorylated) EGFR and its constitutively active mutant EGFR variant III (EGFRvIII) (Al-Nedawi et al., 2008, 2009a, 2010). Breast cancer cells have been found to shed EVs containing HER-2 protein, another member of the EGFR family (Koga et al., 2005). Different cancer cell lines shed EVs containing other oncoproteins including myr-AKT (Di Vizio et al., 2009), LMP1 (Meckes et al., 2010), Ras (Lee and Rak, 2011, unpublished observation), including mutant K-ras (Franklin et al., 2012), BRAF/V600E (Ramachandran et al., 2011), PDGFR, beta-catenin, c-Met, and several others (Al-Nedawi et al., 2010). EVs may also contain tumor suppressor proteins (e.g., PTEN) (Al-Nedawi et al., 2010) and their potential role in horizontal modulation of the malignant phenotype is a subject of an ongoing interest.

Oncogenic nucleic acids have also been identified in the cargo of various EVs, including transcripts for the various aforementioned oncoproteins (Skog et al., 2008; Graner et al., 2009). As mentioned earlier, AB may carry DNA sequences associated with the Epstein-Bar virus-related oncogenes (EBNA1, EBER), as well as those encoding oncogenic H-ras and Myc (Holmgren et al., 1999; Bergsmedh et al., 2001). Cell culture medium and serum of mice harboring human medulloblastoma (MB) xenotransplants may contain EVs with encapsulated DNA corresponding to the amplified oncogenic c-Myc sequences (Balaj et al., 2011), while plasma of colorectal cancer patients was found

to contain functional circulating DNA encoding mutant K-ras (Garcia-Olmo et al., 2010).

Pioneering work of several investigators provided ample evidence as to the presence of multiple miR species in the cargo of EVs emanating from various cell types (Ratajczak et al., 2006b; Valadi et al., 2007; Skog et al., 2008; Taylor and Gercel-Taylor, 2008). Much research on miR detection in samples of blood or cerebrospinal fluid (CSF) collected from cancer patients has focused on the simultaneous isolation of all circulating miRs including their protein- and microparticle-associated fractions (Chen et al., 2012). Taylor profiled miRs in both the tumor tissue and serum-derived, tumor-specific exosomes collected from ovarian cancer patients. Those miRs (miR-21, miR-141, miR-200a, miR-200c, miR-200b, miR-203, miR-205, and miR-214) that were present in both the tumor and exosomes, and which had been previously identified as overexpressed in human ovarian cancer were then validated by qRT-PCR demonstrating a direct correlation between the miR signature of the tumor and that of the tumor-derived exosomes (Taylor and Gercel-Taylor, 2008). All of these miRs were significantly elevated in exosomes collected from patients diagnosed with early and late stage ovarian cancer compared to benign ovarian disease; however, miR-200c and miR-214 were also specifically present in higher copy numbers in late stage malignancies. The levels of circulating let-7a and miR-195 are significantly elevated in plasma samples collected from breast cancer patients compared to healthy women (Heneghan et al., 2010). These miRs were also overexpressed in the tumor relative to normal tissue. Interestingly, both aforementioned miRs may also act as biomarkers of therapeutic response, as post-operative levels were comparable to blood samples collected from healthy women. In prostate cancer patients circulating miR-141 is higher compared to healthy individuals (Mitchell et al., 2008). Furthermore, circulating miR-141 and miR-375 (also elevated in prostate cancer specimens compared to normal tissue) are associated with metastatic disease (Brase et al., 2011). Profiling studies of miRs found in large oncosomes in prostate cancer revealed a pro-invasive signature. A more comprehensive list of circulating miRNAs that may act as diagnostic and/or prognostic biomarkers can be found in past reviews (Kosaka et al., 2010; Cortez et al., 2011).

At least some of these miR species, may possess oncogenic and tumor suppressive characteristics (Garzon et al., 2010). Indeed, we observed that MB cells engineered to express miR-520g shed EVs containing this miR into culture media and the blood of xenograft bearing mice (D'Asti et al., 2012). Mir-520g acts as an oncogene in these and other neuroectodermal tumors (Li et al., 2009). Several other putative oncomirs have also been detected in the cargo of EVs released from human GBM cells including let7a, miR-16-1, miR-92, and miR-21 (Skog et al., 2008).

BIOLOGICAL CONSEQUENCES OF ONCOSOME PRODUCTION

The biological significance of the EV-mediated release of oncogenic molecules is usually inferred from their inherent transforming activity coupled with the ability to undergo intercellular trafficking. While this is an intriguing possibility, there is no formal and conclusive *in vivo* evidence in support of the absolute requirement or the rate-limiting involvement of vesiculation in key aspects of cancer progression. Nonetheless, proof-of-principle experiments *in vitro* or in mouse models suggest several potential pathogenetic mechanisms and the existence of the unexpected, intercellular dimension of oncogenic signalling (**Figure 2**).

Thus, EVs containing oncogenic EGFRvIII are capable of transferring this oncoprotein into indolent glioma cells, in which this influence activates the canonical MAPK and AKT signaling pathways. The biological consequences of this "ectopic signaling" include augmentation of soft agar colony formation, production of angiogenic factors, and changes in gene expression (Al-Nedawi et al., 2008). Tumor cell-derived EVs can also mediate the transfer of EGFR to endothelial cells inducing aberrant signaling and autocrine activation of VEGF receptors (VEGFR2). Similar quasi-transforming *in vitro* consequences are associated with the cellular uptake of EVs containing activated AKT and LMP1 proteins (Di Vizio et al., 2009; Meckes et al., 2010). Exosomes can also mediate a transfer of oncogenic K-ras between aggressive and indolent colorectal cancer cell lines, causing transformation-like changes (Franklin et al., 2012).

Even more dramatic outcomes were observed when non-tumorigenic NIH3T3 fibroblasts were exposed to EVs derived from invasive breast cancer cells containing tissue transglutaminase (tTG) and fibronectin (FN). Uptake of this material *in vitro* and *in vivo* led to overt transformation and tumorigenic conversion of the NIH3T3 recipients (Antonyak et al., 2011). Similarly, DNA sequences containing oncogenic K-ras gene were detected in association with particles circulating in blood of colorectal cancer patients. Again, the uptake of this material by NIH3T3 cells resulted in the onset of their tumorigenic phenotype. This phenomenon was postulated to play a role in the remote transformation of normal cells and formation of distant outgrowths, a process termed "genometastasis" (Garcia-Olmo et al., 2010). Many of these observations are consistent with the pioneering work of Holmgren and colleagues who originally demonstrated that the uptake of oncogenic DNA sequences (H-ras, c-Myc) contained in tumor cell-derived AB (EVs) may lead to the expression of the respective oncoproteins and tumorigenic phenotype in non-transformed recipient cells (mostly NIH3T3 fibroblasts) (Holmgren et al., 1999; Bergsmedh et al., 2001).

While the aforementioned observations raise the spectre of EV-mediated widespread dissemination of oncogenic material and horizontal transformation of normal cells, the likelihood and scope of such events requires some qualification. First, the half-life of oncoproteins and their transcripts in recipient cells is probably somewhat limited due to breakdown and dilution during cell division. Second, cells differ in their ability to take up, retain, and utilize the EV-related material. In fact, shedding of EGFR and other oncoproteins as EV cargo may represent a mechanism of removal of these overabundant molecules from their parental cancer cells, and could be reiterated in EV recipients. Aggregation of proteins at the plasma membrane may serve as a trigger for such protective shedding processes (Shen et al., 2011; van der Vos et al., 2011). Third, the biological effects of the uptake of active oncoproteins by non-transformed cells may not always be tantamount to cellular transformation. This is because normal cells (unlike immortalized NIH3T3 fibroblasts) retain a repertoire

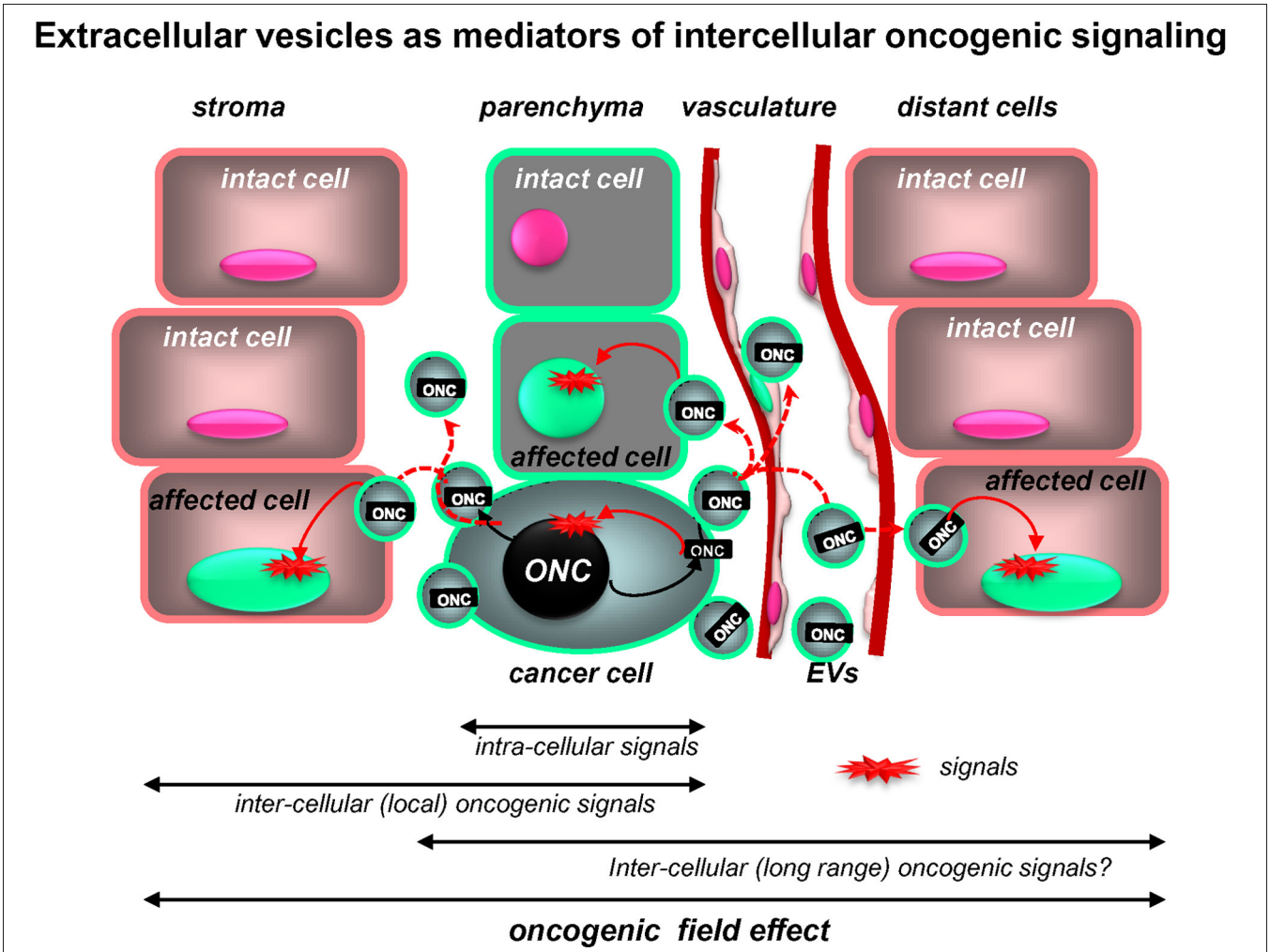

FIGURE 2 | Extracellular vesicles as putative mediators of the intercellular propagation of oncogenic signaling. While intracellular signaling pathways elicited by mutant oncogenes (*ONC*) are increasingly well understood, oncoproteins (ONC) may also operate outside of the confines of cancer cells due to intercellular trafficking of their containing EVs (oncosomes). Uptake of this material by proximal non-transformed cells and at distant organ sites may trigger downstream oncogenic signals in these recipient cells and alter their phenotype and behavior. Thus, intercellular trafficking of oncoproteins (and nucleic acids) extends the range of oncogenic signaling beyond the boundaries of cells harboring the original mutation (see text for details).

of tumor suppressors that may activate apoptotic or senescence programs in the presence of protracted oncogenic signaling, a phenomenon known as "oncogenic stress response" (Serrano et al., 1995). Even established but indolent cancer-derived cells do not necessarily undergo overt tumorigenic conversion upon the uptake of EGFRvIII-containing oncosomes (our unpublished observation), and additional genetic events or molecular predispositions may be required for such a change to take place. However, the potential that at least some cells (e.g., stem cells, premalignant cells, or dormant cancer cells) may be susceptible to malignant conversion via oncosome-mediated molecular transfer cannot be excluded at this time. It is also likely that more transient phenotypic changes (increased angiogenic potential, cellular activation, stress responses) may result from exposure of various normal and indolent cells to circulating oncosomes in cancer patients.

MODULATION OF CANCER CELL VESICULATION BY MICROENVIRONMENT, STRESS, AND DIFFERENTIATION PATHWAYS

Oncogenes and tumor suppressors do not only function as cargo, but also as a part of the regulatory circuitry that controls cellular vesiculation in cancer. Some recent examples to this effect include the modulation of exosome production by p53 tumor suppressor (Yu et al., 2006) and its target known as TSAP6 (Lespagnol et al., 2008). On the other hand, loss of p53 expression may enhance EV-mediated emission of TF from colorectal cancer cells. Oncogenic Ras, EGFRvIII, constitutively activated AKT (myr-AKT) exhibit vesiculation-inducing effects in various settings (Yu et al., 2005; Al-Nedawi et al., 2008). These intrinsic effects epitomize the link between various intracellular pathways and regulation of EV production in response to external stresses and stimuli. For instance, the aforementioned p53-regulated EV emission occurs

prominently in cells undergoing radiation responses, i.e., when this suppressor protein is induced and plays an important biological role (Yu et al., 2006). Analogous alterations in EV production could be expected in cells exposed to other forms of genotoxic or microenvironmental stress, hypoxia, metabolic deprivation, or contact with inflammatory mediators (Svensson et al., 2011), with possible involvement of pathways containing proto-oncogenes and tumor suppressors. Similarly, exposure of various cells to high concentrations of exogenous EGFR ligands (EGF, TGFα) often triggers robust cellular vesiculation (Di Vizio et al., 2009; Garnier et al., 2012).

Cancer cells form clonal hierarchies in which oncogenic, differentiation, and extracellular stimulation pathways blend to control cellular composition and behavior. This includes pathways that define cellular stemness and trans-differentiation events, of which epithelial-to-mesenchymal transition (EMT) represents an important example. EMT is a process whereby cancer cells of epithelial or ectodermal origin (including neuroectodermal cells) transiently acquire a mesenchymal phenotype (e.g., vimentin positivity), as well as more motile and tumor initiating properties (Mani et al., 2008), all of which are implicated in aggressive and metastatic growth (Thiery et al., 2009). Several molecular events are capable of inducing EMT, including cooperation between Ras and TGFb signalling pathways, activation of the MET receptor, induction of several EMT-related transcription factors (e.g., YB1, Twist, or Brachyury) (Fernando et al., 2010), blockade of E-cadherin, and other changes (Thiery et al., 2009).

In A431 squamous cell carcinoma cells harboring an amplified *EGFR* gene, stimulation with EGFR ligands (TGFα) coupled with blockade of E-cadherin results in an EMT-like state characterized by the onset of vimentin expression, and spindle morphology, as well as internalization of cell surface receptors, and a profoundly altered vesiculation profile. The latter includes the overall increase in EV emission, increase in EV-associated TF antigen, as well as elevated emission of exosome-like particles (Garnier et al., 2012). These changes are associated with greater tumor initiating capacity, as measured by increased numbers of metastatic colonies resulting from intravenous injection of A431 cells *in vivo* (Milsom et al., 2008). A reflection of some of these changes could also be found in the proteome of EVs emitted by cells that have entered the mesenchymal state as a result of expression of oncogenic EGFR (Garnier et al., 2012) or H-ras (Tauro et al., 2012).

Interestingly, molecular elements of the EMT-inducing machinery may not only modulate cellular vesiculation, but also are often found in the EV cargo. This has been observed in the case of YB1 (Frye et al., 2009), EGFR, and MET (Al-Nedawi et al., 2008, 2010). Since EMT often co-segregates with the elevated tumor initiating (stem cell) capacity of cancer cells, it is possible that the accompanying changes in vesiculation may contribute to this process in some way; for example, by conditioning the niche environment, influencing the adjacent host cells (Ratajczak et al., 2006a), modulating sites of metastasis (Hood et al., 2011), or impacting the vasculature (Gesierich et al., 2006). Indeed, a link between cancer stem cell vesiculation and angiogenesis has recently been described (Grange et al., 2011). It is presently unclear whether these processes involve intercellular transfer of oncogenic molecules.

ONCOSOMES IN BRAIN TUMORS

As in the case of other malignancies, oncogenic proteins and nucleic acids may be emitted from brain cancer cells as EV cargo (Al-Nedawi et al., 2008; Skog et al., 2008; Balaj et al., 2011). Likewise, oncogenic signaling intermediates and effector molecules may be present in EVs produced by different types of primary and secondary brain tumors, their surrounding parenchyma, microglia, stroma, vasculature, and blood cells. The scope of these processes and their biological impact, however, are far from understood and only a limited number of examples have been published to date (Al-Nedawi et al., 2008; Skog et al., 2008; Graner et al., 2009; Balaj et al., 2011).

Initial reports suggested that biologically active, phosphorylated, and oncogenic EGFRvIII protein is contained in the cargo of small (100–400 nm) EVs produced by EGFRvIII-transformed GBM cells, and this material is emitted into the culture media and plasma of tumor xenograft-bearing mice (Al-Nedawi et al., 2008). These studies documented the aforementioned EV-mediated transfer of EGFRvIII activity to indolent U373 glioma cells and the resulting upregulation of VEGF, BclXL, and changes in levels of other EGFR target genes, as well as increased soft agar colony forming capacity. Co-injections of growth arrested (Mitomycin C-treated) EGFRvIII expressing EV donor cells with GFP-tagged indolent EV recipient glioma cells revealed the expected intercellular transfer of the EGFRvIII immunofluorescence *in vivo*. However, no overt tumorigenic conversion of the indolent cells has been recorded in these experiments [(Al-Nedawi et al., 2008, 2010) and our unpublished data]. The expression of EGFRvIII, EGFR, PDGFR, MET, PTEN, and other GBM-related oncogenic and tumor suppressive proteins was also noted in other models of high grade glioma (U87, U87vIII, U87-PTEN) (Al-Nedawi et al., 2010; Lee et al., 2011b). In addition, a recent study of the phosphoproteome associated with EVs shed by the U373vIII GBM cell line harboring mutant EGFRvIII revealed a rich repertoire of proteins that have undergone this activating post-translational modification, including molecules with oncogenic, signaling, and gene-regulatory potential. This list includes phosphorylated membrane receptors (EGFR, HER2, MET), intracellular protein kinases (PKC, MEK1, Raf1), regulators of apoptosis (BAD), transcription factors (Jun, CREB1), regulators of protein translation (eIF4E, eIF2A), histones (H2B, H3.3), and DNA binding proteins (steroid receptors) (Al-Nedawi et al., 2010). In agreement with these findings, the recent proteomic analysis of GBM-derived exosomes documented the presence of EGFRvIII in samples isolated from culture supernatants and patient plasma (Graner et al., 2009).

Recent elegant studies by Skog, Breakefield and their colleagues brought to light the presence of oncogenic nucleic acids in EVs derived from brain tumors (Skog et al., 2008; Balaj et al., 2011; van der Vos et al., 2011). For example, the EGFRvIII transcript was present in the cargo of EVs isolated from culture medium of primary GBM cell isolates, and in plasma of GBM patients (Skog et al., 2008) as well as in corresponding circulating platelets that appear to take up GBM oncosomes (Nilsson et al., 2011).

Notably, levels of the EGFRvIII mRNA signal in plasma were reduced upon surgical tumor de-bulking, which confirmed the tumor-related origin of this material. These investigators have also demonstrated the functionality of the EV-associated mRNA in driving gene expression (luciferase) upon intercellular transfer. These experiments documented robust biological effects of GBM-derived EVs especially as stimulators of cellular growth and endothelial morphogenesis (Skog et al., 2008). Molecular profiling of GBM-associated EVs unveiled a rather astonishing wealth of molecular species, including mRNA, non-coding RNA (multiple miRs), and proteins, some of which were enriched in EVs in comparison to parental cells (Skog et al., 2008). The mechanism of cargo assembly and molecular enrichment during EV biogenesis remains unclear, but in the case of mRNA this process may depend on a specific "zipcode-like" 25 nucleotide sequence at the 3'UTR. This motif is thought to selectively guide certain transcripts to the regions of EV biogenesis with the help of miR-1289 (Bolukbasi et al., 2012). Various mRNA sequences were detected in EVs isolated from plasma of an independent cohort of GBM patients (Noerholm et al., 2012). With a few aforementioned exceptions, studies do not provide a conclusive picture as to the biological activity *in vivo* and the oncogenic potential of EV-associated molecules found in plasma of patients with GBM, but this remains a disturbing possibility. Moreover, brain tumor cells produce EVs containing oncomirs. This includes the emission of miR-520g, which is a part of the 19q13.41 amplicon associated with a subset of supratentorial primitive neuroectodermal tumors (sPNET) (Li et al., 2009). As mentioned earlier, cells transfected with the corresponding pre-miRNA gene release EVs containing miR-520g (D'Asti et al., 2012). The emission and biological role of other oncomirs involved in primary and secondary brain tumors have not been studied.

EVs isolated from viable brain tumor cells have also been recently shown to contain functional DNA sequences (exoDNA). GBM cells emit EVs that contain retrotransposons and are capable of mediating their transfer to recipient endothelial cells. Several MB cell lines expressing the amplified c-Myc oncogene emit the corresponding genomic sequences as EV cargo, both *in vitro* and *in vivo* (Balaj et al., 2011). It is not clear whether these EVs possess Myc-related biological activity. The corresponding mechanisms by which the relatively large genomic amplicon sequences may be processed into single stranded DNA and inserted into EVs remain to be elucidated.

Similarly, it remains relatively unexplored whether any of the recently uncovered oncogenic mutations in adult and pediatric brain tumors manifest themselves, and contribute to the disease progression via the release of the related mutant proteins (or nucleic acids), as cargo of oncosomes. In this regard, some of the intriguing examples include IDH1 G395 mutations in high grade glioma (Yan et al., 2009), which have recently been detected in EV preparations from GBM culture medium and CSF (Balaj et al., 2012). Similarly, the expression of the gene fusion product KIAA1549/BRAF in juvenile pilocytic astrocytoma (JPA) (Jones et al., 2009) may be detectable in a similar manner, since another form of mutant BRAF (V600E) was found in EVs collected from plasma of melanoma patients (Ramachandran et al., 2011). Rapid progress in molecular characterization of adult and pediatric GBM has recently been extended to RTKs (PDGFR, MET) (Paugh et al., 2011), mutant forms of histone H3.3 and chromatin remodeling genes (Schwartzentruber et al., 2012), as well as several other events involving genetic and epigenetic alterations (Parsons et al., 2008; Lavon et al., 2010). It is noteworthy that by a simple analogy to the aforementioned studies on EGFRvIII, all of these newly discovered molecular changes may result in the emission of EVs endowed with signatures and biological activities resulting from their content of the respective mutant genes and gene products, and thereby may serve as biomarkers for molecular diagnosis of the underlying brain tumors (Al-Nedawi et al., 2009b).

The central nervous system (CNS) is also a site of non-neuroectodermal cancers including hematopoietic malignancies and hemangioma, as well as several secondary brain tumors with distinct molecular underpinnings. The ability of these tumors to elaborate and shed oncosomes still remains to be studied. Metastatic brain tumors often originate from distant cancers such as those of the lung, breast, skin (melanoma), and several other sites, and are associated with high morbidity, mortality, and therapeutic intractability. Virtually nothing is known about the role of oncosomes in such secondary brain tumors, in spite of the fact that these conditions have emerged as a growing therapeutic challenge (Steeg et al., 2011). Correlative studies resulted in detection of tumor-related DNA containing microsatellite markers of chromosome 3p alterations in plasma of patients with non-small cell lung cancer. This finding is indicative of a systemic disease, which often metastasizes to the brain (Lleonart et al., 1998). Also p53 sequences in plasma of ovarian cancer patients may segregate with a higher incidence of brain metastasis (Swisher et al., 2005). In all these cases the association between circulating DNA and the emission of tumor EVs is plausible but unproven. While GBM-derived EVs (Noerholm et al., 2012) and modified exosomes (Alvarez-Erviti et al., 2011) may cross (or circumvent) the blood brain barrier, this may not necessarily apply to the ability of naturally occurring extracranial EVs to access sites of brain metastases, and conversely, it is unknown whether EVs emanating from metastatic brain tumors can freely access systemic circulaton (Steeg et al., 2011). Therefore, it remains to be studied whether formation of metastatic niches in the brain is related to biological activities of cancer or stromal-derived EVs, whether oncosomes participate in these processes, and whether signatures of brain metastases can be found in patient plasma.

THE POSSIBLE ROLE OF EXTRACELLULAR VESICLES AS BIOMARKERS IN BRAIN TUMORS

Brain tumors represent a significant medical challenge due to their anatomical location, functional impact, and biological complexity. Primary brain tumors likely originate from different populations of NSCs and their major types include astrocytoma, oligodendroglioma, meningioma, ependymoma, and embryonal brain tumors such as MB, primitive neuroectodermal tumors (PNET), and atypical teratoid/rhabdoid tumor (AT/RT), each associated with different age-related incidence (Wrensch et al., 2002; Zhu and Parada, 2002; Stiles and Rowitch, 2008). Astrocytic brain tumors constitute the most prevalent and heterogenous brain tumor type in adults and are divided into grades I–IV, according to their histopathological and clinical

characteristics. The most aggressive grade IV tumors are referred to as glioblastoma multiforme (GBM) and presently remain incurable (Wen and Kesari, 2008).

The rapid development of new technologies over the past two decades resulted in the recent explosion of profiling and sequencing studies that have profoundly altered the landscape of primary brain tumors (Li et al., 2012). Perhaps the most notable development in this regard is the subclassification of the traditional, clinically-based nosology into a multitude of molecularly distinct disease subtypes, each characterized by a distinct set of driver mutations, their related oncogenic pathways, and signature changes in the cellular transcriptome, proteome, miR-ome, and epigenome. This complexity carries enormous therapeutic implications as each molecular pathway of brain tumorigenesis and disease subtype may potentially require a different therapeutic paradigm, contain distinct molecular targets for therapy, and could be characterized by separate sets of diagnostic, prognostic, and predictive biomarkers.

Some of the more spectacular examples of recent developments in this regard include the large scale analysis of the mutational status of human GBM with extensive verification of several functional gains and losses (Parsons et al., 2008). In addition to the primary and secondary pathways of GBM progression involving some of the aforementioned genetic events [e.g., EGFR amplification and IDH1 mutation, respectively (Ohgaki and Kleihues, 2009)], high grade glioma is now recognized to consist of at least four major molecular subtypes (neural, proneural, classical, and mesenchymal), which differ in their genetic and epigenetic make-up (Verhaak et al., 2010). Although histologically similar, these tumors also differ from pediatric GBMs, which are characterized by distinct gene expression pattern and unique mutations, involving the growth factor RTKs (PDGFR or MET) (Paugh et al., 2011), mutant histones (H3.3) (Schwartzentruber et al., 2012), and several other genetic and epigenetic abnormalities. Similarly, in MB, several molecular subtypes have recently been discovered, and their molecular drivers (Myc, Wnt, SHH) described in some detail, along with unique genetic events that may separate primary and metastatic tumors in the same patient (Wu et al., 2012).

It is reasonable to predict that, as in the case of EGFRvIII in adult GBM, many (if not all) of these driver, passenger, and signature mutations, in high and low grade adult and pediatric glioma, MB, ependymoma, and other tumors may be present in the corresponding patient plasma as cargo of EVs. Should the appropriate detection methods be developed, EV platform could become invaluable for early diagnosis, subtype determination, longitudinal monitoring of the disease progression, and adaptive following of therapeutic responses (**Figure 3**). These are but a few examples that illustrate the evolving oncogenic landscape of human brain tumors and translational opportunities that EV emission may present in this context.

In addition to the unexpectedly complex molecular nature of brain tumors, they also exhibit considerable intra-lesional heterogeneity. Recent evidence suggests that while certain (classical subgroup) GBM lesions contain regions positive for EGFRvIII expression, this signal may be consistently absent in adjacent tumor tissues (Biernat et al., 2004). Experimental evidence suggests that such oncogenic mosaic is maintained in an active manner by paracrine interactions between cancer cell populations, a process that involves interleukin 6 and other mediators (Inda et al., 2010). Moreover, different regions of GBM may contain clones expressing different oncogenic mutations of cellular RTKs, such as amplified EGFR or PDGFR (or their mixture) (Snuderl et al., 2011). Temporal changes in molecular profiles of brain tumors have also been detected. This spatiotemporal and regional complexity may result in significant sampling errors and diagnostic challenges, especially when coupled with limited and highly invasive access to brain tumor tissue (through surgery or biopsy).

In this regard, the ability of brain tumor cells to shed EVs containing oncogenic, mutant, or otherwise cancer-specific molecular cargo opens a new window of opportunity (**Figure 3**). As we and others have suggested earlier, the access to EVs circulating in blood or CSF may provide an unprecedented glimpse into the repertoire of molecular alterations occurring in individual cancer patients and in real time. As mentioned previously, EVs isolated from plasma of mice harboring GBM xenografts (Al-Nedawi et al., 2008) and from GBM patients (Skog et al., 2008) have already provided a proof of principle in this regard (e.g., detection of EGFRvIII). Preliminary experiments with mouse tumors suggest that this approach may not only permit the analysis of mutant DNA or RNA (and their variations), but also detection of proteins that serve as targets for biological therapeutics (e.g., phospho-EGFR). The related molecular responses may also be reflected in circulating EVs (e.g., EGFR dephosphorylation) (Al-Nedawi et al., 2010). In a few anecdotal cases of GBM, plasma EV samples were found to be positive for the mutant EGFRvIII, while this signal was weak or undetectable in the corresponding surgical tumor tissue (Skog et al., 2008; Al-Nedawi et al., 2010). This may suggest that in those rare cases the EGFRvIII expressing regions of the tumor may have been missed during tissue collection, but the presence of this oncogene could be captured by the analysis of EVs.

The same principle could be extrapolated to tumor-associated stromal cells (microglia, astrocytes, vascular, and inflammatory cells), many of which possess the ability to vesiculate, and their EVs may reveal their functional and molecular states. Since molecular profiling of tumor-associated host cells (*in situ*) has already translated into valuable prognostic information in breast cancer (Finak et al., 2008), so too, at least hypothetically, could the analysis of stromal cells and their related circulating EVs in primary and secondary brain tumors.

While considerable technological barriers still do exist, the analysis of EVs ("vesiculome") in patients with brain tumors may provide unique information as to oncogenic driver mutations, molecular signatures, oncogenic pathways, disease subgroups, drug-resistance-associated mutations (e.g., mutations of EGFR), and other markers. Similarly, temporal changes in the molecular make-up of the tumor could potentially be monitored in individual patients, along with the magnitude of drug responses. In addition the evidence of stem cell markers, microenvironmental

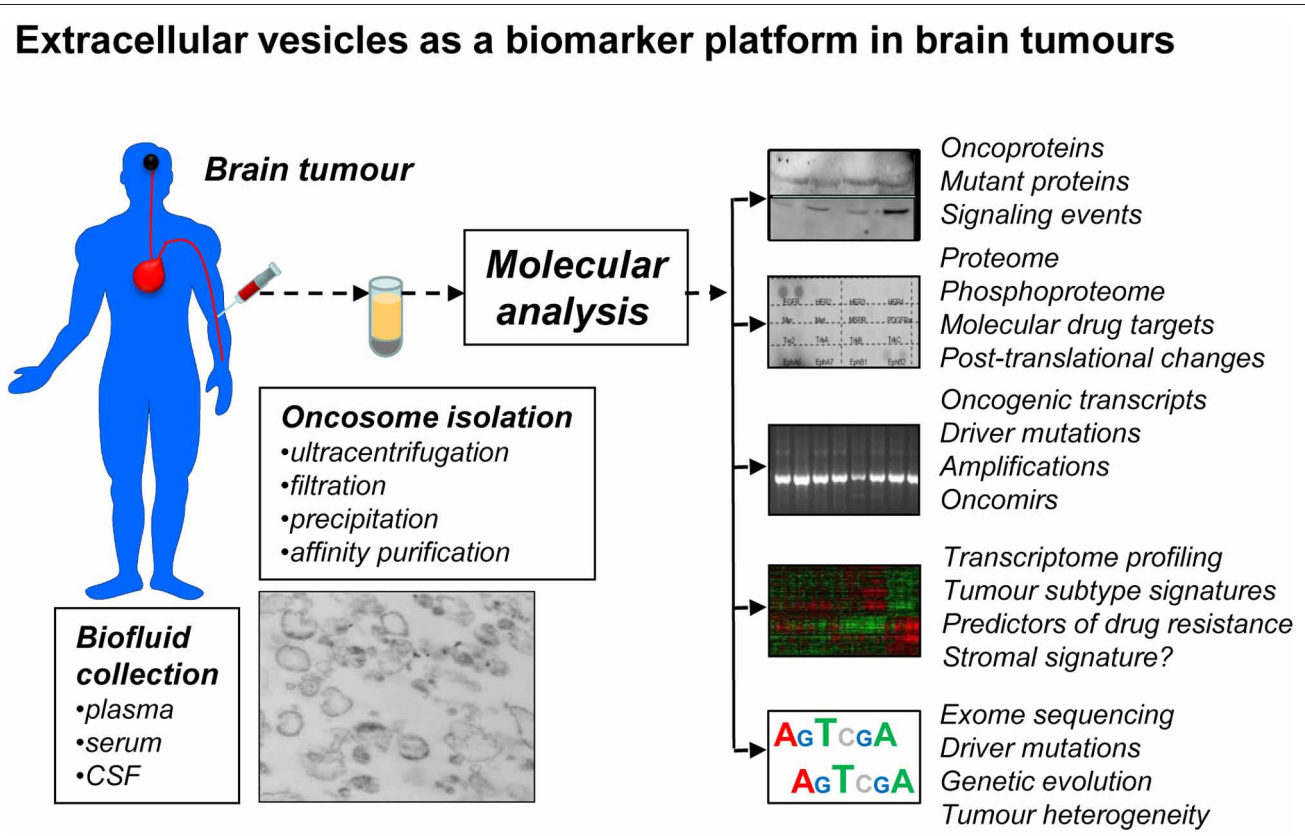

FIGURE 3 | Extracellular vesicles as a prospective biomarker platform in molecular diagnosis of primary and secondary brain tumors. EVs that circulate in peripheral blood or cerebrospinal fluid (CSF) can be readily recovered using several existing methods and various microfluidic and nanotechnology platforms under development. It may be possible to enrich for tumor-derived EVs (oncosomes) using tumor markers (EGFRvIII) and/or specific immunoaffinity techniques. The cargo of oncosomes may be dissected for individual molecules (oncoproteins), their activation, posttranslational processing (phosphorylation) and combinations, including for the purpose of monitoring putative drug targets and their responses to targeted therapies. EVs may provide information as to new, or pre-existing mutations that may occur in brain tumor cells, as well as scope and phylogeny of driver events, for example by sequencing nucleic acids (DNA, RNA) in the cargo. Profiling of proteins and nucleic acids may reveal signatures of brain tumor subgroups and individual variations in gene expression. The challenge is to develop technologies that would ensure sensitivity, specificity, reproducibility and processing of this information for clinical purposes.

responses (e.g., by measuring levels of hypoxia-regulators such as HIF or CAIX) and many other parameters could conceivably be extracted from the molecular cargo of circulating EVs (**Figure 3**). If successful, these approaches could have a major impact on the design of biomarker-driven clinical trials, drug development, and ultimately the outcomes in brain tumors.

SUMMARY

Cellular and regional heterogeneity as well as intercellular communication emerge as key elements in the pathogenesis of brain tumors. In this regard, the involvement of EV-mediated molecular trafficking represents an intriguing aspect, especially as it relates to the horizontal transfer of molecular triggers of cellular transformation: oncogenes, tumor suppressors, and mediators of genetic instability (Rak and Guha, 2012). In so doing, EVs could reprogram cellular phenotypes and recruit indolent and normal cells to participate in angiogenesis, invasion, dissemination, and other events. It is conceivable that a limited numbers of cancer cells that underwent the initial mutation may generate a larger "oncogenic field" by emitting EVs harboring mutant genes (**Figure 2**). It is possible, therefore, that the intercellular trafficking of EVs may serve as a target for new anticancer therapeutics (Al-Nedawi et al., 2009a).

While the relative contribution of EV trafficking to the biology of different types of primary and metastatic brain tumors remains to be thoroughly investigated, the emission of EVs containing molecular signatures may offer unprecedented diagnostic opportunities. Development of new technologies (including microfluidics and nanotechnology) that would secure a non-invasive, remote, and repeated access to biological information encapsulated in circulating EVs has already begun (Shao et al., 2012). Thus, a better understanding of the link between cellular transformation and vesiculation processes may have an enabling influence on the future progress in individualized patient care.

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Exosomes: vehicles for the transfer of toxic proteins associated with neurodegenerative diseases?

Shayne A. Bellingham[1,2,3], Belinda B. Guo[1,2], Bradley M. Coleman[1,2] and Andrew F. Hill[1,2,3]*

[1] *Department of Biochemistry and Molecular Biology, The University of Melbourne, Melbourne, VIC 3010, Australia*
[2] *Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, VIC 3010, Australia*
[3] *Mental Health Research Institute, Melbourne Brain Centre, The University of Melbourne, Melbourne, VIC 3010, Australia*

Edited by:
Claudia Verderio, CNR Institute of Neuroscience, Italy

Reviewed by:
Claudia Verderio, CNR Institute of Neuroscience, Italy
Roberto Chiesa, Istituto di Ricerche Farmacologiche Mario Negri, Italy

***Correspondence:**
Andrew F. Hill, Department of Biochemistry and Molecular Biology, Bio21 Molecular Science and Biotechnology Institute, University of Melbourne, 30 Flemington Road, Parkville, VIC 3010, Australia.
e-mail: a.hill@unimelb.edu.au

Exosomes are small membranous vesicles secreted by a number of cell types including neurons and can be isolated from conditioned cell media or bodily fluids such as urine and plasma. Exosome biogenesis involves the inward budding of endosomes to form multivesicular bodies (MVB). When fused with the plasma membrane, the MVB releases the vesicles into the extracellular environment as exosomes. Proposed functions of these vesicles include roles in cell–cell signaling, removal of unwanted proteins, and the transfer of pathogens between cells. One such pathogen which exploits this pathway is the prion, the infectious particle responsible for the transmissible neurodegenerative diseases such as Creutzfeldt–Jakob disease (CJD) of humans or bovine spongiform encephalopathy (BSE) of cattle. Similarly, exosomes are also involved in the processing of the amyloid precursor protein (APP) which is associated with Alzheimer's disease. Exosomes have been shown to contain full-length APP and several distinct proteolytically cleaved products of APP, including Aβ. In addition, these fragments can be modulated using inhibitors of the proteases involved in APP cleavage. These observations provide further evidence for a novel pathway in which PrP and APP fragments are released from cells. Other proteins such as superoxide dismutase I and alpha-synuclein (involved in amyotrophic lateral sclerosis and Parkinson's disease, respectively) are also found associated with exosomes. This review will focus on the role of exosomes in neurodegenerative disorders and discuss the potential of these vesicles for the spread of neurotoxicity, therapeutics, and diagnostics for these diseases.

Keywords: exosomes, prions, Alzheimer's disease, exosomal shuttle RNA, neurodegenerative diseases

INTRODUCTION

Most, if not all, types of mammalian cells release small membranous vesicles known as exosomes. In addition to their protein content these vesicles have recently been shown to contain messenger RNA (mRNA) and microRNA (miRNA) species. Roles for these vesicles include cell–cell signaling, removal of unwanted proteins, and transfer of pathogens, such as prions, between cells. Prions are the infectious particles that are responsible for transmissible neurodegenerative diseases such as Creutzfeldt–Jakob disease (CJD) of humans or bovine spongiform encephalopathy (BSE) of cattle. Proteins associated with certain neurodegenerative disorders, such as Alzheimer's and Parkinson's disease and the prion diseases CJD and BSE, can be selectively incorporated into intraluminal vesicles of MVBs and subsequently released into the extracellular environment in exosomes. As exosomes can be isolated from circulating fluids such as serum, urine, and cerebrospinal fluid (CSF), they provide a potential source of biomarkers for neurological conditions. This review will describe the roles these vesicles play in neurodegenerative disease and their potential for diagnostics through the analysis of their protein and genetic cargo.

EXOSOME BIOGENESIS AND PROTEIN SORTING

Exosome biogenesis occurs within multivesicular bodies (MVBs) in the endosomal system, which co-ordinates cargo transport between the plasma membrane, trans-Golgi network (TGN), and lysosomes. Collectively, the endosomal system consists of primary endocytic vesicles, early endosomes, and MVBs. Early endosomes are located near the cell membrane where they act as the first port of call for primary endocytosed vesicles which are either recycled to the plasma membrane or targeted to MVBs. Proteins that are sequestered to the limiting membrane of MVBs can be selectively incorporated into intraluminal vesicles (ILVs) by invagination of the MVB membrane. From here, proteins are either degraded by fusion of the MVB with the lysosomal membrane and release of the ILV's into the lysosome, or alternatively, they can be released into the extracellular environment as exosomes when MVBs fuse with the plasma membrane (**Figure 1**). Protein sorting and packaging into ILVs occurs in a regulated manner, involving a variety of mechanisms including mono-ubiquitination and the ESCRT (endosomal sorting complex required for transport) machinery (Hicke, 2001), association with lipid rafts (de Gassart et al., 2003), higher-ordered oligomerization (Vidal et al., 1997; Fang et al., 2007), and segregation into microdomains by ceramide (Trajkovic et al., 2008).

The existence of multiple mechanisms for protein sorting into exosomes has raised the possibility that the various pathways could generate multiple populations of MVBs, containing ILVs with potentially distinct properties and fates (van Niel et al., 2006;

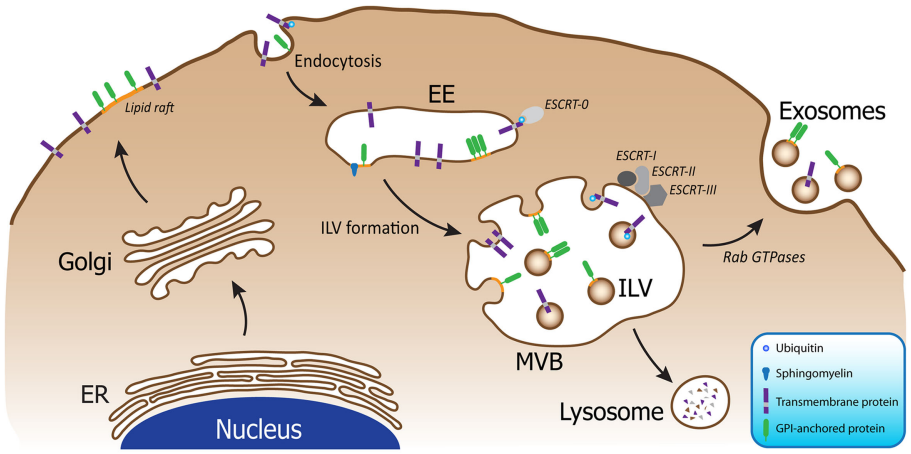

FIGURE 1 | Exosome biogenesis occurs within MVBs of the endosomal system. Following endocytosis into early endosomes (EE), the cargo is packaged into ILVs within MVBs upon inward budding of the membrane. Four different mechanisms have been described to facilitate this process: mono-ubiquitination and the ESCRT machinery; association with lipid rafts; higher-ordered oligomerization; and segregation into microdomains by ceramide. MVBs can then fuse with lysosomes resulting in degradation of the cargo, or alternatively, the MVBs can fuse with the plasma membrane, resulting in release of the ILVs as exosomes, a process which is regulated by Rab GTPases.

Simons and Raposo, 2009). The MVBs could vary in the amount of ILVs that are generated; the content of the ILVs; and also the ultimate fate of the ILVs, either targeted to lysosomes for degradation or released into the extracellular matrix as exosomes. The processes that govern the sorting of neurodegenerative disease related proteins into ILVs could therefore pre-determine the fate of these proteins, and thus play a role in disease progression. However, it is important to keep in mind that redundant pathways are a common phenomenon in biology, and therefore multiple mechanisms may be responsible for sorting various proteins into exosomes. Likewise sorting mechanisms involved may not be mutually exclusive and additional pathways could exist to compensate for disrupted pathways.

THE ROLE OF EXOSOMES IN PRION AND ALZHEIMER'S DISEASES

Prion diseases are fatal, transmissible neurodegenerative disorders that include CJD and Gerstmann–Straüssler–Scheinker Syndrome (GSS) in humans, bovine spongiform encephalopathy (BSE) in cattle and scrapie in sheep. In humans, prion disease occurs in sporadic, familial and acquired etiologies. However, all forms of the disease are transmissible, with possible routes of infection through dietary exposure, medical procedures, and blood transfusion (reviewed in Aguzzi and Heikenwalder, 2006). According to the protein-only hypothesis of prion propagation, an abnormal isoform of the cellular form of the prion protein (PrP^{C}), which is referred to as PrP^{Sc}, is the sole or major component of the infectious prion agent (Prusiner, 1982). The normal prion protein isoform, PrP^{C}, is encoded by *PRNP* and is expressed in all tissues of the human body, with the highest levels of expression observed in tissues of the central nervous system and brain.

Both PrP^{C} and PrP^{Sc} have been isolated in association with exosomes, and PrP^{Sc} containing exosomes were infectious in both animal and cell bioassays (Fevrier et al., 2004; Vella et al., 2007; Alais et al., 2008). In addition, cells loaded with purified PrP^{Sc} have been found to transfer between cells *in vitro* using tunneling nanotubes (Gousset et al., 2009). While tunneling nanotubes can traverse only short distances, exosomes are capable of traveling long distances, and are thus of interest in the peripheral spread of prions. Interestingly, a recent study found exosomes were able to traverse along tunneling nanotubes, suggesting many of these intercellular modes of transport may not be completely independent of one another (Mineo et al., 2012). Although these studies have primarily utilized cultured cell systems to isolate the exosomal vesicles, primary cultured neurons (Faure et al., 2006) and CSF (Vella et al., 2008) have also been used as a source of exosomes in which PrP^{C} has been detected.

The exosome membrane contains lipid rafts enriched in cholesterol, sphingomyelin and ganglioside GM3 (Wubbolts et al., 2003) and externalized phosphatidylserine (Morelli et al., 2004) which are believed to participate in vesicle structure and function, and trafficking of particular proteins to exosomes (de Gassart et al., 2003). PrP^{C} is tethered to the plasma membrane by a glycosylphosphatidyl-inositol (GPI) anchor, and the conversion of PrP^{C} to PrP^{Sc} has been suggested to occur in lipid raft regions (Taylor and Hooper, 2006). Additionally, an interaction between an N-terminal domain of PrP and a postulated lipid raft resident protein or lipid can occur on the membrane (Taylor and Hooper, 2006). GPI tethering is also likely to hold true in exosomes as phase partitioning experiments with Triton X-114 have shown that exosomal PrP^{C} migrates to the detergent phase, consistent with it still containing a GPI anchor (Vella et al., 2007). The presence of lipid rafts in exosomes could also aid in its ability to transmit PrP^{Sc}. One study suggested that the generation of new PrP^{Sc} during infection required the insertion of PrP^{Sc} into lipid rafts (Baron et al., 2002). Hence it is plausible to speculate that the exosome containing PrP^{Sc} may be able to insert its PrP^{Sc} cargo into the membrane of recipient cells upon contact. Another function of the lipid raft nature of exosomes may be to stabilize a particular infectious isoform of PrP^{Sc}. Similar effects have been observed in cell free PrP

conversion systems where lipids have been found to assist in the formation of *de novo* PrP^{Sc}, presumably by acting to stabilize or align intermediary isoforms (Deleault et al., 2007; Wang et al., 2007, 2010).

Alzheimer's disease (AD) is the most common form of dementia in humans and is characterized pathologically by the extracellular deposition of insoluble amyloid plaques comprised of the β-amyloid peptide (Aβ), a 39–43 amino acid peptide produced by proteolytic cleavage of the amyloid precursor protein (APP; Cai et al., 1993; Findeis, 2000; Serpell and Smith, 2000; Murakami et al., 2002). The amyloidogenic pathway of APP processing involves sequential cleavage by β- and γ-secretases. β-Secretase cleaves at the amino-terminus of Aβ (Seubert et al., 1993; Mattson, 1997) resulting in the release of secreted APP, and leaves intact Aβ as a membrane-associated, 99-amino-acid carboxy-terminal fragment (β-CTF). β-CTF can undergo endocytosis via clathrin-coated vesicles (Selkoe, 1996) and is trafficked to various endosomal compartments, including MVBs, from which exosomes are derived (Yamazaki et al., 1996).The initial link between Aβ and its association with exosomes proposed that intracellular-accumulated Aβ in MVBs is incorporated into exosomes and released into the extracellular environment (Rajendran et al., 2006; Sharples et al., 2008). The identification of Aβ in association with exosomes is an important finding, especially as other exosomal proteins such as Alix and Flotillin-1 have been found to accumulate in the plaques of brains from patients with AD (Rajendran et al., 2006). Exosomes could also provide an explanation for transport of Aβ and the equally toxic APP-CTFs around the body to the brain, where they contribute to amyloid deposition.

PRION LIKE MECHANISMS IN OTHER NEURODEGENERATIVE DISEASES

While prion diseases have long been thought to be the only neurodegenerative disease that is infectious and capable of spreading between individuals, key proteins involved in other neurodegenerative diseases such as Alzheimer's, Parkinson's and ALS may share similar behavioral features as prions (**Table 1**). At a basic biochemical level, misfolding and aggregation of these proteins can occur through seeded polymerization which has an initial, lengthy lag phase. However, this lag phase can be largely eliminated through the introduction of an already misfolded seed. Like prions, these aggregates appear to be able to persistently self-propagate, and there is also evidence of spreading from cell-to-cell and throughout the CNS. The spreading of these aggregates is no more obvious than when the neuropathology and deposition of the pathological proteins during these diseases is examined.

A considerable number of *in vivo* studies during the last half century have shown that prions from one diseased source can be introduced into an otherwise healthy animal and cause PrP^{Sc} formation and clinical prion disease. However it wasn't until the mid-nineties when a similar experiment was conducted in which brain extracts from human Alzheimer's patients were injected into otherwise healthy primates and the formation of amyloid plaques was observed in the site of injection, and adjacent brain regions (Baker et al., 1994). These observations have since been reproduced in murine models of Alzheimer's disease (Kane et al., 2000; Meyer-Luehmann et al., 2006; Morales et al., 2011). Similarly, tau misfolding can be induced in transgenic mice by injection of tissue homogenates containing aggregated tau (Clavaguera et al., 2009). This phenomenon has also been observed in humans when patients with Parkinson's disease received grafts of normal neuronal stem cells. Upon post-mortem examination it was found that the grafts contained α-synuclein inclusions that could only have arisen through transmission or spread from diseased brains (Kordower et al., 2008; Li et al., 2008). Interestingly, brain homogenates containing α-synuclein aggregates, when injected into transgenic mice, were also found to be able to initiate both aggregation of α-synuclein and the onset of clinical symptoms (Hansen et al., 2011). Given the intracellular origin of exosomes, they present a potential pathway in which cytosolic neurodegenerative disease related proteins are released into the extracellular space. In fact, a number of neurodegenerative disease related cytosolic proteins have already been found to be contained within exosomes such as α-synuclein (Emmanouilidou et al., 2010; Alvarez-Erviti et al., 2011a) and tau (Saman et al., 2011). SOD1 has also been found released in association with exosomes from a cell model of ALS (Gomes et al., 2007).

Inoculation of permissive cell lines with brain homogenates from prion infected mice results in infection of cells with prions, and continual replication of PrP^{Sc} that amplifies over time with increased passage of cells (Vella et al., 2007; Courageot et al., 2008; Vilette, 2008). Similar observations can be seen with other neurodegenerative diseases. Cultured neuronal cells incubated with tau aggregates were found to take up fibrils in an endocytic manner, and subsequently induce fibrillization of cytoplasmic tau (Frost et al., 2009; Guo and Lee, 2011). The newly aggregated intracellular tau was also capable of transferring between co-culture cells (Frost et al., 2009). Likewise polyglutamine peptide aggregates can also be taken up by cultured cells and sequestered into aggresomes, where they can recruit cytoplasmic proteins and transfer the aggregates between co-cultured cells (Ren et al., 2009). The application of exogenous recombinant aggregated α-synuclein to cultured cells has also been found to be efficient at seeding aggregation of its intracellular counterpart, and subsequent transfer between cells by a number of groups (Danzer et al., 2009; Desplats et al., 2009; Hansen et al., 2011; Volpicelli-Daley et al., 2011).

Most recently, mutant SOD1 aggregates have been shown to be able to enter neuronal cells where they can seed aggregation of the normal, cytoplasmic mutant SOD1 (Munch et al., 2011). Upon removal of the seed, aggregation persists, suggesting these newly formed endogenous aggregates are capable of continually seeding further aggregation in a mechanism similar to PrP^{Sc}. Although it is unclear if exosome associated SOD1 is pathogenic, a mutant form of SOD1 was able to pass from cells through a 0.4 μm filter and propagate in recipient cells (Munch et al., 2011). This suggests that mutant SOD1 aggregate seeds are less than 0.4 μm and/or the aggregates are contained within exosomes. A similar experiment has been performed using these 0.4 μm filters to demonstrate that PrP^{Sc} can be transferred from one cell to another without direct cell contact, thus suggesting involvement of exosomes in this process (Alais et al., 2008).

Prion infectivity and seeding ability has been detected in the CSF of prion infected animals and humans (Atarashi et al., 2007, 2008, 2011; Wilham et al., 2010; Orru et al., 2012), and exosomes

Table 1 | Neurodegenerative diseases associated with exosomes.

Disease protein or peptide in aggregates	Normal protein	Examples of human disease	Human disease transmission	Aggregate seeding in cell culture	Aggregate seeding in mice	Cell-to-cell transfer of aggregates	Seeded clinical disease in mice	Associations of proteins with extracellular transport mechanisms	Reference
PrP^{Sc}	PrP^{C}	Creutzfeldt–Jakob, Fatal familial insomnia, Gerstmann–Straussler Sheinker, Kuru	Yes	Yes	Yes	Yes	Yes (using synthetic PrP^{Sc}, with infected cell lysates, exosomes and tissue extracts)	Direct cell-to-cell, exosomes, tunneling nanotubes	Magalhaes et al. (2005), Kanu et al. (2002), Fevrier et al. (2004), Vella et al. (2007), Gousset et al. (2009), Wang et al. (2010), Alais et al. (2008), Prusiner (1982)
Aβ	APP	Alzheimer's	No	Yes	Yes	No	No	Direct cell-to-cell and minor portion observed in exosomes	Magalhaes et al. (2005), Meyer-Luehmann et al. (2006), Kane et al. (2000), Morales et al. (2011), Rajendran et al. (2006), Sharples et al. (2008)
α-synuclein	α-synuclein	Parkinson's	No	Yes	Yes. Also in human stem cell grafts	Yes	Yes (using brain extract from disease mice)	Observed in exosomes	Luk et al. (2009), Alvarez-Erviti et al. (2011a), Danzer et al. (2009), Desplats et al. (2009), Hansen et al. (2011), Kordower et al. (2008), Li et al. (2008)
Tau	Tau	Alzheimer's, frontotemporal lobar dementia, progressive supranuclear palsy	No	Yes	Yes	Yes	No	Unknown	Clavaguera et al. (2009), Frost et al. (2009), Guo and Lee (2011), Saman et al. (2011)
SOD1	SOD1	Amyotrophic lateral sclerosis (ALS)	No	Yes	Unknown	Yes	Unknown	Released from cells and observed in exosomes	Gomes et al. (2007), Munch et al. (2011)
PolyQ	Huntington	Huntington's disease	No	Yes	Unknown	Yes	Unknown	Unknown	Ren et al. (2009)

isolated from sheep CSF are enriched in PrP^C (Vella et al., 2008). Therefore it is plausible that some infectivity may be released into the CSF in association with exosomes in infected animals. Interestingly, exosomal markers were found to be enriched in amyloid plaques in the brains of mice (Kokubo et al., 2005) and post-mortem human AD patients (Rajendran et al., 2006), suggesting exosomes played a role in trafficking of Aβ aggregates to these sites during disease progression. Monomeric and oligomeric α-synuclein and tau have also been found in blood and CSF in PD and AD patients (Vandermeeren et al., 1993; El-Agnaf et al., 2006; Tokuda et al., 2010; Bruggink et al., 2011), suggesting release from the CNS possibly in association with exosomes. Phosphorylated tau was also found in exosomes from human CSF in early Alzheimer's disease (Saman et al., 2011). Together, these observations provide strong evidence for the *in vivo* involvement of exosomes in neurodegenerative disease. While there is mounting evidence demonstrating exosomes maybe involved in the spread of pathogenic neurodegenerative disease related proteins, it is unknown whether they act purely as a transport mechanism or perhaps their structural and molecular makeup aid in the process.

EXOSOME STRUCTURE AND FUNCTION IN NEURODEGENERATIVE PROTEIN TRANSFER

Exosomes contain an array of different proteins; some are specific to the cell type of origin, while others are common across all exosomes. As a result of their endosomal origins exosomes contain proteins involved in membrane fusion and transport such as the Annexins and Rab proteins. Exosomes also contain heat shock proteins, adhesion molecules, metabolic enzymes, cytoskeletal proteins and are heavily enriched in tetraspanins such as CD63 and CD81 [as discussed in a number of reviews including (Thery et al., 2002; Schorey and Bhatnagar, 2008)] (**Figure 2**). It is suspected that proteins on the surface of exosomes aid in their uptake by recipient cells. Using proteases to shave off surface proteins of both exosomes and/or cells has been shown to reduce cellular uptake of exosomes (Escrevente et al., 2011), suggesting proteins on the surface of cells and exosomes act as mediators for facilitating fusion. It has also been shown that exosome transfer is inhibited at 4°C (Escrevente et al., 2011). These observations suggest exosome uptake is not a passive process, but is energy dependent and mediated by protein receptors.

Unlike the spread of prions via exosomes which need only surface interaction, transfer of the cytosolic neurodegenerative proteins into the recipient cells require the exogenous transporter exosomes to fuse with the plasma membrane. Direct fusion with the plasma membrane and release of contents into cytosol has been demonstrated with the use of luciferin-loaded exosomes which "injected" their intraluminal content into the cytosol of the target dendritic cells (Montecalvo et al., 2012). A similar mechanism is likely to occur when cationic liposomes were used to deliver α-synuclein fibrils to cultured cells (Luk et al., 2009). The liposomes fused with the plasma membrane, delivering their exogenous α-synuclein fibrils and induced formation of Lewy-body like structures in the cytoplasm of the recipient cell. Similarly, it was found that combining purified PrP^{Sc} with microsomes or liposomes improved the efficiency of the PrP^{Sc} as an inoculum in cell infections (Gabizon et al., 1988; Baron et al., 2006). Exosomes could potentially function in a similar manner to microsomes or liposomes by direct fusion with the plasma membrane improving the cellular uptake of prions or other neurodegenerative disease associated proteins. An alternative uptake mechanism of exosomes through endocytosis has been observed in rat neuronal PC12 cells with the endocytosed exosomes being trapped in cytosolic vesicles, and subsequently transported to the perinuclear region where they accumulate as large organelles (Tian et al., 2010). It is also possible that following endocytosis, back fusion can occur between the exosomal membrane and the limiting membrane of the endocytic compartment, thus releasing the exosomes luminal contents into the cytosol. Finally, aggregated proteins can damage lipid bilayers and form pores (Tsigelny et al., 2008) – a process which could occur post endocytosis releasing the misfolded proteins into the cytosol where they can seed further aggregation of the normal endogenous proteins.

ROLE OF EXOSOMAL SHUTTLE RNA IN NEURODEGENERATIVE DISEASE

Since their discovery, exosomes were thought to contain lipids and proteins with no genetic material (Johnstone et al., 1987). However, Ratajczak et al. (2006) reported that microvesicles, which are circular membrane vesicles 100–1000 nm shed directly from the plasma membrane, contained RNA. While Valadi et al. (2007) demonstrated that exosomes isolated from human and mouse mast cells contain both mRNA and miRNA, but no DNA. miRNA's are a class of non-coding RNA (ncRNA) species of approximately 22 nucleotides in length that function by post-translational repression of target mRNA's by binding to their 3′ untranslated regions (Bartel, 2004). Valadi et al. (2007) demonstrated that the exosomal RNA differed from that of donor cells from which they were derived, contained little or no ribosomal RNA and were enriched in small RNA species. Transfer experiments revealed that exosomal RNA is shuttled between donor and recipient mast cells and remained functional upon uptake in the recipient cell cytoplasm. Collectively these miRNA and mRNA species have been termed "exosomal shuttle RNA" (esRNA). esRNA was also shown to be contained and protected from degradation within the exosome vesicles themselves and it was proposed that exosome-mediated transfer of esRNA is a novel mechanism of genetic exchange between cells, either in the microenvironment or over a distance.

esRNA has since been isolated from exosomes derived from cancer cells, stem cells and dendritic cells (Taylor and Gercel-Taylor, 2008; Rabinowits et al., 2009); and biological fluids such as peripheral blood (Hunter et al., 2008), serum (Skog et al., 2008; Taylor and Gercel-Taylor, 2008), and saliva (Michael et al., 2010). Moreover, several studies have demonstrated a functional role of miRNA mediated transfer of exosomes to recipient cells (Valadi et al., 2007; Kosaka et al., 2010; Pegtel et al., 2010; Yang et al., 2011; Montecalvo et al., 2012). Tumor-suppressive miRNA's have been shown to be functional when transferred by exosomes in a ceramide dependent manner in prostate cancer cell lines (Kosaka et al., 2010). While immature and mature dendritic cells can package and release miRNA in exosomes dependent upon their activation state, with released exosomes fusing with recipient cell plasma membranes transferring functional miRNA into the cytosol (Montecalvo et al., 2012). Macrophages can also regulate

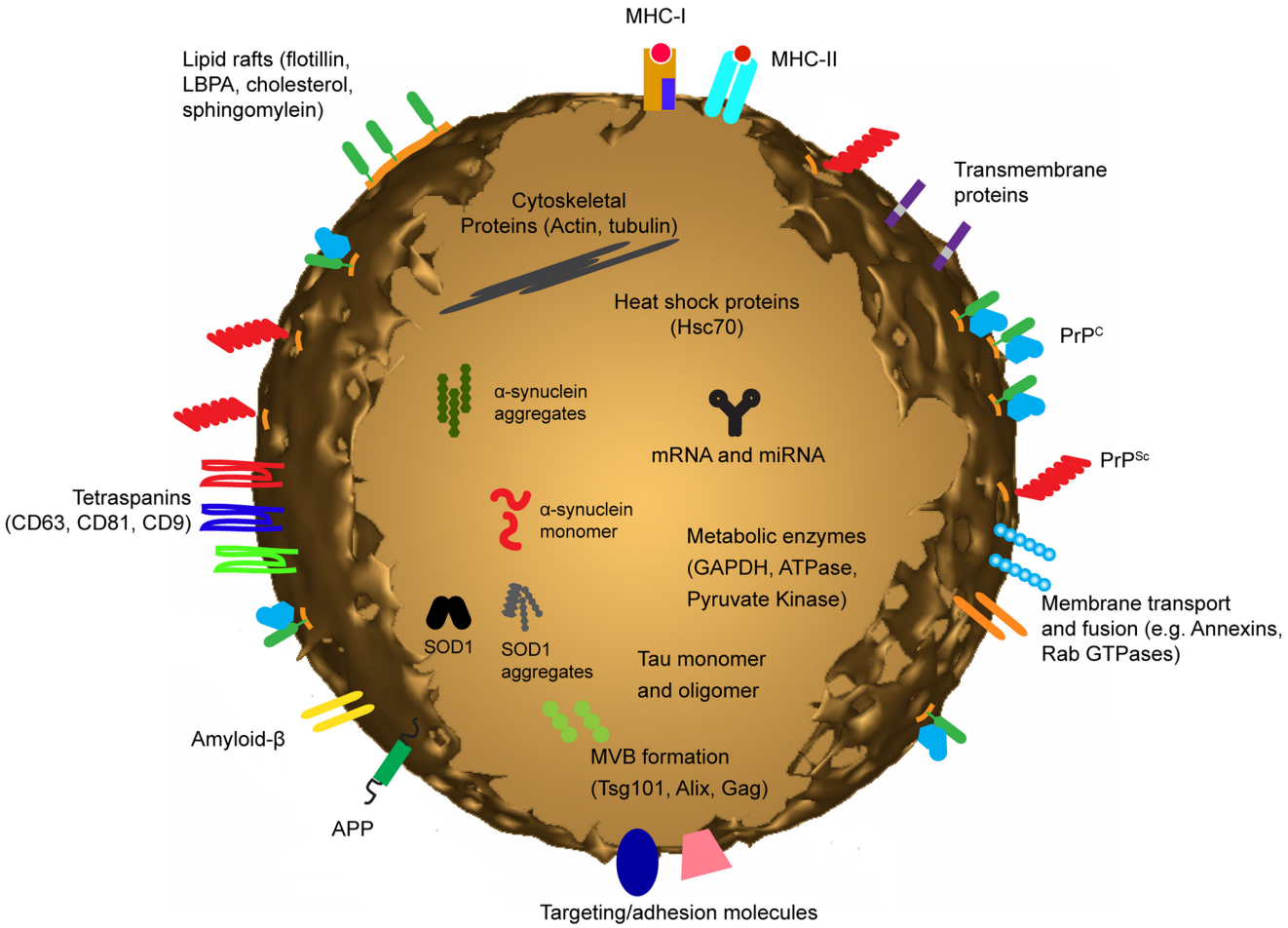

FIGURE 2 | Exosomes are small membrane bound vesicles sharing similar topology to the plasma membrane. They contain mRNA and miRNA, and a vast array of different proteins depending on their host cell. However they are generally all enriched in proteins involved in MVB formation, tetraspanins, membrane transport and fusion and a number of cytosolic proteins. In addition to these generic proteins, proteins associated with neurodegenerative diseases such as Alzheimer's, Prion disease, and Parkinson's disease have been identified in exosomes.

the invasiveness of breast cancer through exosome-mediated delivery of miRNA into cells promoting metastasis (Yang et al., 2011). Exosomes can also functionally deliver ncRNA, retroviral RNA repeats and tRNA sequences which are subsequently incorporated into RNA-silencing pathways (Gibbings et al., 2009; Lee et al., 2009; Haussecker et al., 2010). Likewise, tumor microvesicles have been demonstrated to contain elevated levels of specific coding, ncRNA and retrotransposon RNA transcripts from endogenous retroviruses that could be horizontally transferred during cancer progression (Balaj et al., 2011). These observations suggest that exosomes package and functionally deliver genetic components such as mature miRNA, mRNA, ncRNA, and retroviral RNA to the microenvironment, strongly supporting the role of exosomes in gene regulation mechanisms and intercellular communication.

Exosomes have been described to be released from a variety of neuronal cells types including microglial cells (Potolicchio et al., 2005), developing neurons (Faure et al., 2006), cultured astrocytes (Taylor et al., 2007), and oligodendrocytes (Kramer-Albers et al., 2007). Additionally, they have been shown to play a role in the normal physiology and synaptic plasticity of the central nervous system, with secretion of exosomes being regulated by calcium influx and glutamatergic synaptic activity in cortical and hippocampal neurons (Lachenal et al., 2011). Therefore, it is plausible to suggest that functional transfer of esRNA plays a significant yet uncharacterized role in the progression of neurodegenerative disorders such as Prion diseases, Alzheimer's disease, and other related disorders.

The nervous system is a rich source of miRNA expression, with estimates suggesting that neuronal miRNA's can post-transcriptionally modulate the expression of more than a third of the coding mRNAs (Kosik, 2006). While the functions of many discovered miRNA's so far remain unknown, some miRNA's have been shown to play a role in several biological processes including proliferation, organ development, cell differentiation, apoptosis, and infectious disease (Croce and Calin, 2005). The role of deregulated miRNA has also been implicated in cancer, with evidence suggesting that aberrant miRNA expression can function both as tumor suppressors and oncogenes (Esquela-Kerscher and Slack,

2006). Aberrant miRNA expression has also been identified as a factor in neurodegenerative related disorders (reviewed in Hebert and De Strooper, 2007, 2009); with **Table 2** summarizing miRNA's implicated in Prion diseases, Alzheimer's disease, Parkinson's, and Tauopathies.

In Alzheimer's disease, miRNA's have been shown to target the 3′ UTR of several key genes by regulating the expression and function of APP and BACE (the enzyme responsible for β-secretase cleavage of APP) in cell culture models of AD (Hebert et al., 2008, 2009; Patel et al., 2008; Boissonneault et al., 2009; Vilardo et al., 2010; Long and Lahiri, 2011). Extensive studies on post-mortem human AD brain samples and in transgenic AD mouse models also identified several miRNA that are significantly deregulated during the disease process (Lukiw and Pogue, 2007; Sethi and Lukiw, 2009; Nelson and Wang, 2010; Schonrock et al., 2010, 2012; Shioya et al., 2010; Li et al., 2011; Wang et al., 2011; Lukiw and Alexandrov, 2012).

Deregulated miRNA expression has also been suggested as a mechanism for failure of proteosomal degradation of insoluble- and phosphorylated-tau proteins in tauopathies (Carrettiero et al., 2009), while a number of brain miRNA's have been shown to regulate the ratio of tau 3-repeat and 4-repeat isoforms causing progressive supranuclear palsy (Smith et al., 2011; Wanet et al., 2012). Interestingly genetic ablation of Dicer, which is responsible for mature miRNA biogenesis, in adult forebrain neurons results in hyperphosphorylation of tau, neuronal loss in the hippocampus, and cellular shrinkage in the cortex (Hebert et al., 2010). The hyperphosphorylation of tau was subsequently demonstrated to involve up-regulation of ERK kinases by deregulation of miR-15 in AD brains (Hebert et al., 2010).

Downregulation of miRNA's 133b and miR-34b/34c has been demonstrated in Parkinson's disease mid-brain dopaminergic neurons (Kim et al., 2007; Minones-Moyano et al., 2011), while miR-7 and miR-153 can regulate expression of α-synuclein 3′ UTR (Junn et al., 2009; Doxakis, 2010). Moreover, pathogenic LRRK2 can cause familial as well as sporadic Parkinson's disease characterized by age-dependent degeneration of dopaminergic neurons, possibly due to inhibition of miR-7 mediated translational repression of α-synuclein (Gehrke et al., 2010).

A miRNA signature in prion disease has been reported in prion infected mice and primates (Saba et al., 2008; Montag et al., 2009). Both studies examined the miRNA profile in brain tissue after clinical symptoms of disease were well-established and determined a subset of miRNA's to be significantly deregulated. In prion infected

Table 2 | Dysregulation of miRNA's in neurodegenerative diseases.

Neurodegenerative disease	Deregulated miRNA	Reference
Prion diseases	let-7b, miR-128, miR-139-5p, miR-146a, miR-320, miR-328 and **miR-342-3p**	Saba et al. (2008)
	miR-342-3p and miR-494	Montag et al. (2009)
	miR-146a	Lukiw et al. (2011), Saba et al. (2012)
Alzheimer's disease	miR-9, miR-124, miR-125b, miR-128, miR-132 and miR-219	Lukiw and Pogue (2007)
	miR-9, **miR-29a** and **miR-29b**	Hebert et al. (2008)
	miR-106a and miR-520c	Patel et al. (2008)
	miR-107	Wang et al. (2008), Nelson and Wang (2010)
	miR-298 and miR-328	Boissonneault et al. (2009)
	miR-17-5p, miR-20, and miR-106b	Hebert et al. (2009)
	miR-9, miR-125b and miR-146a	Sethi and Lukiw (2009)
	miR-146a	Li et al. (2011), Lukiw et al. (2011)
	miR-101	Vilardo et al. (2010), Long and Lahiri (2011)
	miR-9 and miR-191c	Schonrock et al. (2010), Schonrock et al. (2012)
	miR-29a	Shioya et al. (2010)
	miR-15, miR-107, **miR-29a**, **miR-29b**, miR-212, miR-424	Wang et al. (2011)
	miR-125b and miR-146a	Lukiw and Alexandrov (2012)
Parkinson's disease	miR-133b	Kim et al. (2007)
	miR-7	Junn et al. (2009)
	miR-7 and miR-153	Doxakis (2010)
	miR-7 and miR-184*	Gehrke et al. (2010)
	miR-34b and miR-34c	Minones-Moyano et al. (2011)
Tauopathies	miR-128	Carrettiero et al. (2009)
	miR-15	Hebert et al. (2010)
	miR-9, miR-124, miR-132 and miR-137	Smith et al. (2011)
	miR-132 and miR-212	Wanet et al. (2012)

miRNA that has been shown to be deregulated in a number of neurodegenerative diseases are underlined.

miRNA deregulated in individual neurodegenerative disease and validated by independent studies are indicated in bold.

mice, Saba et al. (2008) identified 15 miRNA's to be significantly deregulated upon prion infection including up-regulation of miR-342-3p and miR-146a. miR-342-3p was also up-regulated in prion infected primate cynomolgus macaques and in brain tissue of type-1 and type-2 human sporadic CJD cases (Montag et al., 2009). Up-regulation of miR-146a during prion infection is proposed to involve inflammatory response pathways and correlates with deposition of prion plaques and activation of surrounding microglia (Saba et al., 2012). Up-regulated miR-146a was also observed in human neuronal–glial primary cell co-cultures challenged with five different species of single- or double-stranded DNA or RNA neurotrophic viruses, pro-inflammatory cytokines, Aβ42 peptide, metal-induced neurotoxicity, and oxidative stress. Furthermore, miR-146a up-regulation was also observed in murine scrapie, in AD brains, and rare human prion disorders, including sporadic CJD and GSS (Lukiw et al., 2011). These results suggest that miR-146a up-regulation in human brain cells is a general mechanism of innate immune response and antiviral immunity (Lukiw et al., 2011).

Despite the increasing body of evidence implicating dysregulated miRNA expression in a number of neurodegenerative disorders and exosomes are implicated in the pathogenic transfer of neurotoxic proteins in these diseases, very little research has focused on the potential role of esRNA in pathogenesis and diagnosis of neurological diseases. Given that several studies have successfully identified miRNA profiles from circulating exosomes isolated from plasma and serum samples in the diagnosis of human diseases including ovarian cancer, glioblastoma, and lung adenocarcinoma (Skog et al., 2008; Taylor and Gercel-Taylor, 2008; Rabinowits et al., 2009), then it is plausible to suggest that miRNA profiling can be applied to diagnosis in other diseases. Recently, it has been reported that extracellular miRNA released from cells in plasma can associate in two populations, both dependent and independent of exosomes either bound to AGO2 (Arroyo et al., 2011; Turchinovich et al., 2011) or high-density lipoproteins (Vickers et al., 2011). Therefore, targeted exosomal purification strategies for enrichment of circulating miRNA biomarkers may be required to increase biomarker sensitivity. Moreover, circulating exosomes isolated for the study of Prion, Alzheimer's, Parkinson's, and other related disorders represent a unique subset of exosomal populations that can be enriched by targeting the defined toxic protein biomarkers associated with these disorders. This strategy can then be coupled to small RNA next generation sequencing technologies to accurately determine circulating exosomal miRNA signatures specific to individual diseases.

POTENTIAL OF EXOSOMES IN NEURODEGENERATIVE DISEASE THERAPEUTICS?

With the discovery that exosomes are able to transmit protein, mRNA and miRNA between cells, the possibility arose that exosomes could be exploited as vehicles for delivering therapeutic compounds *in vivo* (Valadi et al., 2007; Simons and Raposo, 2009; Sun et al., 2010). Since the development of RNA interference (RNAi), much work has been carried out to utilize this technology for treatment of various diseases. However, many barriers were encountered, plaguing clinical translation of this technology. One of the biggest issues faced is the ability to target specific tissues and at therapeutic doses without eliciting immune responses and inducing toxicity (van den Boorn et al., 2011). Current approaches for clinical application of RNAi include usage of viral and synthetic carrier systems such as liposomes and nanoparticles. However, there are several disadvantages with these systems. Viral particles can be cleared from the body by antibodies and also has the potential to activate immune responses, making repeated administration challenging (Waehler et al., 2007). Harnessing exosomes, the body's own intercellular delivery mechanism, would therefore provide a breakthrough for the field of drug delivery and it would bypass many issues such as immune activation, acceptance by target cells, and prevent degradation of cargo.

In a recent study, the first steps toward the application of exosomes as a drug delivery vehicle was taken. Using exosomes from immature murine dendritic cells, Alvarez-Erviti et al. (2011b) modified the exosomes to express a fusion of the exosomal membrane protein Lamp2b and a neuron-specific RVG peptide. The exosomes were then loaded with siRNA for BACE1, a protein implicated in Alzheimer's disease. Following intravenous injection, the exosomes were targeted to the neurons, microglia and oligodendrocytes in the mouse brain, and was able to induce knockdown of BACE1. This discovery highlighted the therapeutic potential of exosome-mediated RNAi technology, with possible applications in neurodegenerative diseases, such as prion disease and Alzheimer's disease, where the key mediators of these diseases can be targeted for knockdown using this approach.

Despite the appeal, in order to apply this technology clinically, a few issues still need to be addressed. Firstly, a stable source of well characterized exosomes that can be expanded needs to be established (van Dommelen et al., 2011). The process of loading exosomes with siRNA will also require optimization in order to achieve maximum efficiency and reduce the need to administer large amounts of exosomes during therapy. Tissue-specific targeting of exosomes should also be further refined to ensure both a safe and specific delivery to target tissues, as well as efficient diffusion across the blood brain barrier. With further improvements in techniques and technology, exosomes hold great potential to revolutionize RNAi-mediated therapy, opening up a door to an alternative, untapped source of clinical therapy.

The precise mechanisms that govern the packaging of esRNA inside exosomes remain unanswered. While several potential mechanisms can be postulated, the most logical explanation is that RNA is packaged directly from the cytoplasm as a result of initial invagination of the MVB into ILV's. However, several studies have shown that exosomes contain little or no 18S and 28S cellular ribosomal species; not all mRNA and miRNA contained within cells can be detected in exosomes, and that some mRNA and miRNA can be directly targeted and packaged in exosomes (Valadi et al., 2007; Hunter et al., 2008; Taylor and Gercel-Taylor, 2008; Pigati et al., 2010; Montecalvo et al., 2012). These observations suggest that esRNA is selectively incorporated into ILV's as opposed to random events or contamination during the process of exosome isolation.

CONCLUSION

As exosomes are being associated with an increasing number of neurodegenerative disorders they may provide a source

of both protein and genetic biomarkers obtained from circulating exosomes, as well as new insights into the observed spreading of neuropathologic lesions common to these diseases. One current drawback of using exosomes for biomarker discovery is the methods that are used for their isolation (based on ultracentrifugation and filtration) as these methods are not immediately amenable for high-throughput screening. The identification of esRNA in exosomes provides an attractive target for biomarkers as demonstrated by the studies in brain tumors. Applying the genetic analysis of exosomes from neurodegenerative disorders such as prion diseases and AD may identify suitable biomarkers that are applicable for diagnostic applications.

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Potential contribution of exosomes to the prion-like propagation of lesions in Alzheimer's disease

Valérie Vingtdeux*[1], *Nicolas Sergeant*[2,3]* and *Luc Buée*[2,3]

[1] *Litwin-Zucker Research Center for the Study of Alzheimer's Disease, The Feinstein Institute for Medical Research, North Shore-Long Island Jewish, Manhasset, NY, USA*
[2] *UMR 837, Institute National pour la Santé et la Recherche Medical, Alzheimer and Tauopathies, Lille, France*
[3] *Faculty of Medicine, Jean-Pierre Aubert Research Centre, Institute of Predictive Medicine and Therapeutic Research, Université Lille Nord de France, UDSL, Place de Verdun, Lille, France*

Edited by:
Claudia Verderio, CNR Institute of Neuroscience, Italy

Reviewed by:
Lawrence Rajendran, University Zurich, Switzerland
Andrew Hill, The University of Melbourne, Australia
Pascal Kienlen-Campard, Université Catholique de Louvain, Belgium

***Correspondence:**
Nicolas Sergeant and Luc Buée, UMR 837, Institute National Pour la Santé et la Recherche Medical, Alzheimer and Tauopathies, Centre de Recherches Jean-Pierre Aubert, Faculté de Médecine Pole Recherche, Place de Verdun, F-59045 Lille, France.
e-mail: nicolas.sergeant@inserm.fr; luc.buee@inserm.fr

Since the discovery of prion diseases, the concept has emerged that a protein could be a transmissible pathogen. As such, this transmissible pathogen agent can transfer its pathological mis-folded shape to the same but normally folded protein thus leading to the propagation of a disease. This idea is now extrapolated to several neurological diseases associated with protein mis-folding and aggregation, such as Alzheimer's disease (AD). AD is a slowly developing dementing disease characterized by the coexistence of two types of lesions: the parenchymal amyloid deposits and the intraneuronal neurofibrillary tangles (NFT). Amyloid deposits are composed of amyloid-beta peptides that derive from sequential cleavages of its precursor named amyloid protein precursor. NFT are characterized by intraneuronal aggregation of abnormally modified microtubule-associated Tau proteins. A synergistic relationship between the two lesions may trigger the progression of the disease. Thus, starting in the medial temporal lobe and slowly progressing through temporal, frontal, parietal, and occipital cortex, the spreading of NFT is well correlated with clinical expression of the disease and likely follows cortico-cortical neuronal circuitry. However, little is known about the mechanism driving the spatiotemporal propagation of these lesions ultimately leading to the disease. A growing number of studies suggest that amyloid deposits and NFT are resulting from a prion-like spreading. In the present chapter, we will develop the current hypotheses regarding the molecular and cellular mechanisms driving the development and spreading of AD lesions from the window of multivesicular endosomes/bodies and exosomes.

Keywords: Alzheimer's disease, tauopathies, multivesicular bodies, exosomes, amyloid precursor protein, microtubule-associated tau protein

ALZHEIMER'S DISEASE, AMYLOID DEPOSITS, AND APP METABOLISM

Alzheimer's disease (AD) is a slow and progressive disease affecting the brain and characterized by the loss of superior cognitive functions ultimately leading to dementia and death. Two neuropathological brain lesions are found in the brain and their presence is necessary for providing a definite diagnosis of the disease, as firstly described by Alzheimer (1911).

Amyloid deposits are amorphous parenchymal deposits of β-sheet ordered proteinaceous material. They are observed with aging, in AD, Down's syndrome, dementia with Lewy bodies, and vascular dementia, all of which are aged-related neurodegenerative disorders. The major component of amyloid deposits is a small peptide of 39 to 43 amino acid residues, named Aβ for amyloid-beta peptide (Glenner and Wong, 1984). It derives from a sequence of successive cleavages of a larger precursor protein named APP. *APP* gene is located on the long arm of chromosome 21 at position 21q11.2 (Goldgaber et al., 1987; Kang et al., 1987). APP is a type I transmembrane protein with a large extra amino-terminal membrane domain, a transmembrane domain, and a short carboxy-terminal cytosolic tail composed of 59 amino acids (**Figure 1**). The principal role of APP remains elusive but several functions are proposed, for instance APP was recently suggested to contribute to iron cellular homeostasis (Duce et al., 2010), to regulate intracellular transport via its interaction with motor proteins such as kinesin, to be a cell surface receptor. Extracellular fragments derived from the cleavage of APP were suggested to be neuroprotective or to promote axon outgrowth (Chasseigneaux et al., 2011) whereas others functions are associated to an ancestral immunological mechanism of defense and would potentially have antibacterial peptide property (Soscia et al., 2011). However, the full spectrum of APP isoform functions remains to be elucidated.

Proteolytic cleavage of APP brings into play sequential events involving first the release of its ectodomain either by α- or β-secretase activities (**Figure 1**). These cleavages generate carboxy-terminal fragments remaining anchored to the plasma membrane and they shed extracellular soluble fragments, both of which are playing a role in axon outgrowth *in vitro* (Chasseigneaux et al., 2011). APP cleavage by α-secretase generates a soluble APP fragment α (sAPPα) and a carboxy-terminal α fragment composed

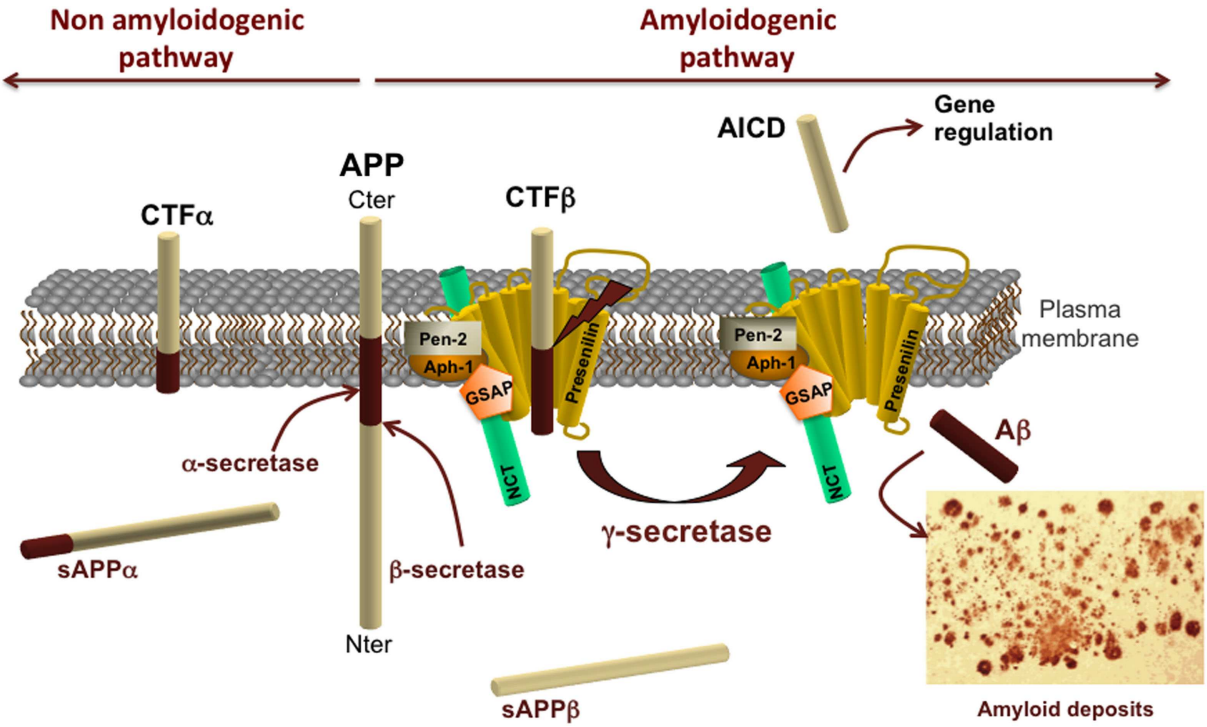

FIGURE 1 | Amyloid protein precursor structure and metabolism. Schematic representation of APP processing by α-, β-, and γ-secretases. APP processing by secretase activities is divided into the non-amyloidogenic pathway on the left and the amyloidogenic pathway on the right. α- and β-secretase activities cleave APP in its extracellular domain to release respectively a soluble fragment sAPPα or sAPPβ in the extracellular space and generate carboxy-terminal fragments CTFα or CTFβ. These CTFs can subsequently be processed by γ-secretase complex to generate AICD and Aβ. The γ-secretase complex is composed of presenilin, nicastrin (NCT), γ-secretase activating protein (GSAP), pen-2, and aph-1.

of 83 amino acids (named C83 or CTFα; for review see Vingtdeux and Marambaud, 2012). This cleavage takes place within the sequence of Aβ peptide thus precluding its formation. This pathway is therefore referred to as the non-amyloidogenic pathway. The α-secretase activity is carried by metalloproteases called A Disintegrin And Metalloprotease (ADAMs). Several ADAM proteases with an α-secretase activity have been identified, including ADAM-17 or TNF-α converting enzyme (TACE; EC 3.4.24.86, peptidase family M12; Buxbaum et al., 1998), ADAM-10 (EC 3.4.24.81, peptidase family M12; (Lammich et al., 1999; Lopez-Perez et al., 2001), and ADAM-9 (EC 3.4.24.; Koike et al., 1999; Hotoda et al., 2002).

The β-secretase cleaves APP at the first amino acid residue of Aβ sequence. The β-cleavage generates a soluble fragment sAPPβ and a CTF comprised of 99 amino acids (C99 or CTFβ). All APP-CTFs (CTFα, β′, and β) can subsequently be cleaved at the juxtamembrane region by the γ-secretase (**Figure 1**). However, ectodomain cleavage of APP is mandatory to intramembrane γ-secretase proteolysis of APP-CTFs. The APP intracellular domain (AICD or C51) is released from both CTFα and CTFβ by the γ-secretase following the cleavage at the ε-site. However, CTFs can also be processed at the γ-sites but yet AICD of 57 or 59 amino acids have not been detected (for review see Pardossi-Piquard and Checler, 2011). The γ-secretase cleavage of CTFβ represents the last step of Aβ production and is currently considered to be the pathway releasing AICD in the cytoplasm, thus having a potential gene regulatory function together with Fe65 and Tip60 (Konietzko et al., 2010). Following cleavages sites are the γ-sites which produce Aβ species of 43, 42, 40, 39, 38, 37 amino acid long following the rule of tri- or tetrapeptide release (Takami et al., 2009; for review see Karran et al., 2011). The γ-secretase is a multiprotein complex composed of at least four proteins, Presenilin, Pen-2, Aph-1, Nicastrin, and one molecule of each is necessary and sufficient to form an active enzymatic complex (Edbauer et al., 2003; Kimberly et al., 2003; Takasugi et al., 2003; Sato et al., 2007).

The α- and β-secretases are sheddases releasing the extracellular domain of APP as well as several others type I transmembrane proteins. The cleavage and localization of enzyme activity is supposed to occur at the plasma membrane or in early endosomes. As for instance, BACE-1 resides within endosomes and APP endocytosis is a prerequisite for cleavage of APP by BACE-1 and generation of Aβ (Vassar et al., 1999; Walter et al., 2001; Ehehalt et al., 2003). BACE-1 optimal protease activity necessitates an acidic pH and acidification of endosome occurs during the route of endosomes to fuse with lysosomes where BACE-1 is degraded (Koh et al., 2005). Cleavage of APP-CTFs by γ-secretase can occur at several places in the cell (e.g., plasma membrane, endosomes. . .). Discrepancies exist regarding the cell localization of γ-secretase by-products. Several APP metabolites including APP, APP-CTFs, Aβ, and AICD have been shown to accumulate in multivesicular bodies (MVBs) following treatment of cells with alkalizing

drugs (Verbeek et al., 2002; Vingtdeux et al., 2007b). Interestingly and similarly to the effect of Gleevec, alkalizing drugs such as chloroquine, ammonium chloride, bafilomycin A1, block Aβ production without affecting AICD generation (Vingtdeux et al., 2007a). More interestingly, following treatment, the AICD amount raise and AICD is also released outside the cell. Inside the cell AICD is reaching the nucleus (Goodger et al., 2009) where it regulates gene expression such as neprelysin (Pardossi-Piquard et al., 2005; for review see Pardossi-Piquard and Checler, 2011). Interestingly, intracellular AICD may be generated from APP-CTFs produced from β-secretase (Belyaev et al., 2010). However, further investigation is needed to determine whether there is one or several AICD and what is the function of AICD. For instance, BACE-1 cleavage of APP and AICD derived from βCTF may contribute to learning, memory, and neuronal plasticity (Ma et al., 2007).

ALZHEIMER'S DISEASE, NEUROFIBRILLARY TANGLES, AND MICROTUBULE-ASSOCIATED TAU

Neurofibrillary tangles are characterized by intraneuronal accumulation of fibrillar material named paired helical filaments (Kurt et al., 1997). Abnormally modified Tau proteins are the major components of this filamentous material. Tau proteins belong to the family of microtubule-associated proteins. A single gene, named *MAPT* located at position 17q21 encoded for several isoforms resulting from alternative splicing of exons 2, 3, and 10 in the human adult brain. There are six isoforms, half of which contains the exon 10 encoding sequence, two-third are having the exon 2 whereas the exon 3 is found in one-third of Tau isoforms and always in association with exon 2. The Tau isoforms differ from each other by the presence of either three (3R) or four repeat-regions (4R) in the carboxy-terminal (C-terminal) part of the molecule and the absence or presence of one or two inserts (29 or 58 amino acids) in the amino-terminal (N-terminal) part (Goedert et al., 1989a,b; Andreadis et al., 1992). Each of these isoforms is likely to have particular physiological roles since they are differentially expressed during development. For instance, only one Tau isoform, characterized by 3R and no N-terminal inserts, is present during fetal stages, while the six isoforms (with one or two N-terminal inserts and 3 or 4R) are expressed during adulthood (Kosik et al., 1989; Goedert and Jakes, 1990). Tau isoforms are differentially distributed in neuronal subpopulations or in yet underdetermined physiological conditions (Goedert et al., 1989a). However, in pathological conditions such as frontotemporal dementia linked to chromosome 17 or myotonic dystrophy, a mis-splicing of Tau is associated to the development of neurofibrillary degeneration (Vermersch et al., 1996; Hutton et al., 1998; Spillantini et al., 1998; Sergeant et al., 2001).

Tau proteins bind microtubules through repetitive regions in their C-terminal part. These repetitive regions are the repeat domains (R1–R4) encoded by exons 9 to 12 (Lee et al., 1989). The three (3R) or four copies (4R) are made of a highly conserved 18-amino acid repeat ending with a PGGG motif (Lee et al., 1988; Goedert et al., 1989b, Himmler et al., 1989; Lee et al., 1989) and separated from each other by less conserved 13- or 14-amino acid inter-repeat domains. Tau proteins are known to act as promoter of tubulin polymerization *in vitro*, and are involved in axonal transport (Weingarten et al., 1975; Cleveland et al., 1977; Brandt and Lee, 1993). Adult Tau isoforms with 4R (R1–R4) are more efficient at promoting microtubule assembly than the fetal isoform with 3R (R1, R3–R4; Goedert and Jakes, 1990; Butner and Kirschner, 1991; Gustke et al., 1994). Besides its major microtubule-binding, -stabilizing, paralleled-ordering functions, Tau also has other functions.

Tau proteins bind to spectrin and actin filaments (Selden and Pollard, 1983; Carlier et al., 1984; Correas et al., 1990; Henriquez et al., 1995). Through these interactions, Tau proteins may allow microtubules to interconnect with other cytoskeletal components such as neurofilaments (Miyata et al., 1986; Andreadis et al., 1995) and may restrict the flexibility of the microtubules (Matus, 1990). There is also evidence that Tau proteins interact with cytoplasmic organelles. Such interactions may allow for binding between microtubules and mitochondria (Jung et al., 1993). The Tau N-terminal projection domain also permits interactions with neural plasma membrane (Brandt et al., 1995). Thus, Tau may act as a mediator between microtubules and plasma membrane. This interaction has been defined as involving a binding between the proline-rich sequence in the N-terminal part of Tau proteins and the SH3 domains of Src-family non-receptor tyrosine kinases, such as Fyn. Studies have determined that human Tau Tyr18 and Tyr29 are phosphorylated by the Src-family tyrosine kinase Fyn (Williamson et al., 2002; Lee et al., 2004). Tau proteins was shown to co-sediment with lipid-raft fractions in response to Aβ and corresponded to phosphorylated Tau proteins at Tyr18 and Ser396/404 (Hernandez et al., 2009). In this latter study, it is suggested that Tau association to lipid-rafts may be necessary to mediate Aβ toxicity through the stabilization of Tau/Cdk5 interaction and thus suggesting that Tau as a potential signal transduction protein. The proline-rich region of Tau proteins is likely involved in the interaction with phospholipase C-γ (PLC-γ) isozymes (Hwang et al., 1996; Jenkins and Johnson, 1998). Hwang and colleagues have demonstrated *in vitro* that Tau proteins complex specifically with the SH3 domain of PLC-γ, and enhance its activity in the presence of unsaturated fatty acids such as arachidonic acid. These results suggest that in cells that express Tau proteins, receptors coupled to cytosolic phospholipase A2 may activate PLC-γ indirectly, in the absence of the usual tyrosine phosphorylation, through the hydrolysis of phosphatidylcholine to generate arachidonic acid (Hwang et al., 1996; Jenkins and Johnson, 1998). Altogether, these data indicate that Tau proteins may also play a role in the signal transduction pathway involving PLC-γ (for review see Rhee, 2001). In line with this idea, recent data demonstrate that Tau is necessary for glutamatergic signaling (Ittner et al., 2010). Overall, there is a growing body of evidence suggesting that tau may be close, interact, or even associate with intracellular vesicular compartment.

THE SPATIOTEMPORAL BRAIN SPREADING OF NEUROFIBRILLARY DEGENERATION

With aging, neurofibrillary tangles (NFT) spread from the transentorhinal cortex to the hippocampal formation. Neuropathological as well as biochemical assessment show that the Tau pathology spreads progressively, invariably, hierarchically, from the transentorhinal cortex to the whole neocortex, along cortico-cortical connections. The brain regions that are sequentially affected explain

well the successive kind of cognitive impairments that characterize the disease: amnesia following the entorhinal and hippocampal degeneration: aphasia, apraxia, and agnosia with the involvement of the neocortex. Of course, amyloid and Tau pathology are present far early before the clinical symptoms (for review see Karran et al., 2011), because neuronal plasticity likely compensate at the first AD stages. Recently, the locus coeruleus has been described as the initiating region of NFD (Braak and Del Tredici, 2011). The Tau pathology is already distributed in the hippocampal formation and the temporal cortex at the "pre-clinical" stage of AD (Delacourte et al., 1999, 2002). Tau pathology, visualized as a triplet of abnormal Tau proteins, is systematically present in variable amounts in the entorhinal and hippocampal regions of non-demented patients aged over 75 years. When Tau pathology is found in other brain areas, it is always along a stereotyped, sequential pathway categorized into 10 stages according to the brain regions successively affected: transentorhinal cortex (S1), entorhinal (S2), hippocampus (S3), anterior temporal cortex (S4), inferior temporal cortex (S5), mid temporal cortex (S6), polymodal association areas (prefrontal, parietal inferior, temporal superior; S7), unimodal areas (S8), primary motor (S9a) or sensory (S9b, S9c) areas, and all neocortical areas (S10). Up to stage 6, the disease could be asymptomatic. In all of the cases at stage 7, individuals with two polymodal association areas affected by Tau pathology are cognitively impaired. This is of importance since it suggests that the spreading process occurs far before the occurrence of clinical symptoms and is also a very slow process likely transmitted through cortico-cortical connections therefore following rules and not randomly and most likely not diffusely. This hypothesis has been recently supported by different experimental works (Clavaguera et al., 2009, 2010; De Calignon et al., 2012; Liu et al., 2012). In contrast, amyloid deposits are diffusely progressing in the brain parenchyma (Braak and Braak, 1991; Duyckaerts and Hauw, 1997). However the mechanisms underlying the spreading and propagation of lesions remains poorly understood and current hypothesis, supported by the most recently published studies, suggested the spreading of AD lesions through interconnected neuronal circuitries. Among several hypotheses, we suggest that exosomes may contribute to this spreading process. Exosomes are produced from multivesicular endosomes and they will be first described followed by exosomes and the potential mechanism for the selectivity of spreading.

MULTIVESICULAR ENDOSOMES AND EXOSOMES

Extracellular components, such as viruses, ligands, or diffusible factors and, part of the plasma membrane proteins are internalized during endocytosis. They are either recycled to the cell surface via early or recycling endosomes or, they are directed to late endosomes and finally delivered to lysosomes for degradation (for review see Gruenberg, 2009). Late endosomes are also known as multivesicular endosomes or MVBs (Gruenberg and Stenmark, 2004; Raposo and Marks, 2007; Rusten et al., 2011). They are required for the degradation of internalized material to fuse with lysosomes and are instrumental to several cellular functions including miRNA activity, mRNA transport, autophagy, cell polarity, receptor signaling, cytokinesis, and migration (Huotari and Helenius, 2011; Rusten et al., 2011). MVBs are large vesicles of several hundred nanometers that are characterized by numerous smaller intraluminal vesicles (ILVs) formed by the inward budding of the endosome limiting membrane. The formation of these ILVs requires sequential steps and the contribution of complex of multi-molecular machinery named Endosomal Sorting Complex Required for Transport (ESCRT). The ESCRT machinery is composed of four ESCRT protein complexes (0, I, II, and III) acting sequentially to sort ubiquitinated cargo and to form a coated subdomain on endosomes that forms the ILVs (**Figure 2**). Evidences for alternative pathways for cargos sorting into MVBs are emerging, which are independent of the ESCRT machinery but seems to depend on the lipid composition of raft-based micro domains. Proper cholesterol levels in late endosomes are required for normal MVBs formation and MVB-mediated membrane protein degradation (Kobuna et al., 2010). It was also shown that the phospholipid LBPA (lysobisphosphatidic acid) and ceramide possess the capacity to drive the formation of membrane invaginations (Matsuo et al., 2004; Trajkovic et al., 2008). Ubiquitination (Ub) is the main sorting signal for cargo entry into the vesicles that bud from the limiting membrane into the lumen of endosomes during the biogenesis of MVBs. A single Ub is sufficient to direct ILV targeting. Ub is recognized by an expanding cohort of endosomal proteins, which may act as Ub-sorting receptors responsible for binding and directing cargo toward ILVs like some ESCRT subunits, including VPS27/Hrs, VPS23/Tsg101, and VPS36/Eap45 (for review see: Piper and Katzmann, 2007). Many integral membrane proteins targeted for lysosomal degradation are ubiquitinated; however, non-ubiquitin sorting signals have also been described. Much less is known about non-Ub signals that sort proteins to ILVs; proteins which have been described to enter ILVs in an Ub-independent manner include Pmel17/Silver (Berson et al., 2003), TfR (Geminard et al., 2004), Nedd4 (Morita and Sundquist, 2004), Sna3 (McNatt et al., 2007; Oestreich et al., 2007). Two motifs "NTR" and "PKD" located on the extracellular part of Pmel17 are responsible for its targeting into the internal vesicles of MVBs (Theos et al., 2006) and COP9 signalosome (CSN)-associated protein Csn5 is involved in protein sorting into ILVs since siRNA of Csn5 causes a significant increase in both ubiquitinated and non-ubiquitinated proteins detected in exosomes (Liu et al., 2009).

Genetics also supports the importance of functional MVB in neurological disease and frontotemporal dementia. The gene encoding *CHMP2B* the ESCRT-III subunit was found to be mutated in a form of frontotemporal dementia (Skibinski et al., 2005) and amyotrophic lateral sclerosis (Cox et al., 2010) suggesting that functional MVBs are required to prevent accumulation of abnormal proteins that can disrupt neural function and ultimately lead to neurodegeneration (Filimonenko et al., 2007). Mutations in *CHMP2B* were first described in Danish and Belgian families but remain rare (Ghanim et al., 2010), yet accounting for less than 1% of Frontotemporal lobar degeneration linked to chromosome 3 (FTD-3; Isaacs et al., 2011; Gijselinck et al., 2012). Mutations $CHMP2B^{Intron5}$ or $CHMP2B^{Delta10}$ are supposed to lead to C-terminal truncation of CHMP2B. Brain tissue examination of patients with *CHMP2B* mutation showed enlarged vacuoles stained with a mannose-6-phosphate receptor antibody. The truncated protein impairs the fusion of endosome with lysosomes without obvious modification of protein sorting to MVB

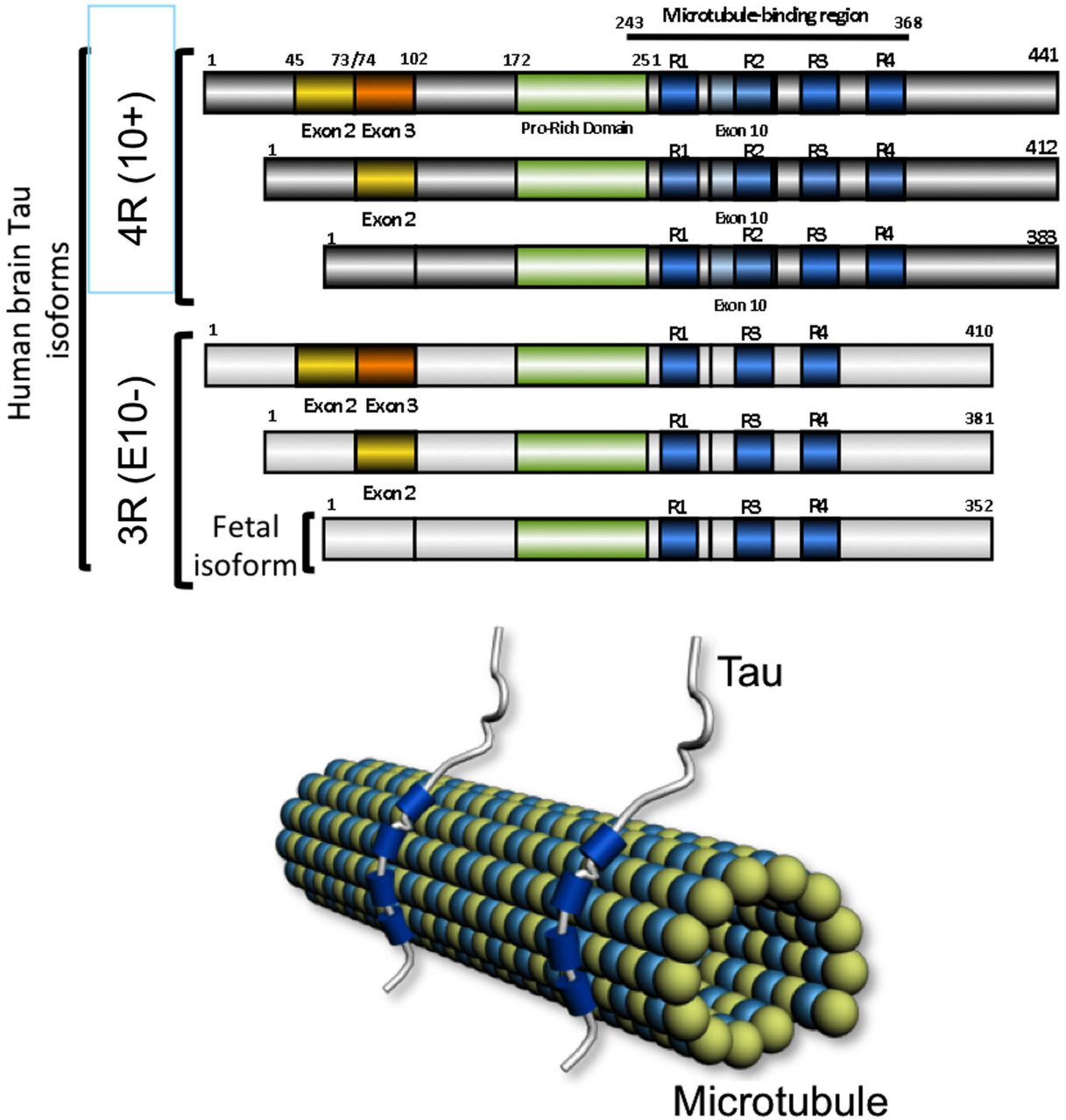

FIGURE 2 | The microtubule-associated protein *tau*. The six human brain isoforms of tau are represented. They differ by the inclusion of exclusion of exons 2 (yellow), exon 3 (orange), and exon 10 (light blue). The microtubule-associated domain are indicated in blue and depending on inclusion of exon 10 there are tau isoforms with 4 (4R) or 3 (3R) microtubule-binding regions. The fetal isoform is lacking the alternative encoding cassettes 2, 3, and 10. Tau protein binds the microtubule lattice through its microtubule-binding domains shown in blue.

(Urwin et al., 2010). Ectopic expression of mutant CHMP2B^{Intron5} in primary cortical rodent neurons promote neuronal cell death through the failure of the mutant protein to dissociate from ESCRT-III complex. In parallel, an increased accumulation of autophagosomes was observed suggesting a defective fusion of autophagosomes with MVB (Lee et al., 2007). Staining of tissue from Alzheimer disease patients with CHMP2B showed an accumulation of the protein in vesicular structures resembling Granulo Vacuolar Degeneration (Yamazaki et al., 2010; Funk et al., 2011) suggestive of a defective autophagic and late endocytic pathways in AD and Frontotemporal lobar degeneration. MVB function is required for the proper clearance of intracellular protein aggregates such as TDP-43 or polyglutamine aggregates observed in Frontotemporal lobar degeneration and amyotrophic lateral sclerosis or Huntington disease, respectively (Filimonenko et al., 2007). Moreover, restoring or enhancing the

lysosomal degradation and rates of autophagic protein turnover in a transgenic animal model of amyloid deposition can rescue the phenotype and decrease the amyloid burden (Yang et al., 2011). Together, a defective function of the endocytic pathway including MVB, autophagy, and lysosome may certainly contribute to the development of several neurodegenerative diseases including AD.

Alternatively to their fusion with lysosomes for degradation of their contents, MVBs have been described to fuse to the plasma membrane and release their content in the extracellular space (Harding et al., 1983; Pan et al., 1985), the ILVs contained in the MVBs when released are referred to as exosomes (Johnstone et al., 1987). Exosomes have a size ranging from 40 to 100 nm and can be secreted by many cell types including neuronal cells (Faure et al., 2006; Rajendran et al., 2006; Vingtdeux et al., 2007b; Lachenal et al., 2011). Exosomes are isolated from the media of cultured cells. However, purification of exosomes is not trivial since membrane fragments or cell debris can easily contaminate exosome preparations. Due to their small size, exosomes are obtained after filtration on 0.22 μm filters and by a series of centrifugation and sucrose gradient (Raposo et al., 1996; Wubbolts et al., 2003; Faure et al., 2006; Thery et al., 2006 for review see Olver and Vidal, 2007). Further immunoisolation can be used (Wubbolts et al., 2003). Several parameters should be evaluated to ascertain the purity of exosomes preparation. The first and likely most important characteristic is the observation of exosomes by transmission electron microscopy. Thus, exosomes have a typical cup-shape form. Several proteins are also common to exosomes and described in exosomes preparation that originate from different sources (for review Vella et al., 2008). Interestingly, several tetraspanins proteins are enriched in exosomes and may contribute to exosomes formation (Wubbolts et al., 2003; De Gassart et al., 2004). Tetraspanins are a growing family of transmembrane proteins with pleiotropic functions found associated with lipid-raft micro domains (for review Hemler, 2005). Interestingly, tetraspanins CD81 and CD9, which are found in exosomes derived from B-cells (Wubbolts et al., 2003), are co-purified with the γ-secretase interactome. Absence of those tetraspanins induces a partial disruption of γ-secretase activity or reduces γ-secretase substrate interaction (Wakabayashi et al., 2009). Although detailed molecular mechanisms remain unknown, together those results further support the idea that MVB and most possibly exosomes are important cellular compartments for APP metabolism regulation and that several γ-secretase regulators may act at this level.

MVBs fate can be affected by macroautophagy (hereafter referred to as autophagy). During autophagy, parts of the cytoplasm and organelles are encapsulated in double-membrane vacuoles called autophagosomes, which eventually fuse with lysosomes for degradation (for review see Levine et al., 2011). Under conditions that stimulate autophagy, MVBs are diverted to autophagic pathway with subsequent inhibition in exosomes secretion (Fader et al., 2008). Conversely, knockdown expression of ESCRT-I, -II, and -III proteins in cell models promotes the accumulation of autophagosomes or autolysosomes, suggesting that MVB and autophagy are intermingled and that loss of MVB function may promote autophagy as well as ineffective fusion of autophagosomes and lysosomes (for review see Rusten et al., 2011). Thus, loss of function of CHMP2B may impair both MVB and autophagosome maturation. With regards to Tau, the autophagy-lysosomal pathway contributes to the degradation of Tau (Wang et al., 2011). However, Tau protein inclusions are seldom detected in FTD-3 (Yancopoulou et al., 2003) and ubiquitin-positive inclusions are observed but TDP-43 negative (Holm et al., 2007).

How exosomes are processed in recipient cells is not yet fully understood. Exosomes can be endocytosed into the endosomal system of recipient cells. Once internalized, exosomes could fuse with the limiting membrane of endosomes to deliver their cytoplasmic content into the host cell cytoplasm. It is also possible that exosomes could directly fuse with the plasma membrane. Although their exact function remains to be discovered, within the extracellular space and in biological fluids such as urine or serum, exosomes have been proposed to participate in different physiological and/or pathological processes such as neurodegenerative diseases (for review see Vella et al., 2008). They could be responsible not only for protein and lipids exchange between cells, but also for mRNA and microRNAs exchange (Valadi et al., 2007). Recently, miRNAs content of purified exosomes produced by dendritic cells were shown to ectopically repress target mRNAs of recipient dendritic cells (Montecalvo et al., 2011). Exosome release and content may be regulated by cellular stress. Thus DNA damage and activation of p53 induce the expression of protein that will be included inside exosomes (Yu et al., 2006). Exosomes may mediate a signal of cellular damage or stress. In the central nervous system, exosomes are proposed to constitute an intercellular communication system (for review see Mathivanan et al., 2011, 2012). Exosomes produced by glia-derived cells stimulate neurite outgrowth through a synapsin and NCAM dependent mechanism. In oxidative stress condition the synapsin released from exosomes is neuroprotective (Wang et al., 2011). AICD and several APP metabolites are found in exosomes derived from primary neuronal cultured cells (Vingtdeux et al., 2007b; **Figure 3**). L1 CAM that is also processed by γ-secretase (Riedle et al., 2009) is recovered in exosomes (Lachenal et al., 2011). Interestingly, modulators of the γ-secretase activity, such as inhibitors, modulate the release of APP-CTFs and Aβ associated to exosomes (Sharples et al., 2008). Altogether, there is a growing body of evidence suggesting that exosomes are small membrane-delineated cell-secreted material that may participate in cell-to-cell communication via both RNAs and proteins. Although speculative, if several intracellular domains of proteins processed by γ-secretase are internalized and secreted within exosomes, the fusion of those exosomes with surrounding cells may regulate gene expression by those intracellular domains and therefore constitute another cell communication system. However, if proteins that are degraded through late endosomes/lysosomes pathway are diverted from this normal degrading route, it may promote their accumulation, shape-transformation, and secretion via the multivesicular and exosome pathway.

PRION-LIKE PROPAGATION OF AMYLOID AND TAU PATHOLOGIES

Besides being a potential system of intercellular communication, exosomes are also known to be instrumental to the dissemination of pathogens, whether those are viruses or proteinaceous pathogens. The first pathological protein described associated with

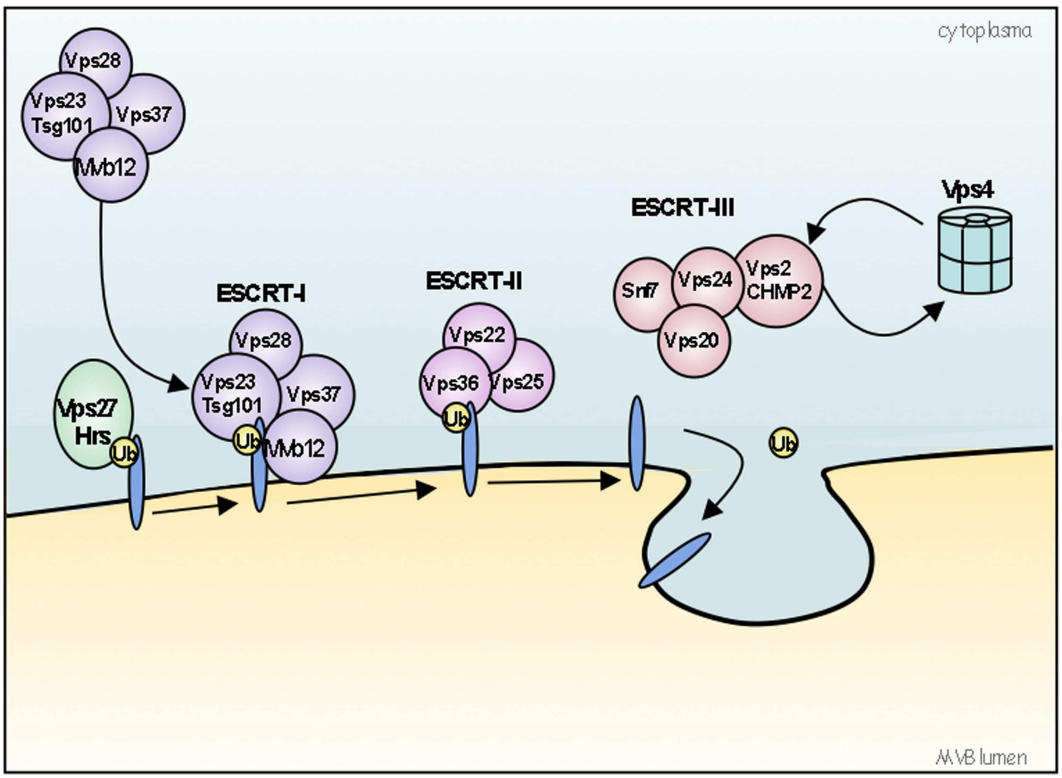

FIGURE 3 | Model for the ubiquitin-dependent sorting of proteins by the ESCRT machinery. The ESCRT machinery is composed of four ESCRT protein complexes (0, I, II, and III) acting sequentially to sort ubiquitinated (ubiquitin is represented as Ub) cargo and to form a coated subdomain on endosomes that forms the ILVs. The VPS27/Hrs-Hse1/STAM complex (ESCRT-0) is first recruited to the endosomes by binding PI(3)P and ubiquitinated cargos. ESCRT-0 then recruits ESCRT-I (composed of Tsg101/VPS23-VPS28-VPS37) to the membrane, where ESCRT-I interacts with ubiquitinated cargos via its VPS23 subunit. Then, ESCRT-I recruits ESCRT-II complex (composed of VPS22/Eap30-VPS25/Eap25-VPS36/Eap45), which in turn initiates the oligomerization of ESCRT-III complex (composed of VPS2/CHMP2-VPS20/CHMP6-VPS24/CHMP3-Snf7/VPS32/CHMP4). ESCRT-I and II initiate the invagination of the limiting endosomal membrane. ESCRT-III deubiquitinating enzymes ensure the dissociation of ubiquitin residues from molecules before sequestration into MVBs. Finally, ESCRT-III recruits supplementary factors like Bro1 and Vsp4 AAA-ATPase. Bro1 will recruit a deubiquitination enzyme whereas VPS4 AAA-ATPase will work to break apart ESCRT-III and other ESCRT complexes, resulting in their dissociation from the membrane.

exosomes was the prion protein (PrP; Fevrier et al., 2004; Alais et al., 2008). Prions diseases are fatal neurodegenerative disorders. They are associated with the conversion of the cellular prion protein (PrPc) into the scrapie PrP (PrPSc), an abnormal conformational state that tends to form amyloid deposits in brain tissue leading to dementia. Into its mis-folded conformation the PrPSc is thought to be infectious (for review see Aguzzi and Rajendran, 2009). Recent findings revealed an unexpected role for exosomes in dissemination of prions: exosomes from prion-infected neuronal cells have been demonstrated to be efficient initiators of prion propagation in uninfected recipient cells and, more importantly, to produce prion disease when inoculated into mice (Vella et al., 2007).

PRION-LIKE PROPAGATION OF AMYLOID PATHOLOGY

Exosomal release instead of lysosomal processing might be of advantage to cells having poor degradative capacities. In the context of AD, exosomes secretion could be a way to dispose of unwanted proteins. Indeed, maturation of autophagolysosomes and their retrograde transport are most possibly impeded in AD (Lee et al., 2011). The underlying mechanism behind the hypothesis that neurodegeneration in AD is triggered by protein spread, cell-to-cell, throughout brain areas could be the shipping of toxic agents such as Aβ or Tau by exosomes. What at the beginning would be beneficial (to bypass a degradation system which is overwhelmed) could become the reason why there is propagation of the disease thorough the brain. Aβ peptides are released by cells in association with exosomes (Rajendran et al., 2006) and interestingly, exosomal proteins such as Alix and flotillin-1 were observed around neuritic plaques, a lesion found in brains from AD patients (Rajendran et al., 2006) suggesting that exosomes-associated Aβ could be involved in plaque formation. MVBs are an intracellular compartment where internalized Aβ can grow into fibrils thereby MVBs may also contribute to amyloid plaque formation (Friedrich et al., 2010). Overall these results suggest that exosomes could play a role in the pathogenesis of AD. The idea of a "prion-like" propagation of Aβ lesions is also supported by results obtained *in vivo* in human wild-type APP transgenic mice (HuAPPwt) which do not develop Aβ deposits. Intracerebral inoculations of AD brain homogenates into the hippocampus of these mice lead

to Aβ deposits which increased with age and spread to areas other than the site of injection (Morales et al., 2011). Propagation of Aβ-induced molecular impairments across synapses is also suggested in a transgenic animal model in which the expression of APP was restricted to the entorhinal cortex. With time and aging, amyloid deposits were observed in connected brain regions such as the dentate gyrus and CA1 pyramidal neurons (Harris et al., 2010). Entorhinal cortex neurons are neither directly connected to the granular cells of the dentate gyrus nor to CA1 pyramidal neurons. Therefore, amyloid deposits that appear with aging in those structures may originate from pathological Aβ species that propagate across synapses. A possible hypothesis would be that the pathological Aβ species are produced and released through the exosome pathway and exosomes are caught by interconnected neurons and trans-synaptically delivered to connected brain regions.

TAU PATHOLOGY SPREADING

The stereotype propagation scheme of neurofibrillary degeneration in AD is evidenced by neuropathological examination as well as biochemical analyses but until recently, hypotheses and experiments trying to address this question remained elusive. Neurofibrillary degeneration is following stereotypical brain circuitry following cortico-cortical connections therefore suggesting a loss of neurotrophic or surviving factor or a diffusible factor responsible for a cascade of molecular events leading to Tau aggregation and neuronal death. However, what is this propagating factor? What if Tau itself wouldn't be the "missing link"? Thus, recent data suggest that neurofibrillary degeneration cortical spreading could follow a transmissible prion-like process. In fact, aggregates of PHF-Tau were purified from a transgenic mice model of neurofibrillary degeneration. Intracranial injection of this preparation was done in a different mouse model, which overexpresses human Tau protein but does not display Tau pathology. Following injection, the development of neurofibrillary degeneration was observed. This Tau pathology progressed from the injection site to neighboring brain structures, suggestive of a diffusible and transmissible propagating mechanism (Clavaguera et al., 2010). Very recently, Frost et al. (2009) have shown using a cell-based system that extracellular Tau aggregates are internalized inside cells and promote the mis-folding and fibrillization of Tau. Internalization of preformed Tau fibrils is facilitated by the use of a lipid-based protein delivery system (BioPorter®) and is likely mediated by endocytosis (Guo and Lee, 2011). The internalized preformed fibrils reduce microtubule-stabilization suggesting a loss-of-function of normal Tau in infected cells. Moreover, two recent studies showed that the tau pathology could spread *in vivo* through neuronal circuitry and trans-synaptic transmission (De Calignon et al., 2012; Liu et al., 2012). Although those transgenic models are valuable to decipher the molecular and cellular mechanisms of tau pathology spreading, the mechanism of tau protein conversion, oligomerization, secretion, trans-synaptic propagation remains elusive. There are some evidences suggesting that Tau may be secreted and secretion of Tau may differ depending on Tau isoform. Thus, Tau isoforms with exon 2 encoding sequence are likely not secreted and this exon 2 sequence is therefore suggested to repress Tau secretion (Kim et al., 2010). A good example of such a dilemma is fibroblast growth factor 2 that is a secreted growth factor without any signal peptide and that is also found in cell nucleus following its interaction with its cognate receptors (Meunier et al., 2009). Tau is likely secreted and is also located into the nucleus following stress conditions (Sultan et al., 2011). Tau secretion, as for Tau nuclear localization, may depend upon yet undefined conditions and therefore, contributions of MVB-exosomes pathways or autophagy-lysosomal pathways (Wang et al., 2009) remain completely open. Recent data strongly suggest that both pathways are possibly interconnected (Sahu et al., 2011). With regards to Tau, the degradation systems may bring insights for the potential routing of Tau to MVB-exosomes or autophagy-lysosome pathway. In NFT or more generally in aggregates, Tau is found ubiquitinated, thus suggesting that Tau may be processed by the proteasome (David et al., 2002). Ubiquitin-independent degradation system, such as caspase or calpain cleavage of Tau has also been described (Berry et al., 2003; Delobel et al., 2005; Ding et al., 2006; Carrettiero et al., 2009; Ferreira and Bigio, 2011). The autophagy-lysosomal pathway contributes to the degradation of Tau via the chaperone-mediated autophagy (CMA; Wang et al., 2009; for review see Wang et al., 2010). The CMA is a lysosome-mediated degradation system of cytosolic protein (for review see Arias and Cuervo, 2011). This system implies the recognition of substrates by a complex of chaperones and translocation of substrates inside lysosomes for further degradation. The CMA malfunction has been connected to the development of several neurodegenerative diseases including Parkinson disease and AD (Arias and Cuervo, 2011). Although speculative and purely hypothetic, through the use of CMA, aggregates of proteins or even oligomers could reach the lysosome and due to their low sensitivity to degradation (e.g., Tau aggregates), the fusion of lysosome with other vesicular structures such as MVB could finally lead to the release of aggregates outside the cell and contribute to their propagation following neuronal connections. Alternatively, proteins such as Tau would normally be addressed to lysosome by the CMA system but a defective lysosome could be the place where oligomers are generated and thereafter route to MVB/exosome pathway. More recently, two consecutive papers described the secretion of Tau protein by the exosome pathway (Saman et al., 2012; Simon et al., 2012). In MC1 neuroblastoma cells overexpressing the four-repeat Tau isoform with no N-terminal insert, a C-terminal truncated form or the full-length Tau protein was found co-purified with exosomes as well as associated with the exosome fraction obtained from human cerebrospinal fluid (Saman et al., 2012). Other Alzheimer associated markers are found in exosome such as Fyn-tyrosine kinase and Aβ (Segura et al., 2005; Rajendran et al., 2006). These proteins were also found in exosomes secreted by MC1 neuroblastoma cells (Saman et al., 2012). Interestingly, while the full-length Tau is recovered from COS cells overexpressing Tau, in HEK stably expressing Tau protein, a fragment encompassing Tau microtubule-binding repeat domains is principally found (Simon et al., 2012). However, endogenous Tau was not detected in exosomes derived from primary embryonic neuronal culture cells (Faure et al., 2006). Exosomes-associated secretion of Tau is only observed in overexpressing systems suggesting that the release of Tau by the exosomes pathway may contribute to eliminate the excess of intracellular Tau. Recent studies suggest that many transmissible pathogens such as PrP, α-synuclein, Huntingtin, Aβ are

shuttle from a cell to another by secretion or tunneling nanotubes (for review see Goedert et al., 2010), however the mechanism of secretion or transmission and their regulation remain poorly understood.

HOW TAU PATHOLOGY TRANSMISSION IS SELECTIVE IN SPORADIC TAUOPATHIES?

Conceptually, what mechanism can we propose to explain the prion-like spreading of Tau pathology affecting selective patterns of neurodegeneration and skipping nearby "less vulnerable" neuronal targets. That's certainly a major fundamental question to address. Why in the scheme of spatiotemporal spreading and propagation of lesions in AD and other sporadic Tauopathies such as Pick's disease, progressive supranuclear palsy or corticobasal degeneration only selective neuronal subpopulations are affected (for review see Sergeant et al., 2008). As for instance, affected neurons in AD essentially belong to the cholinergic system whereas those degenerating in PSP are dopamine neurons (Murphy et al., 2008). One possibility would be that selectivity of propagation could follow neuronal circuitry through synaptic transmission. This would be possible if exosomes were preferentially released at the synaptic junction, as suggested by Smalheiser (2007). There are emerging evidence that exosomes are produced and secreted by neurons and that synaptic activity could enhance exosomes secretion (Lachenal et al., 2011). However, the demonstration derives from *in vitro* experiments using primary neuronal embryonic culture cells. Study of exosomes in tissue yet remains highly challenging although recently it was shown that exosomes could be detected in synaptic boutons at the *Drosophila* larval neuromuscular junction (Koles et al., 2012). Consequently, little if not nothing is known about the neuronal localization, regulation of release and their propensity of diffusion *in vivo*.

There are therefore other possibilities, such as the tunneling nanotubes (for review see Goedert et al., 2010). Tunneling nanotubes are fine membrane channels that have recently been described in mammalian cells for communication between cells but also for cell-to-cell propagation of mis-folded PrPs (for review see Gerdes et al., 2007; Gousset et al., 2009; Zhang, 2011). These tunneling nanotubes could also propagate other transmissible mis-folded proteins such as Aβ (Zhang, 2011) but the question of selectivity of transmission remains open. However, it is note of worthy that tunneling nanotubes is induced following oxidative stress in rodent hippocampal neurons and astrocytes. Cell-to-cell connection and communication of intracellular organelles or Aβ could be trigged by cellular stress (Zhang, 2011). Following this scheme, the stressed cell, such as a degenerating neuron, would connect via tunneling nanotubes to closely surrounding or connected neurons to deliver the pathogenic protein. However hypothetic, tunneling nanotubes is an emerging mechanism of cell communication under stress conditions that may or could contribute to neurodegenerative diseases (Goedert et al., 2010; Zhang, 2011).

Coming back to exosomes and now considering that exosome release and secretion is controlled and localized to pre- or post-synaptic locations then several hypotheses can be postulated. In both pre- and post-synaptic situations propagation through exosomes would be closely dependent upon neuronal connections, as far as the diffusion of exosomes is following a paracrine or "juxtacrine" rule of diffusion (Mathivanan et al., 2011). Thus, only interconnected neurons would disseminate toxic species via exosomes. We can also hypothesize that exosomes originating from different type of neurons (e.g., cholinergic, GABAergic, glutamatergic, dopaminergic. . .) may contain specific membrane-associated biomarkers. Intercellular communication mediated by exosomes may result from passive fusion of exosome membrane with the plasma membrane of the targeted cell or may use a ligand receptor system. In line with the latter system, the selectivity of intercellular communication could result from specific interaction between ligand and receptor. There are several examples that could illustrate a selectivity of propagation of exosomes using this ligand receptor selectivity. For instance, protocadherin is a cluster of 52 cadherin-like genes with a singular organization. The amino-terminal region of protocadherins is encoded by three sets of separate exons arranged in three clusters (alpha, beta, and gamma). N-terminal encoding exons are spliced with one of three carboxy-terminal encoding exons. Alternative splicing generates an extraordinary diversity of protocadherin isoforms suggested to confer selective and specific intermolecular membrane-associated protein interactions (Wu and Maniatis, 2000; Wang et al., 2002). The second example is DSCAM, the *Drosophila* homolog of human Down syndrome cell adhesion molecule that belongs to the axonal guidance receptor family. Alternative splicing of DSCAM can generate as many as 38016 mRNA isoforms and therefore lead to expression of huge protein diversity (Schmucker et al., 2000). More interestingly, one DSCAM protein isoform binds exactly to the same isoform but not a slightly different one, making the binding of DSCAM isoforms very stringent (Wojtowicz et al., 2004). As for DSCAM, the selectivity of transmission pattern could be mediated following an axonal guidance-like system. In a very simplified view, axonal guidance is driven by equilibrium between attractive and repulsive signals through specific signaling pathways, allowing axonal growth and connection to its specific neuronal target (for review see Bashaw and Klein, 2010). Thus exosome release from one type of cell will be attracted by its target cell and repulsed by surrounding cells. Altogether, examples provided could contribute to neuronal communication and propagation of mis-folded proteins along specific identified neuronal circuitries. Although all these hypotheses could be envisioned a better knowledge of the metabolism of exosomes *in vitro* and *in vivo* is necessary to address this problematic.

CONCLUSION

Among pathophysiological mechanisms of neurodegenerative diseases leading to intra or extracellular protein aggregates, a consensual mechanism support a prion-like propagation of mis-folded proteins. However, when this mechanism implies the propagation from cell-to-cell, shuttling pathways incriminated remains poorly understood (see **Figures 4** and **5** for potential hypothesis). A growing body of evidence suggests that the endocytic – multivesicular endosome and exosome pathways may contribute to this process and to the development of several neurodegenerative diseases. Much is known about the routing of proteins through those recycling or degradative pathway but much less is

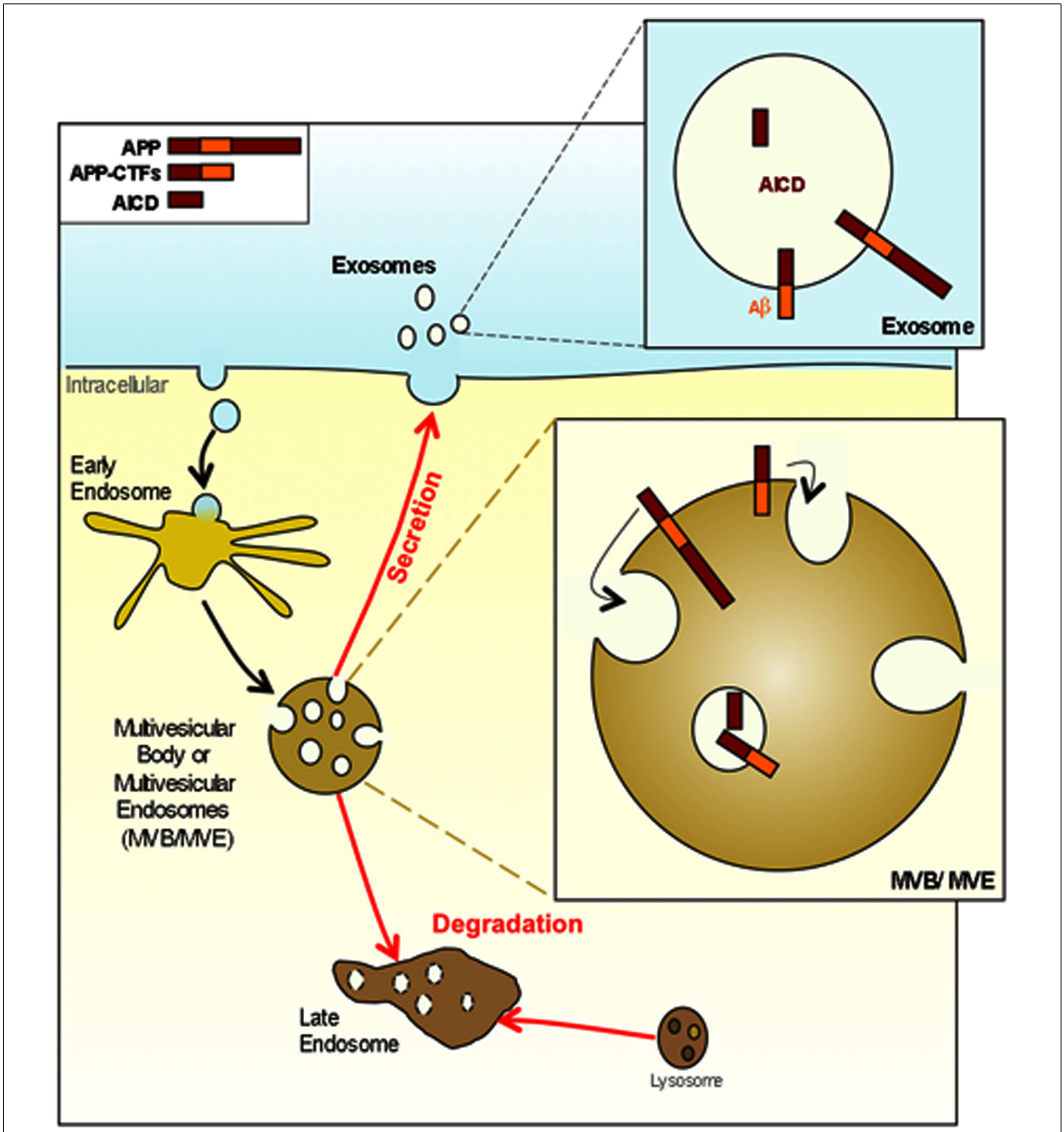

FIGURE 4 | *APP* and its metabolites are present in multivesicular bodies and exosomes. APP and APP-CTFs are internalized and directed into the internal vesicles of multivesicular bodies (MVB). At this point APP and its metabolites can either be degraded after the fusion of MVB with lysosomes or can be released in the extracellular space in association with exosomes consecutively to the fusion of MVB with the plasma membrane.

known about the contribution of those systems to the development of neurodegenerative diseases. However, this MVB – exosome system can be diverted from its physiological function as for instance to produce human immunodeficiency viral particles (Nguyen et al., 2003; for review see Gould et al., 2003). Following this hypothesis, the autophagy-lysosome and/or MVB – exosome pathways could also be diverted to deliver and propagate toxic oligomers or aggregates in neurodegenerative diseases such as AD. Blocking the diffusion of those toxic and mis-folded species by this secretory pathway could also represent a potential therapeutic

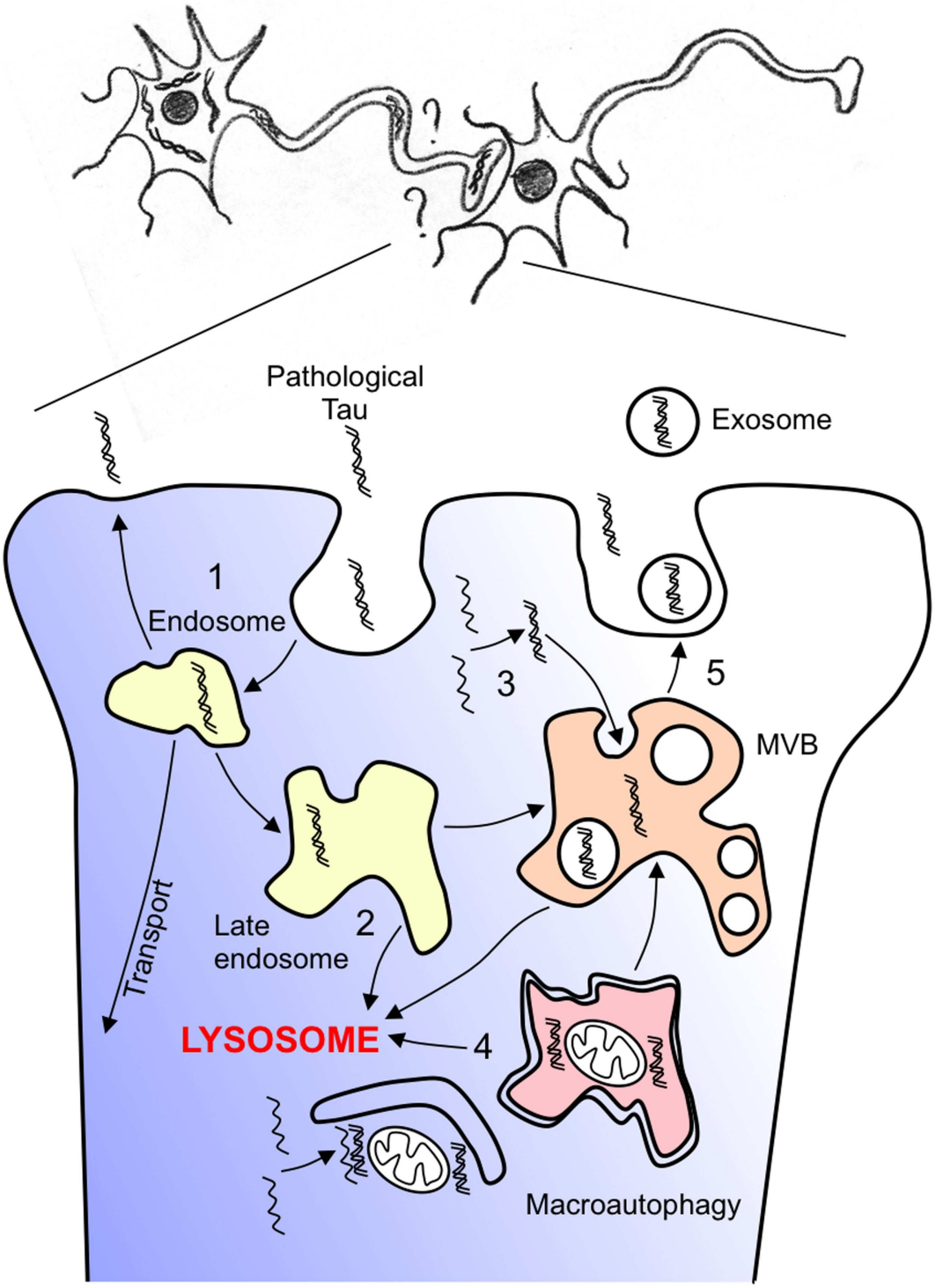

FIGURE 5 | Hypothesis of pathological tau spreading. How can pathological tau species spreads through trans-synaptic connections? A non-exhaustive representation of several hypotheses are given. (1) Pathological species can be endocytosed recycled, amplified, transport, and secreted (2) Endocytosed species can follow the endosome lysosome routing and either be addressed to lysosomes or to multivesicular endosomes/bodies (3) Pathological tau species that are produced in the cell soma are included into multivesicular bodies by inward budding of the late endosome membrane (4) Pathological tau species can be included into large autophagic vesicles by macroautophagy and further fused with multivesicular bodies (5) pathological tau species can be release by fusion of multivesicular bodies with the plasma membrane at the synaptic junction and capture by the connected dendrite.

approach of neurodegenerative diseases such as Amyloidopathies, Tauopathies, Synucleinopathies, or more largely Foldopathies all of which are likely sharing a "prion-like" propagation of toxic mis-folded proteins.

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Microvesicles: novel biomarkers for neurological disorders

Elisa Colombo[1], Bruno Borgiani[1], Claudia Verderio[2] and Roberto Furlan[1]*

[1] *Clinical Neuroimmunology Unit, Division of Neuroscience, Institute of Experimental Neurology, San Raffaele Scientific Institute, Milan, Italy*
[2] *Department of Medical Pharmacology, CNR Institute of Neuroscience, University of Milano, Milano, Italy*

Edited by:
Mauro Maccarrone, University of Teramo, Italy

Reviewed by:
Mauro Maccarrone, University of Teramo, Italy
Monica Bari, University of Roma Tor Vergata, Italy

****Correspondence:***
Roberto Furlan, Clinical Neuroimmunology Unit – Dibit, Secretary-Treasurer of the International Society of Neuroimmunology, San Raffaele Scientific Institute, Via Olgettina 58, 20132 Milano, Italy.
e-mail: furlan.roberto@hsr.it

Microvesicles (MVs) are released by most cell types in physiological conditions, but their number is often increased upon cellular activation or neoplastic transformation. This suggests that their detection may be helpful in pathological conditions to have information on activated cell types and, possibly, on the nature of the activation. This could be of paramount importance in districts and tissues that are not accessible to direct examination, such as the central nervous system. Increased release of MVs has been described to be associated to the acute or active phase of several neurological disorders. While the subcellular origin of MVs (exosome or ectosomes) is basically never addressed in these studies because of technical limitations, the cell of origin is always identified. Endothelium- or platelet-derived MVs, detected in plasma or serum, are linked to neurological pathologies with a vascular or ischemic pathogenic component, and may represent a very useful marker to support therapeutic choices in stroke. In neuroinflammatory disorders, such as multiple sclerosis, MVs of oligodendroglial, or microglial origin have been described in the cerebrospinal fluid and may carry, in perspective, additional information on the biological alterations in their cell of origin. Little specific evidence is available in neurodegenerative disorders and, specifically, MVs of neural origin have never been investigated in these pathologies. Few data have been reported for neuroinfection and brain trauma. In brain tumors, despite the limited number of studies performed, results are very promising and potentially close to clinical translation. We here review all currently available data on the detection of MVs in neurological diseases, limiting our search to exclusively human studies. Current literature and our own data indicate that MVs detection may represent a very promising strategy to gain pathogenic information, identify therapeutic targets, and select specific biomarkers for neurological disorders.

Keywords: microvesicles, neuroinflammation, neurodegeneration, brain tumors, neurological disorders

INTRODUCTION

Microvesicles (MVs) have gained recently large attention as both potential biomarkers, and a tool to investigate the biology of cells from sites difficult to reach *in vivo*. Solid tumors, for example, display an elusive nature of transformed cells, grow into organs, and re-appear in unpredictable sites when producing metastases. By releasing massive amounts of MVs, however, they may reveal their presence. Investigating the content of these vesicles, information may be gained on biological processes occurring within the tumor mass. Similarly, in diseases of the central nervous system (CNS), a part from post-mortem examination, neuroscientists, and neurologists do not have access to the diseased tissue with the extreme exception of cases that need a cerebral biopsy, which are usually not representative of the most common neurological disorders. Therefore, here also, MVs, that are physiologically released by all neural and non-neural cells within the CNS (**Figure 1**), hold promise as possible vehicle of clinical and biological information. The difficulties related to the detection and analysis of MVs in neurological disorders are partially overlapping with those found in other diseases, and partially peculiar. In fact there are common problems of general inadequacy of available detection techniques. It is now a general consensus that flow cytometry (FACS) is unable to detect properly small exosomes (**Figure 2**), but only can reliably analyze ectosomes (**Figure 2**), also called shed vesicles. Further, as compared to tumor cells, platelets, or endothelial cells, neural cells release very reduced amounts of MVs, posing also a problem of detection limit. Finally, the most interesting compartment to examine, the cerebrospinal fluid (CSF), cannot be sampled serially without posing an ethical problem. Nevertheless, the possibility to access these complex cargo structures, storing a multiplicity of signals derived from un-accessible cells, gives the possibility to get very relevant information on their cell of origin during disease. Upon proper interpretation, these evidences may yield data with clinical diagnostic and prognostic value, provide information to stratify patients concerning response to treatments, or even suggest new therapeutic targets.

Investigations have already been performed in this respect and we are going to review available evidence for MVs of different cellular and subcellular origin, and detected by different techniques, in different compartments, as potential biomarkers in neurological disorders (**Table 1**).

CEREBROVASCULAR DISORDERS

The number of MVs derived from endothelial cells or platelets have been linked to the extent of myocardial infarcts (Mallat et al., 2000;

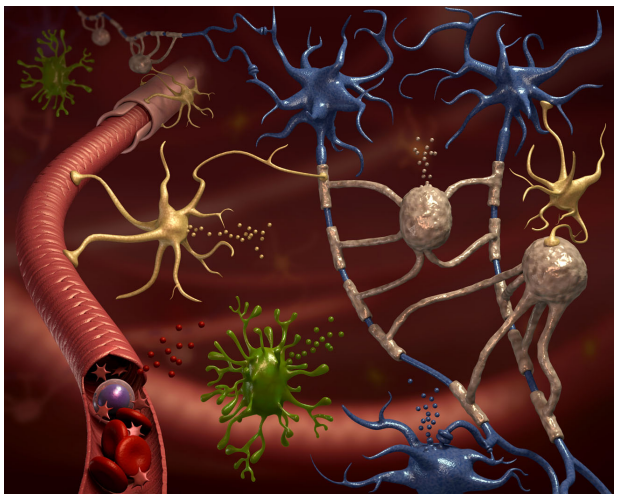

FIGURE 1 | All neural cell types release microvesicles (MVs). The CNS parenchyma is very complex in terms of cellular composition. This cartoon depicts neurons (blue cells), their axons surrounded by myelin produced by oligodendrocytes (gray cells), ramified microglia (green cells), astrocytes (yellow cells), and blood vessels (in red). Still, this is a very simplified representation of CNS tissue. All represented, and not represented, cell types are able to release MVs delivering signals to neighboring cells and into the environment (van der Vos et al., 2011). Some of these MVs are drained to accessible biological fluids like the blood or the cerebrospinal fluid, where they might constitute a new class of biomarkers.

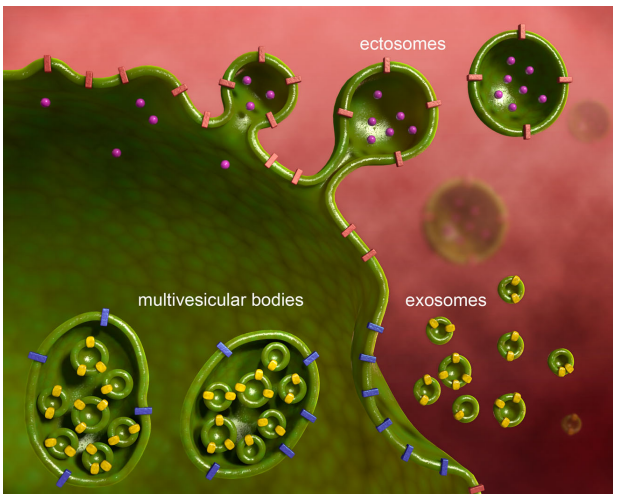

FIGURE 2 | Microvesicles are of different subcellular origin. MVs released from various neural cell types have different subcellular origin. In the present review we consider ectosomes, also called shed vesicles, and exosomes. Ectosomes shed from the plasma membrane carrying along transmembrane proteins, and soluble proteins, nucleic acids, and metabolites present in the cytoplasm. Ectosomes are large and heterogeneous in size. Exosomes derive from the release of multivesicular bodies, an intracellular organel along the endocytic pathway, that controls membrane composition and content. Exosomes are small and homogenous in size.

Jung et al., 2012). Similarly, their number has been investigated by several groups in cerebral ischemia.

Already in the early 1990s, Ahn and co-workers found that platelet-derived MVs, stained for CD42 and detected by FACS, are increased in plasma of patients with ischemic stroke, especially in those with transitory ischemic attacks or with lacunar infarcts, as compared to those with thrombosis of large vessels (Lee et al., 1993). In this first, pioneering study, however, no correlations had been drawn with the extent of the ischemic area, or the severity of the outcome (Lee et al., 1993). These results have been confirmed, over 10 years later, using CD61 and CD62P to identify MVs of platelets origin, in whole blood of patients with ischemic stroke (Cherian et al., 2003; Pawelczyk et al., 2009). These two reports discuss their results associating increased platelet activation, testified by the increased release of MVs, with higher risk of developing stroke. They therefore propose to use the detection of platelet-derived MVs as a biomarker to be used in a population at risk to have a stroke, to identify individuals with higher chance, or particularly close to develop the event. Two groups in Japan have used an interesting, alternative, technical approach to overcome some of the limitations of flow cytometry in measuring platelet-derived MVs. By ELISA they quantify the platelet marker CD42a or CD42 on ultracentrifugated plasma MVs, confirming the positive association of these markers with the occurrence of ischemic stroke (Shirafuji et al., 2008; Kuriyama et al., 2010). From these studies we can conclude that platelet-derived MVs are studied to dissect the contribution of platelets to the pro-thrombotic state leading to stroke, but, judging from available literature, appear of limited clinical usefulness as biomarkers.

Endothelium-derived MVs, recently identified and linked to cerebral ischemia, may be more promising. The first available report associated the number of endothelial MVs, identified in plasma by FACS staining for CD105, CD144, phosphaditYl serine (PS), and CD54, with several clinical parameters, including stroke size, severity, and outcome (Simak et al., 2006). In particular, lesion volume appeared to be in direct correlation with the number of $CD105^+$, $CD54^+$, PS^+, but not $CD144^+$ MVs. $CD144^+$ MVs, on the other hand, predict the hemorrhagic transformation of the ischemic lesion (Simak et al., 2006). Contrasting results were reported 1 year later, when similar $CD31^+$ or $CD62E^+$endothelial MVs levels were described in acute ischemic stroke patients and in stroke mimics, i.e., patients with stroke-like symptoms but apparently without ischemic lesions (Williams et al., 2007). The lack of a real consensus on the definition of stroke mimics (potentially affected by transitory ischemic attack?), and several technical limitations, including the use of archival samples stored frozen for over 1 year, however, limit the interpretation of these data. More recently, Jung et al. (2009) have substantially confirmed the original finding. In fact, they describe elevated endothelium-derived MVs to be significantly associated to stenosis of both intra- and extra-cranial portions of cerebral arteries. Further they associate distinct MVs markers for extra-cranial ($CD62E^+$) and intra-cranial ($CD31^+CD42b^-PS^+$) localization of the stenosis. This study also confirms a positive correlation of the number of plasma endothelial MVs and infarct size and clinical severity. Analysis of predictive parameters in patients already carrying risk factors showed that plasma levels of endothelial MVs were inversely correlated with the time of occurrence of an ischemic

Table 1 | MVs in neurological diseases.

Disease	Site and detection	Cell of origin	MPs modulation	References
CEREBROVASCULAR DISORDERS				
Ischemic stroke	Plasma by FACS	Platelets	↑ $CD42^+$	Lee et al. (1993)
Ischemic stroke	Blood by FACS	Platelets	↑ $CD61^+$ ↑ $CD62P^+$	Cherian et al. (2003), Pawelczyk et al. (2008)
Ischemic stroke	Plasma by ELISA	Platelets	↑ $CD42a^+$	Shirafuji et al., 2008
Ischemic stroke	Plasma by ELISA	Platelets	↑ $CD42^+$	Kuriyama et al. (2010)
Ischemic stroke	Plasma by FACS	Endothelium	↑ $CD105^+CD41a^-CD45^-$ ↑ $CD105^+CD144^+$ ↑ $CD105^+PS^+CD41a$ ↑ $CD105^+CD54^+CD45$	Simak et al. (2006)
Strokes mimics	Plasma by FACS	Endothelium	↑ $CD31^+$ ↑ $CD62E^+$	Williams et al. (2007)
Intracranial arterial stenosis	Plasma by FACS	Endothelium	↑ $CD31^+CD42b$ ↑ $CD31^+PS^+$	Jung et al. (2009)
Extracranial arterial stenosis	Plasma by FACS	Endothelium	↑ $CD62E^+$	Jung et al. (2009)
Cerebral vasospasm	Plasma by FACS	Endothelium	↑ $CD105^+PS^+$ ↑ $CD62E^+$ ↑ $CD106^+$	Lackner et al. (2010)
Cerebral infarction following vasospasm	Plasma by FACS	Platelets	↑ $CD41^+$	Lackner et al. (2010)
NEUROINFLAMMATORY DISEASES				
Multiple sclerosis	CSF by electron microscopy	Oligodendrocytes	↑ Unknown	Scolding et al. (1989)
Multiple sclerosis	Plasma by FACS	Endothelium	↑ $CD31^+CD42^-$ ↑ $CD51^+$	Minagar et al. (2001)
Multiple sclerosis	Blood by FACS	Endothelium	↑ $CD54^+$ ↑ $CD62E^+$	Jy et al. (2004)
Multiple sclerosis	Plasma by FACS	Platelets	↑ $CD62P^+$	Sheremata et al. (2008)
Cerebral malaria	Plasma by FACS	Endothelium	↑ $CD51^+$	Combes et al. (2004)
NEURODEGENERATIVE DISORDERS				
Alzheimer	CSF by WB	Neurons?	↑ Phospho-tau	Saman et al. (2012)
Alzheimer	Blood by FACS	Platelets	No modulation	Lee et al. (1993), Sevush et al. (1998)
Vascular dementia	Plasma by FACS	Platelets	↑ $CD42^+$	Lee et al. (1993)
EPILEPSY				
Temporal lobe epilepsy	CSF by immunoblotting	Stem cells	↑ $CD133^+$	Huttner et al. (2012)
BRAIN TUMORS				
Glioblastoma	Biopsies by electron microscopy	Tumor cells	↑ Membrane blebs	González-Cámpora et al. (1978)
Glioblastoma	CSF by immunoblotting	Stem cells	↑ $CD133^+$	Huttner et al. (2008)
Glioblastoma	Serum and biopsies by RT-PCR	Tumor cells	↑ $EGFRvIII^+$	Skog et al. (2008)
TRAUMA				
Traumatic brain injury	CSF and plasma by prothrombinase assay	Platelets and endothelium	↑ $CD42^+$ ↑ $CD31^+$	Morel et al. (2008)

stroke (Jung et al., 2009). The predictive value of endothelial MVs (defined as $CD105^+PS^+$, $CD62E^+$, or $CD106^+$), has been confirmed in a different clinical setting, namely the risk to develop cerebral vasospasm in patients with spontaneous subarachnoid hemorrhage (Lackner et al., 2010), in which also platelet-derived MVs may play a role (Lackner et al., 2010). The recent introduction of treatments of the acute phase of ischemic stroke, for example systemic thrombolysis, indicates the need for biomarkers able to stratify patients and minimize side effects of these therapies. Further, the identification of patients in which the risk for ischemic stroke is so high that it might be worth treating with anticoagulants could be a very powerful preventive strategy. Plasma endothelial MVs levels may potentially represent a solid biomarker for these two conditions. Investigations to validate this concept are conducted in several centers.

NEUROINFLAMMATORY DISEASES

With the introduction of MRI, and especially gadolinium-enhanced MRI, the field of neuroinflammation has found a gold standard biomarker providing localization, structural (or even

ultrastructural), molecular, and functional information. Performances of MRI are still increasing and its potential has not been fully exploited, since new sequences, providing new information, are constantly developed. What is the need, then, for new biomarkers in neuroinflammation? MRI is very costly, difficult to perform on all patients in the emergency room, and, for the moment, provides very little information on the biological status of single cell types. MVs hold the potential to fill this gap, providing quantitative and qualitative information on distinct cell types selectively involved in CNS pathologies.

The pioneering work in this field was published over 20 years ago, describing MVs of oligodendroglial origin in the CSF of patients affected by multiple sclerosis (MS; Scolding et al., 1989). Authors discussed their findings in the perspective of dissecting the effector mechanisms leading to myelin destruction, rather than as potential biomarkers for MS. The reason, among others, may rely on the fact that CSF is not a readily accessible biological fluid and repeated sampling, as mentioned above, poses an ethical issue. On the other hand, inflammatory events occurring in the CNS may produce biomarkers that are rapidly diluted in the circulation and difficult to detect in peripheral fluids, such as plasma, displaying high background noise levels for most markers. Nevertheless, $CD31^+$ endothelial MVs, identified in plasma samples by FACS, have been associated to clinical and neuroradiological exacerbation of MS, while $CD51^+$ endothelial MVs have been found elevated in both relapsing and remitting MS patients as compared to controls (Minagar et al., 2001). The same group has confirmed their findings in 2004, further describing that most endothelial MVs can be detected in the blood in the form of conjugates with other cells, especially monocytes (Jy et al., 2004), while described that, similarly to stroke, platelet-derived MVs, despite elevated in the plasma of MS patients as compared to controls, display a reduced discriminating power between health and disease (Sheremata et al., 2008).

Endothelium-derived MVs have been investigated also in cerebral malaria, a complication of malaria occurring in about 1% of patients infected with *Plasmodium Falciparum* and in which the endothelium of small cerebral vessels appears to play a crucial role (Milner, 2010). Indeed, endothelial MVs, detected by FACS staining for CD51, are selectively increased in plasma of patients with cerebral malaria and in patients with both coma and severe anemia, as compared to patients with uncomplicated malaria or only with severe anemia (Combes et al., 2004). Further, the number of plasma endothelial MVs normalizes upon treatment, suggesting a possible role also as biomarkers for therapeutic efficacy (Combes et al., 2004).

A common feature of all neuroinflammatory diseases is the primary involvement of the prototypical immune neural cell type: microglia. Practically indistinguishable from peripheral tissue macrophages using common markers, not accessible due to anatomical reasons, the only current way to gain information on microglia activation *in vivo* is by positron emission tomography using the tracer [11C](R)-PK11195 (Kannan et al., 2009). We have recently described that microglia derived MVs, identified by FACS staining for IB4, are dramatically increased in the CSF of patients with neuroinflammatory diseases such as patients affected by relapsing MS, neuromyelitis optica, meningitis (RF and CV personal communication). Our clinical and experimental data suggest that microglial MVs may be a solid marker for disease status and response to therapies, with all the limitations of biomarkers in the CSF that we have discussed above.

NEURODEGENERATIVE DISORDERS

Little evidence is available in the literature for MVs alterations in neurodegeneration. In Alzheimer's disease, negative results for platelet-derived MVs have been reported, demented patients displaying MVs levels overlapping to healthy individuals (Lee et al., 1993; Sevush et al., 1998). A very recent report, however, describes that in early Alzheimer it is possible to detect increased levels of phosphorylated tau protein in the exosome fraction of the CSF (Saman et al., 2012). This is a very promising finding, and points to the possibility for an early diagnosis of AD through CSF MVs content. For amyotrophic lateral sclerosis, experimental data *in vitro* on mice tissue suggest the possible increase in motoneuron-derived apoptotic MVs (Appert-Collin et al., 2006), but no human follow-up studies have been performed. Thus, the only positive available evidence is for vascular, multi-infarct, dementia in which elevated platelet-derived MVV have been reported (Lee et al., 1993), in line with data available for cerebral ischemia, of which vascular dementia is a chronic form. Since so little work has been done so far, neurodegeneration appears, in perspective, an interesting field to investigate MVs.

EPILEPSY

Epilepsy is, of course, mostly a clinical diagnosis and is monitored by electroencephalography. Stratification of patients in clinical subtypes is, however, not always trivial. Notably, MVs positive for the stem cell marker prominin-1/CD133, likely derived from neural stem cells or ependymal cells, have been found elevated in the CSF of patients with partial temporal, but not extra-temporal, epilepsy (Huttner et al., 2012). $CD133^+$ MVs CSF levels were similarly increased in cryptogenetic forms or in patients were temporal epilepsy was secondary to neoplasms dysplasia, or hippocampal sclerosis (Huttner et al., 2012).

BRAIN TUMORS

Brain tumor diagnosis and monitoring is usually performed by neuroradiology, with the need in certain circumstances to perform an invasive cerebral biopsy. A pioneering work by Roy Weller and collaborators, suggested already in 1978 the existence of glioblastoma-derived MVs (Gonzalez-Campora et al., 1978). In fact, by scanning electron microscopy they described, on human glioblastoma biopsy samples, the presence of several membrane alterations such as microvilli, blebs, and ruffles, suggesting a high motility of cell membranes. MVs close to shed from the cell membrane are clearly depicted in electron scans of this work (Gonzalez-Campora et al., 1978). Evidence for glioblastoma-derived MVs in biological fluids are, however, very recent. Huttner et al. (2008) have quantified, by immunoblotting, prominin-1/$CD133^+$ in MVs purified by ultracentrifugation from CSF samples. They show that prominin-1/CD133 levels are very high in CSF MVs from patients with glioblastoma as compared to healthy individuals. More useful in a clinical setting may be the finding by Skog et al. (2008) that it is possible to detect, by nested PCR, the transcript coding

for the oncogenic form of the epidermal growth factor receptor EGFRvIII in MVs purified by ultracentrifugation from serum of a subgroup of patients with glioblastoma. This opens the possibility to use a serum biomarker, carried by glioblastoma-derived MVs, to support diagnosis in case of uncertain neuroradiological images, by avoiding invasive procedures such as cerebral biopsy, and to monitor therapeutic efficacy or the appearance of recurrences.

TRAUMATIC BRAIN INJURY

Traumatic brain injury may, besides acute lesions, determine secondary cellular and vascular damage leading to poor clinical outcome. Morel and co-authors described that in the CSF and in the plasma of patients with traumatic brain injury, PS^+ MVs, defined as pro-coagulant by a functional assay, increase and peak in the acute phase, returning to basal levels within 10 days (Morel et al., 2008). The cellular origin of these MVs has been defined by capturing them with platelet and endothelial plastic-bound specific antibodies. Patients with persistent high CSF levels of these MVs displayed a poor clinical outcome. Thus, CSF endothelial or platelet-derived MVs may help identify those traumatic patients that need special care because of their high risk to develop secondary events leading to a fatal outcome or severe neurological deficit.

CONCLUSION

As can be learned from this review, MVs have not been widely investigated as potential biomarkers in neurological disorders, although the first evidence of their existence in the CNS was provided several decenniums ago. The most striking limit of current available reports is that, apart from glioblastoma tumor cells, MVs from very few, actually only two CNS specific cell types have been investigated for release in humans, namely oligodendrocytes and microglia. In fact, most studies mentioned in this review deal with MVs released by endothelium or platelets, and translate to the brain concepts that had been developed principally for myocardial infarct. The main reason for this may rely on the fact that, in general, neural cells generally release low amounts of MVs as compared to endothelium, platelets, stem cells, or tumor cells. Therefore, brain specific MVs are very diluted, if present, in peripheral biological fluids such as blood, plasma, or serum, thus making very difficult their detection by current available technologies. On the other hand, endothelium and platelets, and even microglia, are involved in most pathological processes occurring within the CNS. Thus, even if detected in the CSF, MVs derived from these cell types are likely to be non-specifically altered in the course of different neurological diseases.

Nevertheless, detection of MVs as biomarkers for neurological disorders is very promising in perspective. The development of new detection systems, specifically designed for MVs, with increased sensitivity and able to stratify MVs according to size and cellular origin, may considerably improve our ability to associate a certain MVs pattern to a specific pathological condition. The main goal to gain disease specificity is, however, identification of the content of MVs. It is reasonable to think that MVs of neural origin carry different molecules in different diseases and even in different disease phases. Further, the unique possibility to obtain from MVs *in vivo* information on brain cells involved in pathological processes may shed light on the pathogenesis of currently elusive pathologies such as primary neurodegenerative disorders, i.e., Alzheimer's, Parkinson's, ALS, etc. In some of these disorders MVs themselves may play a role in pathogenesis, and thus constitute a novel therapeutic target, once the biology of their release will be dissected in more detail (Bianco et al., 2009).

From the neurologist's perspective, validated, routinely available, detection techniques for MVs may be very interesting in stroke, where they may constitute an additional paraclinical parameter to evaluate when deciding therapeutic strategies, or to identify patients at high risk for a poor clinical outcome. In brain tumors the possibility to avoid brain biopsy and to monitor disease progression through MVs only depends from the detection limits, since tumor-specific MVs bearing specific markers have already been identified, and may indeed constitute a valuable tool in future clinical neurology. In neuroinflammation the role for MVs may be more difficult to define, since solid biomarkers, such as MRI, are already available, and microglial MVs in the CSF, despite holding promise, may remain a non-specific parameter, helpful but not decisive to make diagnosis and difficult to use for monitoring. Detection of MVs from neural cell origin may be extremely useful in neuroinfection, especially in those cases were the pathogen may be elusive, since it is known that MVs are carriers for infectious agents, and isolation of MVs from the CSF may help to increase significantly the sensitivity of available tests.

In conclusion, MVs already represent an interesting biomarker in neurology. To make their detection a routine procedure in the clinics, we need a change of gears in the development of specific technologies able to increase the performances of currently available assays. This would probably allow also more investigations in neglected fields like neurodegeneration, that however constitute one of the major challenges for research in medical neurosciences.

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Role of exosomes/microvesicles in the nervous system and use in emerging therapies

Charles Pin-Kuang Lai [1,2] ***and Xandra Owen Breakefield*** [1,2] *******

[1] *Department of Neurology, Neuroscience Center, Massachusetts General Hospital and Program in Neuroscience, Harvard Medical School, Boston, MA, USA*
[2] *Department of Radiology, Center for Molecular Imaging Research, Massachusetts General Hospital, Boston, MA, USA*

Edited by:
Claudia Verderio, CNR Institute of Neuroscience, Italy

Reviewed by:
Patrizia Rosa, Italian National Research Council, Italy
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****Correspondence:***
Xandra Owen Breakefield, Molecular Neurogenetics Unit, Massachusetts General Hospital-East, 13th Street, Building 149, Charlestown, MA 02129, USA.
e-mail: breakefield@hms.harvard.edu

Extracellular membrane vesicles (EMVs) are nanometer sized vesicles, including exosomes and microvesicles capable of transferring DNAs, mRNAs, microRNAs, non-coding RNAs, proteins, and lipids among cells without direct cell-to-cell contact, thereby representing a novel form of intercellular communication. Many cells in the nervous system have been shown to release EMVs, implicating their active roles in development, function, and pathologies of this system. While substantial progress has been made in understanding the biogenesis, biophysical properties, and involvement of EMVs in diseases, relatively less information is known about their biological function in the normal nervous system. In addition, since EMVs are endogenous vehicles with low immunogenicity, they have also been actively investigated for the delivery of therapeutic genes/molecules in treatment of cancer and neurological diseases. The present review summarizes current knowledge about EMV functions in the nervous system under both physiological and pathological conditions, as well as emerging EMV-based therapies that could be applied to the nervous system in the foreseeable future.

Keywords: microvesicles, exosomes, neuron, neuroregeneration, neurodegeneration, development, cancer, therapy

INTRODUCTION

Ligand-receptor interaction and direct cell–cell contacts via specialized physical conduits, such as gap junctions and membrane nanotubes, have long been considered as the predominant means of intercellular communication (Davis and Sowinski, 2008; Goodenough and Paul, 2009). Yet, a novel method of cell-to-cell communication has recently emerged from groundbreaking discoveries in the past few years on nucleic acid content of extracellular membrane vesicles (EMVs). EMVs have been demonstrated to facilitate horizontal transfer of mRNAs, microRNAs (miRNAs), and proteins between cells without direct cell-to-cell contact (Bergsmedh et al., 2001; Ratajczak et al., 2006a; Valadi et al., 2007; Al-Nedawi et al., 2008; Skog et al., 2008; Balaj et al., 2011; Ramachandran and Palanisamy, 2011; Turchinovich et al., 2011; Chen et al., 2012). There are several EMV categories known to-date, which are included under the general terms exosomes, microvesicles (MVs), and apoptotic blebs (ABs).

Exosomes are the smallest EMVs (40–100 nm in diameter), and homogenous in shape (cup-shaped after fixation under electron microscopy with a buoyant density of 1.13–1.19 g/cm^3 (Théry et al., 2001; Hristov et al., 2004). Unlike other types of EMVs that are directly shed/released from the plasma membrane, exosomes are formed by a series of processes beginning with inward invagination of clathrin-coated microdomains on the plasma membrane (Denzer et al., 2000). Once these vacuoles have entered the cell, the Endosomal Sorting Complex Required for Transport (ESCRT) facilitates the development of the invaginated vacuoles carrying ubiquitinated cargos into early endosomes. This is followed by a secondary invagination of vesicles (termed intraluminal vesicles, ILVs), into the endosomes where they accumulate with subsequent maturation of the complex into large multivesicular bodies (MVBs; Denzer et al., 2000). At this stage, MVBs may be trafficked to lysosomes for degradation ("degradative MVBs") or, instead, fuse with the plasma membrane ("exocytic MVBs") for the release of ILVs into the extracellular space, where upon they are referred to as exosomes (Mathivanan et al., 2010). A study on oligodendrocytes suggested that ILV release is ESCRT-independent and relies on the distribution of sphingolipid ceramide in MVBs, which directs the extracellular release of ILVs as exosomes (Trajkovic et al., 2008). Additional investigations are needed to determine if distinct MVB or ILV populations destined for degradation or exocytic release are present, as well as whether a common exosomal trafficking mechanism exists in all cell types (Mathivanan et al., 2010). Understanding the biogenesis and trafficking of exosomes will provide insight into how cells employ these extracellular organelles for intercellular communication. In some studies, release of exosomes appears to depend of Rab27 (Ostrowski et al., 2010) and Rab 35 (Hsu et al., 2010), and can be blocked with an inhibitor of neutral sphingomyelinase (Trajkovic et al., 2008). In addition, elevated $[Ca^{2+}]_i$, following Ca^{2+} and ionophore A23187 treatment was found to induce exosome and microvesicle release from erythrocytes (Allan et al., 1980; Salzer et al., 2002), further supporting a role of EMVs in response to different stimuli.

Microvesicles (MVs) are irregularly shaped, larger EMVs with a 100–1,000 nm diameter (Pilzer et al., 2005; Cocucci et al., 2009). A defined buoyant density of MVs has not yet been determined, but it may overlap that of exosomes (Théry et al., 2009; van Dommelen et al., 2011). In contrast to the endocytotic origin of

exosomes, release of MVs results from outward budding at the plasma membrane followed by fission of their connecting membrane stalks (Kobayashi et al., 1984; Dolo et al., 2000; Cocucci et al., 2007; Piccin et al., 2007). While MV biogenesis remains to be defined, microdomains on the plasma membrane containing a high cholesterol level and signaling complexes, or lipid rafts, have been suggested to selectively sequester lipids for MV generation (Del Conde et al., 2005). Work by Gould and collaborators indicates that MV release may be triggered by oligomerizing proteins on the cell surface and may share mechanistic elements with release of enveloped viruses (Gould et al., 2003; Shen et al., 2011). MV production is observed in a variety of cells in a resting state, but can be significantly elevated under various stimulations, including increased $[Ca^{2+}]_i$, cellular stress (e.g., DNA damage), decreased cholesterol levels, cytokine exposure, and anticancer drug treatment (Salzer et al., 2002; Shedden et al., 2003; Yu et al., 2006; Llorente et al., 2007; Lehmann et al., 2008; Bianco et al., 2009). Notably, even larger EMVs (1–5 μm in diameter) are released from tumor cells, especially in response to EGF stimulation (Di Vizio et al., 2009). Altogether, these findings suggest an active physiological role of MVs under different cellular conditions.

Apoptotic blebs are 50–4,000 nm in diameter with a buoyant density of 1.16–1.28 g/cm^3 (Hristov et al., 2004; Simak and Gelderman, 2006). Similar to MVs, ABs are also irregularly shaped, making them difficult to discern from one another based on their morphology. ABs, as its name suggests, are released from condensed and fragmented apoptotic cells during late stages of cell death (Henson et al., 2001; Hristov et al., 2004). ABs retain DNA fragments from the deceased cells, and can be taken up by neighboring cells for horizontal gene transfer as a form of intercellular communication (Bergsmedh et al., 2001; Holmgren, 2010).

EMV TERMINOLOGY VIS-À-VIS CELL OF ORIGIN

Most cells throughout the body, including those in the nervous system are believed to release EMVs. Early pioneering studies named EMVs based on their cellular origins, and to some extent their biogenesis, such as: archaeosomes, argosomes, dexosomes, epididymosomes, prostasomes, and oncosomes (Brody et al., 1983; Quaite-Randall et al., 1995; Zitvogel et al., 1998; Greco et al., 2001; Simpson et al., 2008; Al-Nedawi et al., 2009; Krishnan and Sprott, 2008; Di Vizio et al., 2009). These EMVs include ones isolated from a variety of cells/tissues in human body, i.e., dendritic cells (DCs), prostate gland, and cancer cells, as well as other species such as *Drosophila* (Simpson et al., 2008). As in many rapidly expanding fields in research, standardization of nomenclature for the different categories of EMVs remains to be resolved by an official organization, and is being undertaken by the International Society for Extracellular Vesicles. As it stands now, MVs/ectosomes/microparticles generally refer to extracellular vesicles released via a direct budding/shedding from the cellular plasma membrane, whereas exosomes are those released from MVBs following their fusion with the plasma membrane. Categorizing EMVs has been intrinsically challenging due to the multiple variable characteristics of EMVs, including: (1) cellular origin and physiological state of cell; (2) biophysical properties and lipid composition; (3) nucleic acids and protein content; and (4) size. For the purpose of this review, EMVs will be used to encompass these extracellular vesicle types.

BIOPHYSICAL PROPERTIES AND LIPID COMPOSITION

Aside from the different biophysical properties (i.e., size, shape, buoyant density) mentioned above for exosomes, MVs, and ABs (**Table 1**), different types of EMVs also have different lipid compositions. By using liquid chromatography and mass spectrometry, a variety of lipid components constituting EMVs isolated from different cells have been identified, including phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine (PS), lyso-bisphosphatidic acid, ceramide, cholesterol, and spingomyelin (Chu et al., 2005; Subra et al., 2007). The particular lipid composition of each EMV type likely contributes to its biophysical properties. Indeed, Parolini et al. (2009) recently reported that different lipid compositions, namely those containing sphingomyelin and *N*-acetylneuraminyl-galactosylglucosylceramide (GM3), are directly related to rigidity and delivery efficiency of exosomes to other cells. In addition, the level of PS exposed on the outer leaflet of exosomes appears to be lower than that of MVs and ABs (Mathivanan et al., 2011). This observation may correlate with the different biogenesis of EMV populations wherein exosomes are of endocytic origin, and MVs and ABs are derived from outward budding from the plasma membrane. PS is displayed on the outer exosome leaflet through floppase, flippase, and scramblase activities, and appears to mediate docking of proteins involved in signaling and fusion to the plasma membrane (Piccin et al., 2007). Therefore, the varying level of PS may affect communication functions of EMVs. Furthermore, ongoing studies indicate that EMVs may have a conserved glycan signature as compared to the cells from which they derive, suggesting a role of glycosylation in EMV protein sorting (Batista et al., 2011).

CONTENTS OF EMVs

Extracellular membrane vesicles are known to contain a broad spectrum of proteins, including transcriptional factors, surface receptors, and "marker" proteins, including tetraspanins (CD63, CD81), integrins, Tsg101, Alix, heat shock protein (HSP70, 90), and flotillin-1 (Lee et al., 2011). In addition, they contain a range of nucleic acids including mRNAs, which can be translated in recipient cells, microRNA (miRNA), and non-coding RNAs (ncRNA) which can regulate gene/mRNA expression, as well as DNA of as yet unvalidated function (Valadi et al., 2007; Skog et al., 2008; Guescini et al., 2010; Balaj et al., 2011; Waldenström et al., 2012). An interactive database will be needed to accommodate these increasing findings in the field of EMVs. Efforts are indeed underway, and a manually curated, web-based community database, ExoCarta, has been introduced to record exosomal proteins, RNAs, and lipids found in different EMV studies (Mathivanan et al., 2012). In addition, an Urinary Exosome Protein Database was created, as its name suggests, to provide a database of identified proteins from urinary exosomes via protein mass spectrometry (http://dir.nhlbi.nih.gov/papers/lkem/exosome/.)

FUNCTIONS OF EMVs IN THE NERVOUS SYSTEM

Most cells in the nervous system are believed to release EMVs. Given that EMVs are capable of transferring genetic information,

Table 1 | Biophysical properties of extracellular vesicles.

	Exosomes	MVs	ABs
Size (diameter)	40–100 nm	100–1,000 nm	50–4,000 nm
Buoyant density	1.13–1.19 g/cm^3	Not defined	1.16–1.28 g/cm^3
Lipid composition	Low PS exposed on the outer leaflet Lyso-bisphosphatidic acid Cholesterol Ceramide	High PS exposed on the outer leaflet Cholesterol	High PS exposed on the outer leaflet
Reference	Vidal et al. (1989), Heijnen et al. (1999), Subra et al. (2007), Trajkovic et al. (2008)	Scott et al. (1984), Zwaal et al. (1992), Bucki et al. (1998),	Stuart et al. (1998)

ABs, apoptotic blebs; PS, phosphatidylserine; MVs, microvesicles adapted from Subra et al. (2007), Mathivanan et al. (2010).

proteins, and lipids without direct cell-to-cell contact, researchers are focusing on the potential role of EMVs during development of the nervous system and as part of neural functions, as well as in disease. In this review, we will cover some of the recent findings on both the physiologic and pathophysiologic roles of EMVs in the nervous system, as well as the emergence of EMV-mediated therapies which may be applicable for treatment of neurological diseases in the foreseeable future. Recent reviews by Frühbeis et al. and Bellingham also provide extended updates into EMVs' participation in neuron-glia communication and neurodegenerative diseases, respectively (Bellingham et al., 2012; Frühbeis et al., 2012).

NORMAL FUNCTIONS

A number of studies have implicated EMVs in neuronal development, synaptic activity, protective mechanisms, and nerve regeneration with various reports indicating EMV release by neural stem/progenitor cells (Marzesco et al., 2005), neurons (Fauré et al., 2006), astrocytes (Taylor et al., 2007), microglia (Potolicchio et al., 2005), and oligodendrocytes (Krämer-Albers et al., 2007) in the brain and Schwann cells in the peripheral nervous system (Court et al., 2008).

Development

In the developing mouse brain during early neurogenesis there is a peak (E10.5–E13.5) of EMV release into the ventricular fluid in the neural tube of small (50–80 nm) and large (600 nm) vesicles which are positive for the stem cell marker, prominin-1 (CD133), although their function is not known (Marzesco et al., 2005). Other studies suggest that these EMVs may be involved in transfer of mRNAs encoding pluripotent transcription factors which can reprogram phenotypes of other cells (Ratajczak et al., 2006a). EMVs have the capacity to participate in the spatial and temporal gradients critical in development. Consistent with this role, non-neuronal floor plate cells in the ventral midline of mouse embryos are able to transfer β-galactosidase to neighboring axons, suggesting EMV transfer as an aspect of axonal path finding (Campbell and Peterson, 1993; **Figure 1A**). In a temporal patterning motif, oligodendrocytes appear to release EMVs as a means of auto-inhibiting myelination until appropriate signals are released from neurons during development, indicating that neuronal maturation is complete (Bakhti et al., 2011). Then as myelination commences EMVs are released from oligodendrocytes in a ceramide-triggered cascade that may be critical for transfer of the major myelin protein, proteolipoprotein (Trajkovic et al., 2008). EMVs may also participate in the genetic changes in the genome (genomic plasticity) of embryonic cells by supporting novel retrotransposon integrations. Both neural progenitor cells in normal embryos (Coufal et al., 2009) and brain tumor cells (Balaj et al., 2011) have high levels of retrotransposon expression, indicating a broadly transcriptionally active genome with the potential for retrotransposon integration events creating novel genotype/phenotypes. At least in the case of tumors, these EMVs have high levels of retrotransposon sequences and reverse transcriptase which may allow cell-to-cell transfer of this genomic plasticity leading to changes in gene expression, with pro-proliferative events being selected for.

Several studies in *Drosophila* have begun to tease out the role of EMVs in different developmental processes. EMV-like vesicles, termed argosomes (Greco et al., 2001) are used to transport a morphogenic Wnt signaling protein along spatial and temporal gradients in wing development, and may also carry Hedgehog, Notch, decapentaplegic (dpp), and Wingless (Wg) signaling proteins involved in setting up developmental gradients in other tissues (for review see Cadigan, 2002; Lakkaraju and Rodriguez-Boulan, 2008). Recently, EMVs were found to be involved in transfer of Wnt-1/Wg at the neuromuscular junction in *Drosophila* both during development and in mature neurons (Korkut et al., 2009). In this case, a multipass transmembrane protein, Evi, assists in presynaptic trafficking of Wnt-1/Wg into vesicles within the synaptic cleft, as well as in subsequent interactions of this signaling ligand with its receptor in postsynaptic cells. When Evi was rendered non-functioning in *evi* mutant *Drosophila* model, Wnt signaling across synapses is disrupted (Korkut et al., 2009).

Synaptic activity

In pioneering studies, Fauré et al. (2006) showed that undifferentiated cortical neurons in culture released EMVs containing L1, a neuronal cell adhesion protein, and GluR2/3 subunits of glutamate AMPA receptors, with release of EMVs being stimulated by depolarization, thus suggesting a role in synaptic function. Subsequent studies confirmed this phenomenon in fully differentiated cortical neurons in culture, and in addition showed that release was stimulated by a calcium ionophore, as well as by an antagonist of $GABA_A$ receptors, both of which result in increased spontaneous neuronal activity (Lachenal et al., 2011). Further, EMVs were found to incorporate the neuronal specific heavy chain of tetanus toxin

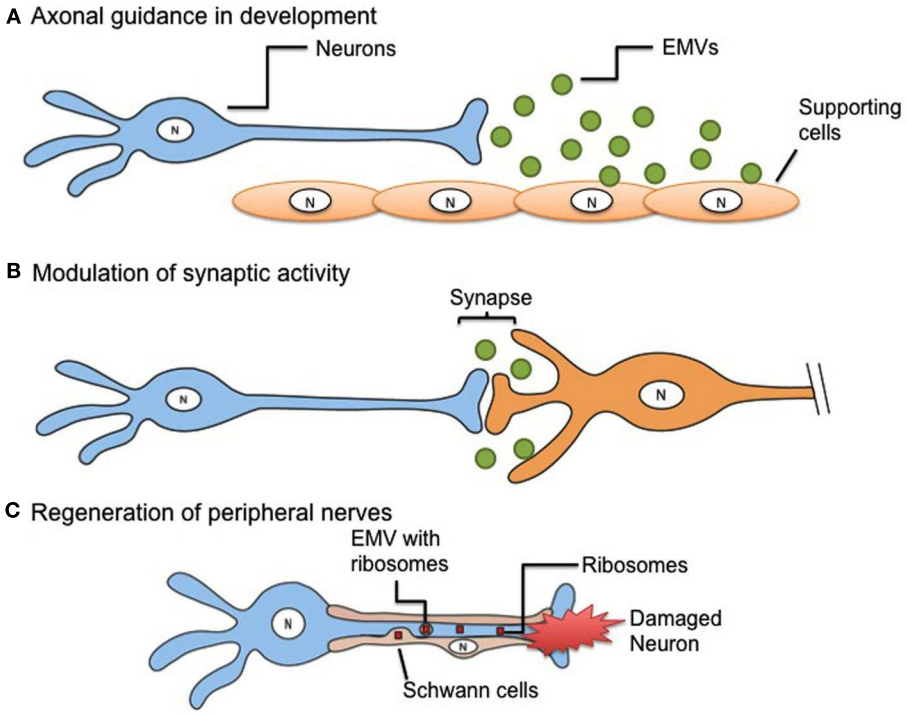

FIGURE 1 | Extracellular membrane vesicles-mediated mechanisms in neurons. (A) A gradient of EMVs in the developing nervous system can serve as a directional guide to axonal growth. **(B)** EMVs released from presynaptic nerve terminals and taken up by their postsynaptic partners can carry informational content which can modulate the strength of synaptic activity. **(C)** Regeneration of peripheral nerves in enhanced by the EMV transfer of ribosomes and mRNA directly from surrounding Schwann cells into the injured nerve to promote protein synthesis.

and reasoned that the presence of GluR2 subunits in EMVs and increased release associated with depolarization could modulate synaptic activity (Smalheiser, 2007; **Figure 1B**).

Injury and regeneration

In general, studies to-date indicate that EMVs primarily serve a protective role in the nervous system. For example, oligodendrocytes release exosomes containing myelin and stress-protective proteins, which serve in the trophic support of neurons (Krämer-Albers et al., 2007). Although synapsin I is usually thought of as a neuronal specific protein associated with synaptic vesicles, it is also produced in lower amounts by astrocytes and released from them within EMVs in response to stress conditions (Wang et al., 2011). These glia-derived EMVs promote neurite outgrowth and increase neuron survival through association between synapsin I and oligomannosidic glycans in response to depolarization and toxic insults to neurons. Brain injury leads to accumulation of toxic proteins in neurons with survival promoted by expression of Ndfip1, an interacting protein with Nedd4 ubiquitin ligases which mediate protein degradation (Sang et al., 2006). Both Ndfip1 and Nedd4 proteins are found in EMVs released by neurons and are hypothesized to serve as a means of rapid removal of toxic proteins after injury (Putz et al., 2008). In a case where EMVs can cause damage to nervous tissue, during brain injury increase in extracellular ATP leads to release of EMVs from microglia and astrocytes through a sphingomyelinase-dependent process (Bianco et al., 2009). These EMVs contain and release IL-1β, a cytokine which inducing inflammatory responses that are damaging to brain tissue.

In a variation on the EMV release mechanism, Schwann cells surrounding a degenerating or damaged peripheral nerve, translocate vesicles surrounded by two membranes containing polyribosomes into the axon where the contents are released (Court et al., 2008; Twiss and Fainzilber, 2009; **Figure 1C**). This serves as a means of delivering mRNA and ribosomes to injured neurons to promote local protein synthesis needed for regeneration, with recent studies *in vivo* showing that labeled ribosomes in the nerve are derived from Schwann cells (Court et al., 2011).

PATHOLOGY

Neurodegeneration

Extracellular membrane vesicles have been implicated in the spread of toxic proteins within the nervous system in a number of neurodegenerative diseases, including transmissible spongiform encephalopathies, Alzheimer's disease (AD), Parkinson's disease (PD), tauopathies, and amyotrophic lateral sclerosis (ALS; for review see Vingtdeux et al., 2007; Vella et al., 2008; Guest et al., 2011; Frühbeis et al., 2012). In all these diseases mutated or "misfolded" proteins serve as templates for formation of protein oligomers that accumulate and interfere with neuronal function, eventually leading to cell death. Possibly in an attempt to rid themselves of these proteins, neurons process them through the endosomal pathway leading either to degradation in lysosomes or to incorporation into MVBs, with the latter serving as a release hatch into the

extracellular space within EMVs. In early studies of the role of EMVs in this process, two groups described the incorporation of both the normal prion protein (PrP) and the misfolded pathogenic prion protein (PrPsc) into EMVs (Ecroyd et al., 2004; Fevrier et al., 2004; Février et al., 2005). Further studies showed that EMV-associated PrPsc was transmitted to normal cells with initiation of prion propagation involving PrP in those cells (Vella et al., 2007). This concept of the tendency of some proteins to seed their own aggregation with "infectious" delivery via EMVs has been implicated in a number of neurodegenerative diseases. Interestingly, in many of these diseases there is a spatiotemporal propagation of the pathology suggesting cell-to-cell spread (Guest et al., 2011), which for non-secreted proteins could be mediated by EMV transfer or nanotubes (Rustom et al., 2004; Gousset and Zurzolo, 2009).

Alzheimer's disease

The concept of EMV-mediated transfer of aggregation-prone proteins has been the most studied for the amyloid-β (Aβ) peptide associated with AD. Although the neuropathologic plaques characteristic of the disease are extracellular, it is currently believed that the plaques may represent a "disposal dump" and it is really the soluble oligomeric fibrils of the Aβ peptide which are neurotoxic (Lublin and Gandy, 2010), as they can serve as a "seeding" center for AD pathology in naive mice (Eisele et al., 2010). These peptides are generated when the amyloid precursor protein is proteolytically processed at the plasma membrane with peptides being re-taken up into endosomes where they can enter MVBs and be released from the cell in EMVs (Rajendran et al., 2006). The possibility that Aβ EMVs can serve as infectious agents is supported by orally transmitted amyloid A1 (AA) amyloidosis among cheetahs (Zhang et al., 2008) with EMVs present in saliva and fecal matter – although, to-date, the role of EMVs in either disease process has not been confirmed. EMVs can also serve as a means of degradation of toxic Aβ when taken up by microglia, but when that clearance pathway is overwhelmed pathologic accumulation of Aβ neurons commences (Yuyama et al., 2012).

Other neurodegenerative conditions

In other neurodegenerative diseases, proteins capable of seeding pathology have also been found in EMVs and shown to have spatiotemporal spread within the nervous system. These include the microtubule-associated tau protein which aggregates in a number of dementia states (Saman et al., 2012), the mutant SOD1 protein underlying some familial forms of ALS (Gomes et al., 2007) and α-synuclein which plays a central role in PD pathogenesis (Emmanouilidou et al., 2010). Studies in PD mouse models show that grafted cells containing aggregated alpha α-synuclein can transfer this aggregate to host cells in the brain (Hansen et al., 2011). These aggregates appear to be passed between cells through the extracellular space as independent entities or via EMVs or nanotubes (Hansen and Li, 2012) with the relative contribution of each of these pathways in the brain still undetermined. It remains an intriguing possibility that EMVs can act as "infectious" agents to spread toxic oligomerizing proteins not only within an individual, but between individuals through fluid contacts, as in amyloidosis in cheetahs (Zhang et al., 2008).

Other disease states

Roles are emerging for EMVs in a number of neurologic disease states. They are capable of transmitting death signals, for example, incorporation of caspase-1 into EMVs produced by monocytes and with transfer to surrounding cells can be the "kiss of death" (Sakar et al., 2009). Apoptotic bodies which form during cell death are also caspase-containing EMVs that can deliver contents to other cells (Simpson et al., 2008). EMVs have also been implicated in autoimmune diseases, such as multiple sclerosis, with those derived from DCs leading to activation of inflammatory NF-κB in microglia and recruitment of major histocompatibility complex (MHC) class II for presentation of self-antigens (Teo and Wong, 2010). In contrast, EMVs can also be protective, with those shed from endothelial cells and astrocytes containing nucleoside triphosphate diphosphohydrolases which can degrade toxic levels of ATP released during breach of the blood brain barrier (BBB) in ischemia (Ceruti et al., 2011). As an apparently common form of intercellular communication, EMVs are undoubtedly a critical player in many different events in the nervous system – providing protection from neurodegeneration, as well as propagation of toxic influences.

TUMORS

Early on investigators noticed that glioblastoma (GBM) cells were covered with "microparticles" (EMVs; Tani et al., 1978). During transformation and progression to malignancy brain tumor cells appear to increase the number and types of EMVs released, as for example when EMV content is compared in conditioned media from GBM cells with normal cells in culture (Balaj et al., 2011). EMVs are believed to be used by tumor cells to modify normal cells in their vicinity so as to promote tumor growth, with most studies carried out in non-nervous system tumors. Pro-active mechanisms include suppression of immune responses to the tumor, opening up of the extracellular space to facilitate tumor cell invasion, stimulation of angiogenesis and modulation of cellular phenotypes (for review see Ichim et al., 2008; Al-Nedawi et al., 2009; Muralidharan-Chari et al., 2010). In addition to proteins within EMVs which modulate these responses, EMVs from GBM cells (as well as other tumor cell types) are also enriched in RNAs associated with proliferation, invasion and immune repression (Skog et al., 2008). Other tumor-related aspects of EMVs include their ability to expel chemotherapeutic drugs from tumor cells (Shedden et al., 2003) and to carry tissue factor leading to hypercoagulation in cancer patients (Zwicker et al., 2009). It seems likely that most of these tumor-enhancing functions of EMVs are associated with tumors of the nervous system as in tumors in other tissues.

Immune responses

There is an extensive literature on the role of EMVs both in suppression and enhancement of immune responses in cancer, most of which have been studied in peripheral tumors. In general, EMVs released by tumor cells serve to suppress the immune response to tumor antigens, including acting as decoys in the tumor environs, switching off T cell responses, eliminating antitumor effector cells, and preventing differentiation of immature DCs into antigen-presenting cells (for review see Taylor and Gerçel-Taylor, 2005; Iero et al., 2008; Théry et al., 2009).

Angiogenesis and invasion

Extracellular membrane vesicles from GBM cells in culture promote angiogenesis of human brain microvascular endothelial cells, which is mediated in part by their relatively high concentrations of angiogenic factors – VEGF, IL-8, TIMP-1, IL-6, and angiogenin as compared to the tumor cells of origin (Skog et al., 2008; Graner et al., 2009). Another EMV protein in this angiogenic cascade includes delta-like 4 Zigand (D114), a recently identified partner for the Notch receptor, that inhibits Notch signaling thereby increasing vessel density and branching in glioma xenograft tumors *in vivo* (Sheldon et al., 2010). Other components of EMVs which promote angiogenesis include sphingomyelin (Kim et al., 2002) and CD147/extracellular matrix metalloprotease inducer (EMMPRIN; Millimaggi et al., 2007). In addition, the acidic environment of the tumor can cause lysis of EMVs and release of vesicular proteins, such as VEGF (Taraboletti et al., 2006). Several studies using other tumor cell types have also indicated possible transfer of functional miRNAs via EMVs (e.g., Collino et al., 2010; Kosaka et al., 2010; Zhang et al., 2010; Mittelbrunn et al., 2011; Yang et al., 2011). miRNA-296 is known to be elevated in brain microvascular endothelial cells as part of an angiogenic response to the presence of glioma cells (Würdinger et al., 2008) and this miRNA is contained in GBM EMVs (J. Skog, unpublished data), so it seems likely that glioma EMVs may contribute to elevated miRNA-296 in endothelial cells. Tumor-derived EMVs also express matrix metalloproteinases (MMPs) and an extracellular MMP inducer on their surface to degrade the extracellular matrix and thereby facilitate invasion of tumor cells into surrounding normal brain tissue (e.g., Castellana et al., 2009).

Phenotypic modification

Extracellular membrane vesicles released by brain tumors alter the phenotype of surrounding cells, presumably through a complex of factors including transcriptional regulators (both proteins and ncRNA), miRNAs, mRNAs, and surface receptors (for review see van der Vos et al., 2011). An early example of this was the demonstration that glioma cells expressing the mutant epidermal growth factor receptor variant III (EGFRvIII) on their plasma membrane pass this onto the membrane of EMVs from where is incorporated into recipient cell membranes, leading to an increased transformative phenotype of the recipient cells (Al-Nedawi et al., 2008). Parallel studies showed that the mRNA for EGFRvIII is also present in EMVs from mutant-positive tumor cells and can be detected in serum EMVs from patients who harbor the corresponding mutation in their tumors, thereby providing a biomarker for genetic status of the tumors (Skog et al., 2008). Others have shown that oligodendroglioma cells send out EMVs containing the apoptotic protein, TRAIL, which leads to death of normal astrocytes and neurons (Lo Cicero et al., 2011).

Genotypic modification

Interestingly, a number of tumors release retroviral-like particles contained within the EMV pool that contain the RNA from human endogenous retroviral (HERV) sequences and reverse transcriptase (Lavie et al., 2005; Contreras-Galindo et al., 2008; Balaj et al., 2011), and may thus mediate abortive infections which disrupt the recipient cell genome. GBM and medulloblastoma brain tumor cells have been found to harbor high levels of HERV RNAs, as well as reverse transcriptase activity (Balaj et al., 2011). It seems likely that a component of increased EMV production by these tumor cells comprises retroviral-like particles, which though non-replicative may still have the capacity to "infect" other cells and integrate into the recipient cell genome, thereby causing potential mutagenesis and oncogene activation. Further, HERVs encode fusogenic proteins which may increase the ability of tumor-derived EMVs to fuse with and enter recipient cells (Duelli and Lazebnik, 2007).

Other studies have shown that during tumor cell death, ABs which fractionate with EMVs, contain oncogene DNA for H-ras and c-Myc which are taken up by other cells (Holmgren et al., 1999; Bergsmedh et al., 2001). Even non-dying tumors cells with amplified c-Myc release this DNA into EMVs (Balaj et al., 2011). It remains to be determined if these EMV-transferred oncogenes can be integrated into the genome of recipient cells.

VIRUSES

Three viruses associated with disruption of brain functions have been found to use EMVs to promote infection and avoid immune rejection by the host, as well as in some cases to confer resistance to infection (for review see Meckes and Raab-Traub, 2011; Wurdinger et al., 2012). These three viruses are – herpes simplex virus type 1 (HSV-1), which in immune compromised patients can cause viral encephalitis (Steiner, 2011); the tumorigenic herpes virus, Epstein–Barr virus (EBV), which can cause central nervous system (CNS) lymphomas (Gerstner and Batchelor, 2010); and human immunodeficiency virus (HIV), which can lead to neurocognitive deficits, dementia, and premature brain aging (Gannon et al., 2011).

Herpes simplex virus type 1

Early during HSV-1 replicative infection/activation, prior to production of infectious virions, EMVs are released from infected cells and serve to prime surrounding cells for productive infection and to reduce immune rejection of the virus (McLauchlan et al., 1992). These EMVs contain viral tegument proteins, some of which serve as immediate early transcription factors to "jump start" secondary infection (Dargan and Subak-Sharpe, 1997). A viral glycoprotein contained in the EMVs, glycoprotein B (gB) also acts on MHCII molecules to prevent presentation of viral peptide antigens to the immune system (Temme et al., 2010).

Epstein–Barr virus-infected cells also use EMVs to reduce the immune response through incorporation of immune suppressive proteins, LMP1 (Flanagan et al., 2003), and galectin-9 (Klibi et al., 2009). In addition, EBV transfers viral miRNAs via EMVs to repress translation of cell proteins which promote resistance to infection (Pegtel et al., 2010; Meckes et al., 2010). EMVs produced by different cell types may have different effects, for example those released by B cells containing glycoprotein 350 can block EBV infection of other cells (Vallhov et al., 2011).

Human immunodeficiency virus retroviral particles bud from the plasma membrane in a similar manner to MVs (Gould et al., 2003; Jouvenet et al., 2011). EMVs released from cells harboring HIV can confer increased infectivity to other cells through transfer of CCR5 co-receptors (Mack et al., 2000) and CXCR4, a

chemokine receptor that interacts with CD4 on the cell surface to facilitate HIV entry (Rozmyslowicz et al., 2003). EMVs derived from infected cells also contain the HIV protein, Nef, which can induce apoptosis of $CD4^+$ cells (Lenassi et al., 2010), thus suppressing the immune response. In a bit of a turnaround these EMVs from HIV infected cells also contain an anti-viral cytidine deaminase which can inhibit viral replication (Khatua et al., 2009).

EMVs AS THERAPEUTIC DELIVERY VEHICLES

Before the recent landmark discoveries of EMVs as a new conduit for cell-to-cell genetic communication (Ratajczak et al., 2006b; Valadi et al., 2007; Ramachandran and Palanisamy, 2011; Turchinovich et al., 2011; Chen et al., 2012), the artificial counterpart of EMVs, liposomes, had been well studied as a nanodelivery system over past decades. Liposomes are spherical vesicles composed of one or multiple natural and/or synthetic lipid bilayers with an aqueous core and a diameter ranging from 50 nm to 5 μm. By taking advantage of these structural properties, liposomes have been investigated as a means to "load and deliver" pharmaceutical drugs and peptides (reviewed in Malam et al., 2009). However, a major conundrum in liposome-mediated delivery lies in its biocompatibility and biodegradability properties, such that ideal liposomes should evade detection by the immune system and have a longer half-life in the circulation for therapeutic cargo delivery (reviewed in Immordino et al., 2006). While remarkable advances have been made in reducing immunogenicity of liposomes, described as "stealth liposomes" and in increasing their half-life in the circulation by coating them with poly-(ethylene glycol, PEG; Allen et al., 1989, 1991), researchers continue to seek endogenous nanodelivery systems to overcome the obstacles faced by artificial vesicles. With emerging understanding of their biological functions, EMVs have been suggested as an ideal candidate to fulfill this role as "physiologic liposomes."

Investigators have made significant progress in the use of EMVs for therapy by taking advantage of their low immunogenicity and unique delivering capability (Zhang et al., 2007; Sun et al., 2009; Alvarez-Erviti et al., 2011; Zhuang et al., 2011; Bolukbasi et al., 2012). By genetic engineering of EMV producer cells or direct modification of EMVs, they can be used to transport therapeutic molecules and agents via insertion into the lipid bilayer and/or loading into their aqueous core (**Figure 2**). Analogous to liposomes, EMVs also serve as an excellent means of protection of "therapeutic cargoes" wherein packaged mRNA, small interfering RNAs (siRNA), proteins, and drugs are better preserved from degradation when compared to their unshielded counterparts. Using these advantages, EMV-mediated therapy is being actively

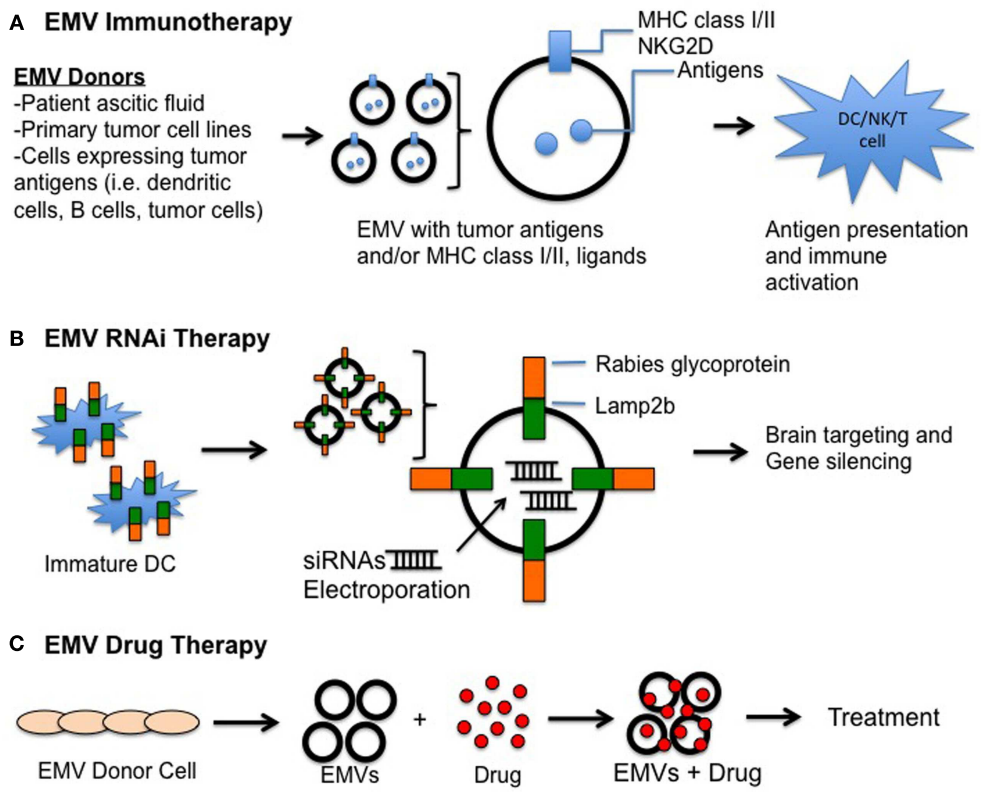

FIGURE 2 | Extracellular membrane vesicles-based therapies. (A) EMV immunotherapy. EMVs containing tumor-antigen within and/or on the membrane surface are isolated from different sources and introduced *in vivo* to elicit targeted immune responses. **(B)** EMV RNAi therapy. EMVs derived from immature dendritic cells (DCs) expressing Rabies glycoprotein-Lamp2b fusion protein were electroporated with siRNAs for targeting against neurons, microglia, and oligodendrocytes for subsequent gene silencing (Alvarez-Erviti et al., 2011). **(C)** EMV drug therapy. Therapeutic compounds can be packaged into/onto EMVs isolated from donor cells to minimize degradation and increase delivery to intended sites.

studied in three main fields: immunotherapy, RNA-interference (RNAi) and drug delivery.

EMVs in cancer immunotherapy

Immunotherapy represents one of the most investigated aspects in EMV-mediated therapy. In immunotherapy, an ideal cancer vaccine serves as an antigen-presenting medium to prime the immune system to recognize tumor-specific antigens, thereby eliciting immune responses against the tumor cells while leaving normal cells unharmed (Trumpfheller et al., 2012). A study by Raposo et al. (1996) first showed B lymphocyte-secreted EMVs contained MHC class II by immunoelectron microscopy and could induce MHC class II-dependent $CD4^+$ T cell responses *in vitro*. Since DCs are the most potent antigen-presenting cells in the adaptive immune system, numerous studies ensued focusing primarily on the immuno-modulating effect of EMVs on DCs in search of more effective cancer vaccines (see review in Tan et al., 2010). Briefly, researchers have used EMVs isolated from various sources to pulse DCs with antigens (André et al., 2004; Chaput et al., 2004; Cho et al., 2005; Hao et al., 2007; Taïeb et al., 2006; Bai et al., 2007; Beauvillain et al., 2007; Guo et al., 2008; Temchura et al., 2008; Viaud et al., 2009; Bu et al., 2011) in order to activate immune cells (i.e., T lymphocytes and natural killer cells) against tumor cells, including those in the ascitic fluid of cancer patients (André et al., 2002), and wild-type or engineered cancer cells *in vitro* (Wolfers et al., 2001; Hegmans et al., 2005; Chen et al., 2006; Yang et al., 2007; Xiu et al., 2007; Ristorcelli et al., 2008, 2009; Cho et al., 2009; Xie et al., 2010; Rountree et al., 2011; Zeelenberg et al., 2011). Furthermore, Viaud et al. (2011) recently reported that highly immunogenic, clinical grade EMVs isolated from interferon-γ treated monocyte-derived DCs express CD40, CD80, CD86, and ICAM-1 on their membranes and can prime $CD8^+$ T cells in a peptide-dependent manner (i.e., MART1, melanoma antigen recognized by T cells) both *in vitro* and *in vivo*.

Despite the recent progress made in developing EMVs as vaccines against different types of cancer, a brain cancer-specific EMV vaccine has not yet been reported. This is not surprising since the CNS is considered an immune-privileged site as it lacks a lymphatic system, has low numbers of circulating T lymphocytes, and possesses a BBB consisting of endothelial cells joined by tight junctions, which restricts passage of larger molecules and cells. In addition, high grade gliomas induce immunosuppression in patients, constituting a further challenge to CNS tumor immunotherapy (Bodmer et al., 1989; Misra et al., 2003; Fecci et al., 2006; Avril et al., 2010; Gustafson et al., 2010). To counter this phenomenon, active immunotherapy using autologous DCs pulsed with autologous tumor antigens, as well as GBM-specific antigens including EGFRvIII peptides have been shown to prolong survival in some patients with primary or recurrent GBMs (reviewed in Thomas et al., 2012). Given that EMVs are capable of ferrying antigens that can subsequently pulse DCs, they may serve as a means to administer active immunotherapy against brain cancers. Notably, a recent paper by Alvarez-Erviti et al. (2011) reported brain-targeting EMVs, which cross the BBB following systemic injection into mice with EMVs derived from syngeneic DCs engineered to express a targeting ligand (Alvarez-Erviti et al., 2011; discussed below in *EMVs in RNAi therapy*). Altogether, these encouraging findings warrant future investigation to examine if EMVs can be employed as an effective CNS cancer vaccine vehicle, thus overcoming immune-privileged properties of the CNS.

EMVs in RNAi therapy

RNA-interference therapies have been actively investigated in the past few decades to target various human diseases, including genetic disorders, HIV infection, and cancers (Burnett and Rossi, 2012). While RNA-based therapies can involve the use of ribozymes, aptamers, and siRNAs, this section will focus on the emerging application of EMVs as a delivery vehicle for therapeutic siRNAs.

Small interfering RNAs are short (∼21–23 nt), single-stranded RNA molecules that target mRNAs with perfect or near-perfect Watson–Crick base-pairing to initiate posttranscriptional gene silencing. In brief, siRNAs can be produced from exogenously introduced double-stranded RNAs (dsRNAs) or short hairpin RNAs (shRNAs) expressed in cells. Upon binding to a pre-RNA-Induced Silencing Complex (RISC) containing Dicer and TAR RNA-binding protein, dsRNAs and shRNAs are processed into siRNAs (passenger or guide) and loaded into RISC complex for mRNA targeting via Watson–Crick based-pairing by the guide strand (Bernstein et al., 2001; Martinez et al., 2002). Argonaute 2 (AGO2), an endonuclease, of the RISC complex then cleaves the target mRNA to inhibit gene expression (Matranga et al., 2005; Rand et al., 2005). Importantly, since the guide strand remains protected from degradation within the RISC complex, it can be used repeatedly to degrade other targeted mRNAs (Matranga et al., 2005; Rand et al., 2005). With these properties, siRNA has been considered as an ideal candidate for RNAi therapy (Burnett and Rossi, 2012).

However, aside from its off-target effects, one of the major challenges confronted by siRNA therapy lies in its delivery formulation *in vivo*. siRNAs can be immunogenic and are inherently prone to degradation due to ribonucleases present in the extracellular space, serum and cells (Whitehead et al., 2011). While strategies such as chemical modification of siRNAs to counter degradation have been developed to minimize these drawbacks, a vehicle to deliver and shelter siRNAs from external environments, as well as to mediate targeting to specific cells has long been sought-after (Castanotto and Rossi, 2009). Vehicles such as liposomes and nanoparticles have been recruited to serve these functions but are faced by immunogenicity issues (see *EMVs as therapeutic delivery vehicles*) and difficulty in transversing the BBB in the CNS, making siRNA delivery to the brain particularly difficult (Shim and Kwon, 2010; see *EMVs in cancer immunotherapy*).

Extracellular membrane vesicles, on the other hand, have been shown to preserve mRNAs and miRNAs within their "aqueous" proteinaceous core even under external RNase treatment, and subsequently to deliver functional RNAs to recipient cells (Valadi et al., 2007; Skog et al., 2008; Zomer et al., 2010; Mathivanan et al., 2011). Remarkably, Alvarez-Erviti et al. (2011) recently reported an exciting strategy targeting EMVs to the brain via systemic injection in mice. By fusing neuron-targeting rabies viral glycoprotein (RVG) peptides to the N-terminus of Lamp2b, a murine exosomal membrane protein, and expressing it in immature DC derived from mouse bone marrow, this team successfully isolated

brain-targeting EMVs (Alvarez-Erviti et al., 2011). The EMVs were loaded with siRNAs by electroporation which targeted either *GAPDH* or *BACE1* mRNAs, the latter being a therapeutic target in AD. These EMVs were injected intravenously into syngeneic mice, resulting in a significant knock-down of expression of the targeted mRNAs in the brain when compared to other body tissues (Alvarez-Erviti et al., 2011). Furthermore, two studies have recently described a "zipcode"-like sequence in the 3′ untranslated region (3′UTR) of enriched mRNAs in EMVs derived from human primary GBM and melanoma cells (Batagov et al., 2011; Bolukbasi et al., 2012). This suggests that mRNAs, and perhaps therapeutic siRNAs, can be specifically targeted for EMV packaging in cells. While more studies are required to address the use of EMVs in clinical trials, these novel findings shed light on the promising potential of EMV-mediated RNAi therapy.

EMVs in drug therapy

Like other therapeutic strategies, *in vivo* delivery of conventional therapeutic drugs has also faced similar challenges, including targeted delivery to tissues/cells, poor drug stability and rapid metabolic degradation. To explore EMV's potential as a drug delivery vehicle, Sun et al. (2009) first reported successful loading of curcumin, a polyphenol anti-inflammatory compound, into EMVs ("exosomal curcumin"). "Exosomal curcumin" exhibited higher solubility and bioavailability than curcumin alone, and "exosomal curcumin" significantly decreased lipopolysaccharide (LPS)-induced inflammatory activity both *in vitro* and *in vivo* more effectively than curcumin itself. It is worth noting, however, that unlike "typical exosomes" which band between 30 and 45% sucrose after gradient density centrifugation, "exosomal curcumin" banded between 45 and 60% sucrose (Sun et al., 2009), suggesting that "exosomal curcumin" may comprise a subpopulation of EMVs and/or the added molecular weight from curcumin loads. Following these encouraging findings, the same group reported successful delivery of "exosomal curcumin" and "exosomal JSI124," a signal transducer and activator of transcription 3 (Stat3) inhibitor, to the rodent brain via intranasal injection, thereby bypassing the BBB (Zhuang et al., 2011). Remarkably, EMV-mediated delivery of curcumin significantly suppressed LPS-induced inflammation, as well as myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis (Zhuang et al., 2011), an animal model for human CNS demyelinating diseases such as multiple sclerosis and acute disseminated encephalomyelitis (Miller and Karpus, 2007). Additionally, "exosomal JSI124" delivered via intranasal administration suppressed GL26 glioma growth in the brain (Zhuang et al., 2011). Although the authors reported no apparent toxicity or aberrant behavior in the animals during and after treatment, more detailed studies are required to establish the safety parameters of intranasal administration of EMVs, such as dosage and potential immunogenicity. Encouraging findings from these studies support a new means for drug delivery and warrant upcoming investigations to test EMV packaging of other therapeutic compounds, EMV immunogenicity, as well as route of delivery across the BBB for future clinical considerations.

CONCLUSION

With the emergence of EMVs as a *de novo* extracellular organelle for cell-to-cell communication, researchers have gathered and studied the role of EMVs under both physiological and pathological conditions, as well as their applications for therapies. In the present review, we have focused on recent discoveries of EMVs' involvement in the nervous system and EMV-mediated therapies developed to-date. While an impressive number of exciting findings have been made in the past few years, many questions still remain to be answered with respect to different aspects of EMV biology. Due to the different cellular origins and biogenesis of EMVs, standardized nomenclature and isolation protocols for EMVs need to be established by the research community for better advancement of ongoing EMV research. Meanwhile, although a majority of the studies to-date has focused on their involvement in diseases, relatively few have reported on EMV's physiological role during development and adult functions in the nervous system. Understanding EMVs' half-life, circulation, and release of cargoes *in vivo* will also be needed to illuminate this intricate intercellular communication system within the body. Altogether, future investigations and exciting findings in EMVs should further reveal how multiple cellular populations communicate and interact, as well as how EMVs can be employed in therapies.

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Impact of biofluid viscosity on size and sedimentation efficiency of the isolated microvesicles

Fatemeh Momen-Heravi[1†], Leonora Balaj[2,3†], Sara Alian[4], Alexander J. Trachtenberg[1], Fred H. Hochberg[2], Johan Skog[5] and Winston Patrick Kuo[1,6]*

[1] *Harvard Catalyst Laboratory for Innovative Translational Technologies, Harvard Medical School, Boston, MA, USA*
[2] *Department of Neurology and Radiology, Massachusetts General Hospital, Boston, MA, USA*
[3] *Neuro-Oncology Research Group, Cancer Center Amsterdam, Amsterdam, Netherlands*
[4] *Biopolymers Facility, Harvard Medical School, Boston, MA, USA*
[5] *Exosome Diagnostics Inc, New York, NY, USA*
[6] *Department of Developmental Biology, Harvard School of Dental Medicine, Boston, MA, USA*

Edited by:
Claudia Verderio, CNR Institute of Neuroscience, Italy

Reviewed by:
Michael Graner, University of Colorado Denver, USA
Antonio Chiesi, HansaBioMed OU, Italy

***Correspondence:**
Winston Patrick Kuo, Harvard Catalyst Laboratory for Innovative Translational Technologies, Harvard Medical School, 4 Blackfan Circle, Room 144, Boston, 02115 MA, USA.
e-mail: wkuo@catalyst.harvard.edu

†Fatemeh Momen-Heravi and Leonora Balaj have contributed equally to this work.

Microvesicles are nano-sized lipid vesicles released by all cells *in vivo* and *in vitro*. They are released physiologically under normal conditions but their rate of release is higher under pathological conditions such as tumors. Once released they end up in the systemic circulation and have been found and characterized in all biofluids such as plasma, serum, cerebrospinal fluid, breast milk, ascites, and urine. Microvesicles represent the status of the donor cell they are released from and they are currently under intense investigation as a potential source for disease biomarkers. Currently, the "gold standard" for isolating microvesicles is ultracentrifugation, although alternative techniques such as affinity purification have been explored. Viscosity is the resistance of a fluid to a deforming force by either shear or tensile stress. The different chemical and molecular compositions of biofluids have an effect on its viscosity and this could affect movements of the particles inside the fluid. In this manuscript we addressed the issue of whether viscosity has an effect on sedimentation efficiency of microvesicles using ultracentrifugation. We used different biofluids and spiked them with polystyrene beads and assessed their recovery using the Nanoparticle Tracking Analysis. We demonstrate that MVs recovery inversely correlates with viscosity and as a result, sample dilutions should be considered prior to ultracentrifugation when processing any biofluids.

Keywords: biofluids, viscosity, micovesicles, sedimentation efficiency, size, ultracentrifugation

INTRODUCTION

Exosomes are nano-sized vesicles (MVs; 30–100 nm) of endosomal origin produced by different parental cells (Keller et al., 2006; Skog et al., 2008; Muralidharan-Chari et al., 2010). Nanoparticles formed through membrane budding are also called microvesicles and their corresponding process of formation is called microvesiculation (Muralidharan-Chari et al., 2010). Their sizes differ from 30 nm in diameter and have been reported up to 5 μm, the former including the more homogenous population of exosomes released from multivesicular bodies (MVBs) and the latter shedding from the plasma membrane which are commonly referred to as MVs (Di Vizio et al., 2009; Théry et al., 2009). In this article, we will refer to all types of shed vesicles under the common term of microvesicles (MVs).

Microvesicles have been extensively studied in serum and culture media from a variety of tumors (Balaj et al., 2011; Koumangoye et al., 2011); a great body of evidence shows that they can be secreted into the extracellular space and are involved in intercellular communication by transferring functional proteins and RNA molecules between cells (Skog et al., 2008; Grange et al., 2011; Yang et al., 2011). MVs are also known to carry antigens from microorganisms like viruses and bacteria and can be potential biomarkers for a variety of diseases (Jayachandran et al., 2011; Raymond et al., 2011). MVs are found in different biofluids such as plasma (Ashcroft et al., 2012), serum (Dalton, 1975), cultured media (CM; Bastida et al., 1984), saliva (Keller et al., 2011), breast milk (Hata et al., 2010), amniotic fluid (Keller et al., 2011), and urine (Wiggins et al., 1987).

A variety of methods have been utilized to isolate microvesicles including sucrose gradient, ultracentrifugation, Exoquick™, microfiltration, and immune affinity capture method (Taylor et al., 2011; Tauro et al., 2012). A standardized method for isolation and assessment of MVs from various body fluids and culture media has not yet been established and hinders reproducible studies for downstream analysis of isolated MVs (Yuana et al., 2011). Ultracentrifugation is considered the "gold standard" for harvesting microvesicles, though inconsistencies have been reported in reproducibility and repeatability of the data. Ultracentrifugation protocols vary across users and this leads to inconsistencies in recovery of MVs (Sustar et al., 2011; Taylor et al., 2011; Tauro et al., 2012).

Viscosity of a fluid is the resistance of a fluid that is being deformed by either shear or tensile stress. Due to different chemical and/or molecular compositions, the makeup of different biofluids

will result in their varying viscosities. This manuscript explores the recovery of MVs derived from different biofluids (serum, plasma, and culture media) with different viscosities, using ultracentrifugation. To the best of our knowledge, this is the first study to assess this parameter.

MATERIALS AND METHODS

SAMPLES AND PRELIMINARY SAMPLE PREPARATION

In this study we used biobanked plasma and serum as well as CM from HEK-293T cells. Whole blood samples were obtained from healthy volunteers upon approved IRB protocols at Massachusetts General Hospital and Harvard Medical School. Serum samples were collected in 10 ml tiger top tubes (BD vacutainer), allowed to coagulate at room temperature for 30 min and spun at 1,300 × *g* for 10 min to separate serum from coagulated blood. The serum was then filtered through a 0.8-μm filter, aliquoted into 2 ml cryovials, and stored at −80°C. Plasma was collected into EDTA-containing tubes (BD vacutainer), spun at 1,500 × *g* for 10 min to separate plasma from the buffy coat. Plasma was then transferred to a clean tube and filtered through a 0.8-μm filter and stored at −80°C until further processing. Culture media was collected from HEK-293T cells, cultured for 48 h in MV-depleted media, and spun at 300 × *g* for 10 min. The supernatant was transferred to a clean tube and spun at 2,000 × *g* for 15 min, filtered through a 0.8-μm filter and stored at −80°C. As controls, polystyrene beads (Thermo Scientific, Fremont, USA) with the specific diameter of 100 nm were used to make control samples (plasma + beads, serum + beads, CM + beads, PBS + beads). Two microliters of serum, plasma, CM, and PBS were spiked with a total of 7.22×10^{10} polystyrene beads without any pretreatment and used for ultracentrifugation. A total of seven samples which included three samples and four controls were used in this study. We use the term MVs for the plasma, CM, and serum without beads and microparticles (MPs) as a term for mixture of MVs of each biofluid plus synthetic added beads.

ULTRACENTRIFUGATION

At the time of analysis 2 ml of serum, plasma, CM, serum + beads, plasma + beads, and PBS + beads were thawed at room temperature for ultracentrifugation. We defined "pre-ultracentrifugation" (pre-UC) as aliquots of each sample prior ultracentrifugation, obtained after vortexing and used for quantity measurement of MVs/MPs. All samples were ultracentrifuged at 100,000 × *g* for 90 min in a Optima Max-XP, fixed angle MLA-55 rotor (*k* factor = 116; Beckman Coulter, Miami, FL, USA), at 4°C. After ultracentrifugation, pellets of samples were collected, and re-suspended in 50 μl PBS and now considered as "post-ultracentrifugation" (post-UC) aliquots of each sample post-ultracentrifugation, used for quantity measurement of MVs/MPs.

NANOSIGHT

Concentration and size analysis of MVs/MPs

The concentration of MVs/MPs for pre-UC samples and post-UC was identified by measuring the rate of Brownian motion using the NanoSight LM10 system (NanoSight, Amesbury, UK) supplemented with a fast video capture and Nanoparticle Tracking Analysis (NTA) software. The instrument was calibrated based on NanoSights's protocol. The samples were measured for 30 s with manual shutter and gain adjustments. Measurements were made for each sample in triplicate after re-calibration of instrument as suggested by NanoSight. NTA was used to measure particle size (measured in nanometers); Pre-UC, Post-UC, and supernatant samples were measured at room temperature in triplicate after calibration of the instrument based on the manufacturer's protocol. Each measurement repeated for three times.

VISCOMETER

Relative viscosities of pre-UC samples (serum, plasma, CM, and PBS) were measured using an Ostwald-type viscometer (Cannon Instrument Co., State College, PA, USA) at constant temperature as described by Fahey et al. (1965), based on time of flow through a volumetric capillary. The viscosity of each liquid (η_1) was determined using the following equation:

$$\eta_1 = \left(\frac{\rho_1 t_1}{\rho_2 t_2}\right) \eta_2,$$

where, ρ_1 = density of unknown liquid, ρ_2 = density of other liquids (water), t_1 = time of the other liquids, t_2 = time of the known liquid, η_2 = viscosity of known liquid. We used the American Society for Testing and Materials (ASTM) standards for measuring dynamic viscosity, *centipoise* (cP).

Statistical analysis

Three measurements (concentration, size, and diffusion coefficient) per sample were generated from the NanoSight instrument for pre-UC and post-UC. Data was averaged and the standard deviation was calculated. The sedimentation efficiency is defined as the difference between initial MVs'/MPs' amount and resulting pellet amount of microvesicles. The sedimentation efficiency of MVs/MPs in the samples was analyzed by one-way ANOVA (*Post hoc* Tukey). Only the data with normal distribution (assessed by the Kolmogorov–Smirnov test) were used. A value of $p < 0.05$ was considered significant. Statistical analyses were performed by using SPSS 15.0 (SPSS Inc., Chicago, USA). The error bars displayed on the NTA graphs were obtained by the standard deviation of the different measurements of each sample. All data is represented as mean ± standard deviation (SD).

RESULTS

HIGHER VISCOSITY RESULTS IN LOWER SEDIMENTATION EFFICIENCY

Table 1 summarizes the mean and standard deviation of pre-UC concentration, post-UC concentration, and sedimentation efficiency for each experimental sample. We noticed a significant difference between sedimentation efficiency of plasma, serum and culture media ($p < 0.001$). The viscosity of the plasma, serum, CM, and PBS were 1.65, 1.4, 1.1, and 1.0 cP, respectively. The Pearson correlation was −0.912 ($p < 0.001$), indicating that a greater viscosity leads to lower sedimentation efficiency. The sedimentation efficiency of plasma with 1.65 cP viscosity was lower because of higher viscosity in comparison to serum (1.4 cP), but the difference was not statistically significant ($p > 0.05$). **Figure 1**, illustrates the comparison of pre-UC MVs/MPs with post-UC concentration. It shows that in spite of lower initial concentration

Table 1 | Evaluation of microvesicles/microparticles concentration (particles/ml) and viscosity before and after ultracentrifugation.

Biofluids (particles/ml)	Mean of pre-UC concentration	Mean of post-UC concentration	Mean of sedimentation efficiency	Std. deviation	Viscosity (cP)
SAMPLES					
Plasma	3.1×10^{12}	7.3×10^{10}	-3.0×10^{12}	1.9×10^{11}	1.65
Serum	3.0×10^{12}	7.4×10^{10}	-2.9×10^{12}	4.0×10^{10}	1.4
Culture media	5.3×10^{10}	2.0×10^{10}	-3.3×10^{10}	5.7×10^{9}	1.1
CONTROLS					
Plasma + beads	3.5×10^{12}	2.0×10^{10}	-3.5×10^{12}	4.0×10^{11}	1.65
Serum + beads	3.5×10^{12}	1.1×10^{11}	-3.4×10^{12}	5.5×10^{10}	1.4
Culture media + beads	9.0×10^{10}	2.2×10^{10}	-6.8×10^{10}	1.1×10^{10}	1.1
PBS + beads	6.9×10^{10}	1.5×10^{10}	-5.4×10^{10}	1.6×10^{10}	1.0

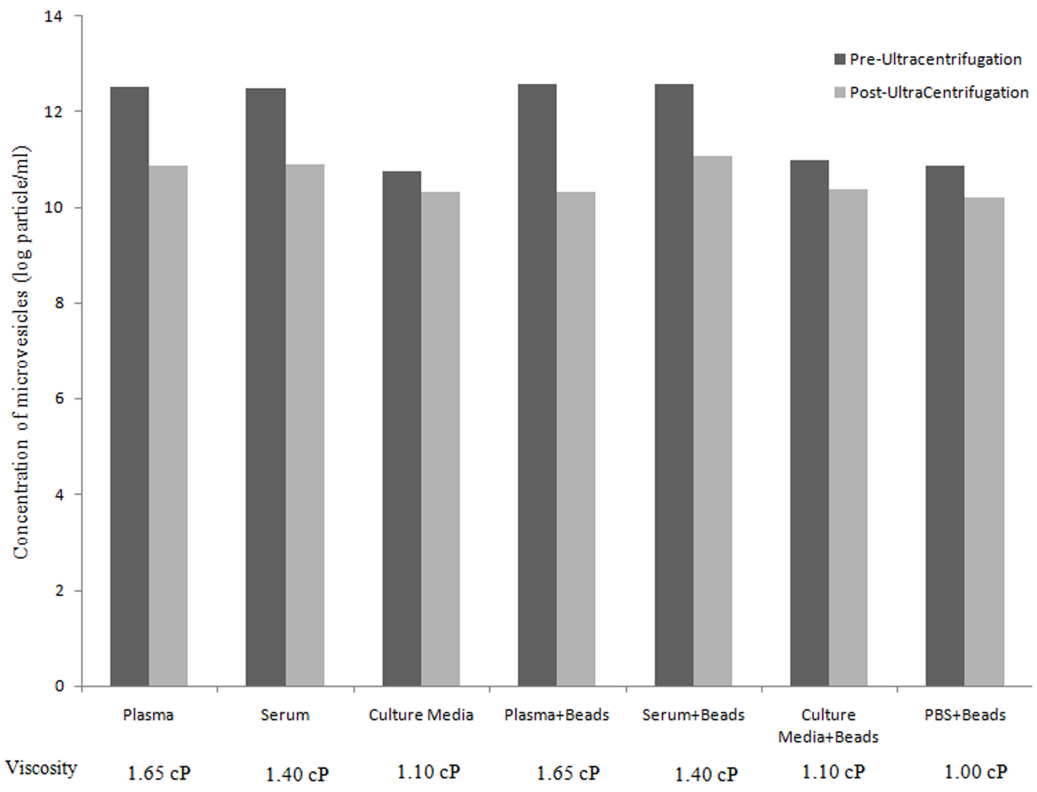

FIGURE 1 | Assessment of microvesicles/microparticles concentration (particles/ml) before and after ultracentrifugation. Bar graph represent the concentration (particles/ml) of MVs/MPs pre-UC (black) and post-UC (gray; *Y* axis) for different tested samples and controls (*X* axis) along with viscosity of each fluid (cP).

of pre-UC MVs, the sedimentation efficiency was higher in CM when comparing MVs' quantities pre-UC with post-UC. Also depicted in **Figure 1**, due to differences in viscosity of plasma and serum when compared to CM, the differences between pre-UC and post-UC was higher in CM when compared to MV quantities ($p < 0.05$). This trend is also seen in plasma + beads and serum + beads versus media + beads ($p < 0.05$), because of plasma and serum's greater viscosity. There were no significant differences between sedimentation efficiency of PBS + beads and culture media + beads pre-UC and post-UC. As presented by **Table 1**, the less viscous fluids (PBS and CM) have higher sedimentation efficiency.

SIZE DISTRIBUTION OF PRE-UC, POST-UC, AND SUPERNATANT

As shown in **Table 2**, the mean size ± SD (nm) of the MVs/MPs in both plasma and serum were found to be significantly larger in Post-UC (plasma = 134.3 ± 11.2 nm; serum = 131.3 ± 2.9 nm) compared to Pre-UC (plasma = 84.0 ± 2.6 nm; serum = 102.0 ± 6.0 nm; $p < 0.05$). Difference between the size of MVs in CM pre-UC (mean ± SD of 107.0 ± 7.0 nm) and post-UC (mean ± SD

Table 2 | Evaluation of microvesicles/microparticles size pre-UC and post-UC.

	Pre-UC MVs/MPs size (mean ± SD) nm	Post-UC MVs/MPs size (mean ± SD) nm	*p* Value	Supernatant MVs/MPs size (mean ± SD) nm
SAMPLES				
Plasma	84.0 ± 2.6	134.3 ± 11.2	0.011[a]	93.7 ± 8.9
Serum	102.0 ± 6.0	131.3 ± 2.9	0.028[a]	100.3 ± 2.1
Culture media	107.0 ± 7.0	118.0 ± 7.9	0.283	111.3 ± 1.2
CONTROLS				
Plasma + beads	96.0 ± 19.31	139.0 ± 6.6	0.028[a]	97.0 ± 22.8
Serum + beads	106.0 ± 5.59	120.7 ± 5.8	0.075	104.7 ± 4.9
Culture Media + beads	113.0 ± 3.0	129.3 ± 2.3	0.003[a]	116.3 ± 3.8
PBS + beads	160.0 ± 13.0	115.7 ± 7.4	0.017[a]	122.0 ± 1.7

[a] *Indicates significant differences in p value.*

of 118.0 ± 7.9 nm) were insignificant. Additionally, MVs in the supernatant of plasma and serum samples were smaller in diameter when compared to same MVs post-UC (mean ± SD of supernatant: plasma = 93.7 ± 8.9 nm, serum = 100.3 ± 2.1 nm; mean ± SD of Post-UC samples: plasma = 134.3 ± 11.2 nm; serum = 131.3 ± 2.9 nm; **Table 2**). Also, PBS + beads showed a significant decrease in average size of MPs post-UC (mean ± SD of pre-UC = 160.0 ± 13.0 versus mean ± SD of post-UC = 115.7 ± 7.4; $p < 0.05$). **Figure 2** shows the NanoSight distribution of MVs for plasma pre-UC and post-UC.

DISCUSSION

Microvesicles are emerging as a source of potential biomarkers with putative prognostic and diagnostic value. One of the interests in the field is to use MVs in a format that could detect initial stages of disease, and accurately predict risk assessment and patient response to therapy. In this study we have examined how viscosity affects sedimentation of MVs using ultracentrifugation. A fluid is termed viscous when the internal frictions are high and as a result, it takes a great deal of energy for particles to initiate and sustain their motion. Viscosity increases with decreasing temperature and most ultracentrifugation steps are carried at +4°C, the highest water density, which suggests that viscosity is at its highest. Viscosity also increases with pressure. Hydrostatic pressure increases up to 200 bar/min in a sample spun at 50,000 rpm (Wattiaux et al., 1971) and this should be taken into account when spinning/comparing different biofluids, assuming all other conditions are kept equal. Here we used a viscometer to determine the "fluid's resistance to flow" which is defined as viscosity. The strain rates are defined by the geometry of the instrument and the corresponding stresses are defined by the fluid's resistance to flow. When one variable is fixed and known, the other force will depend on the viscosity of the fluid. Our results demonstrated that ultracentrifugation of MVs is greatly affected by the viscosity of the biofluid used. Plasma had the highest viscosity (1.65 cP), followed by serum (1.4 cP), culture media (1.1 cP), and lastly PBS (1.0 cP). The viscosity of serum and plasma were concordant with Tangney et al. (1997).

We found that viscosity has a significant correlation with the recovery of MVs/MPs. Because plasma has more proteins, e.g., fibrinogen and other clotting factors, the internal frictions are high and as a result, it requires more energy for particles to move (Tangney et al., 1997). The same extrapolation may be attributed to serum because, although it lacks clotting factors, it has other proteins that increase its internal friction when compared with less viscous fluids like culture media and PBS. The sedimentation efficiency of plasma was lower because of higher viscosity in comparison to serum and culture media. Culture media had a viscosity very close to that of PBS and a higher number of MVs were pelleted in culture media. These results were confirmed when the samples were spiked with 100 nm polystyrene beads. The data suggests that viscosity is an important parameter to consider when working with a biofluid where a lower viscous fluid yields more MVs in the pellet, and that comparison of different biofluids should be avoided unless samples have been diluted to reach similar viscosity values.

Additionally, the result of this study showed that the average size of the MVs increased significantly after ultracentrifugation in plasma and serum ($p < 0.05$); while average size of culture media derived MVs increased insignificantly. The average size of pelleted beads derived from PBS + beads, the less viscous fluid, decreased significantly ($p < 0.05$). This finding contradicts the belief that plasma/serum has MVs that are larger in size in comparison with cell lines. It may indicate that longer ultracentrifugation time is needed because of viscosity, providing the capability of extracting smaller particles from plasma and serum. Another factor that should be taken into account is sedimentation stability (streaming) which affects both accuracy and resolution. Streaming, a factor that is related to Brownian motion of small particles, causes the reported size distribution to be larger than actual size distribution (Scott et al., 2005). MVs derived from plasma and serum had smaller sizes that reflect more Brownian motion during sedimentation, which could lead to reduced resolution and sedimentation efficiency. Another factor that could lead to greater MVs' diameter is lipoprotein fusion; Ala-Korpela et al. (1998) assessed particle fusion based on fluorescent resonance energy transfer and showed that lipoprotein particle fusion could occur after sequential ultracentrifugation.

The following formula considers the centrifugal force, buoyancy, and Stokes law which governs the sedimentation velocity of a spherical particle:

$$v = \frac{\Delta \rho d^2 a}{18\eta}$$

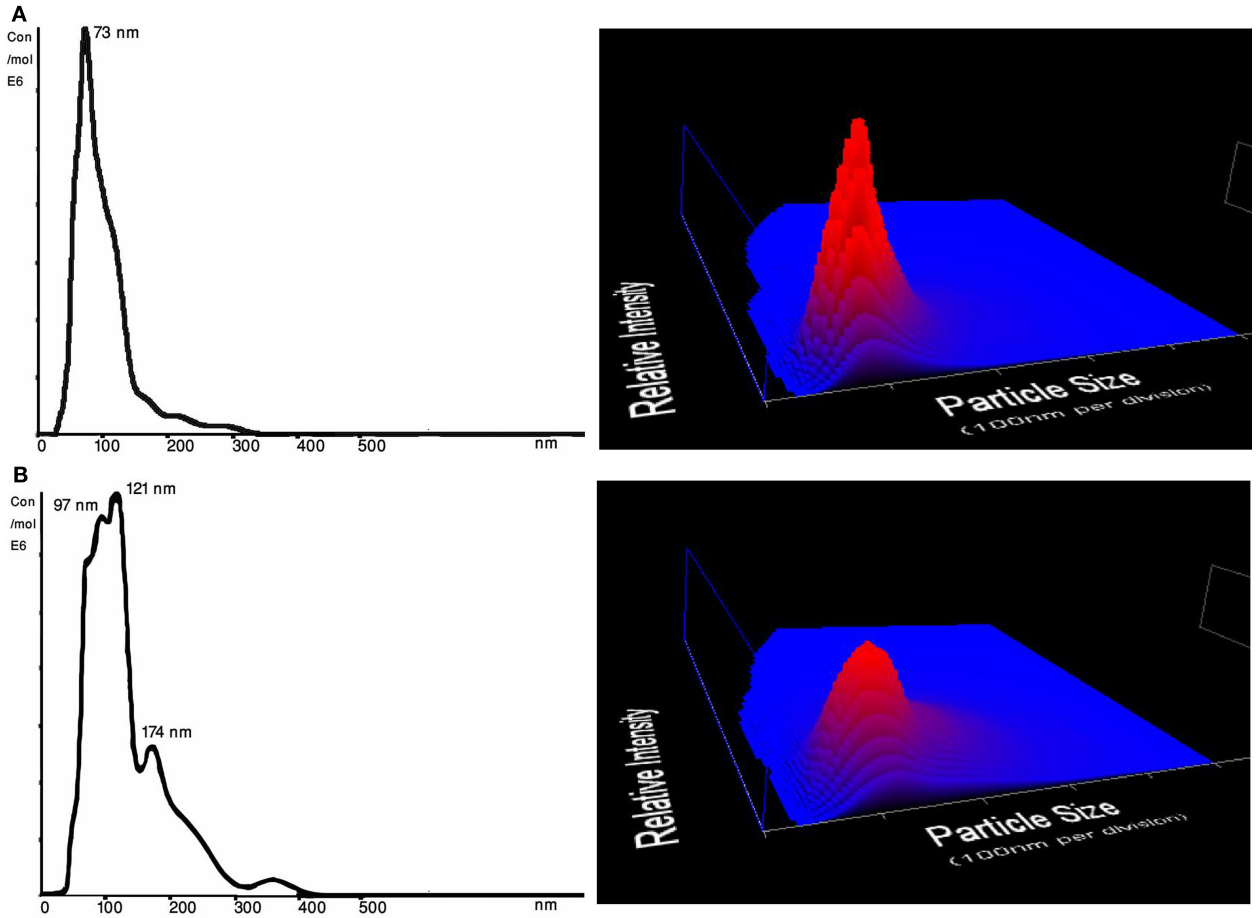

FIGURE 2 | Size distribution (nm) and concentration (particles/ml) from NTA measurements of a representative plasma samples. Three dimensional graph illustrating size versus intensity (relative frequency of each size range among the entire population of MVs) versus concentration (particles/ml) of microvesicles from plasma. **(A)** Plasma MVs Pre-UC – Average size of plasma MVs were 73 nm before ultracentrifugation; 3D graph representing particle size versus intensity versus concentration (particles/ml) of microvesicles before ultracentrifugation **(B)** Plasma MVs Post-UC – Average size of plasma MVs were 137 nm after ultracentrifugation; 3D graph representing particle size versus intensity versus concentration (particles/ml) of microvesicles before ultracentrifugation.

Where $\Delta\rho$ is the difference in densities of the microparticles and the medium, d is the effectual diameter of the MVs, a is the acceleration of the centrifugal force generated in the centrifuge rotor, and η is the viscosity of the medium (Sustar et al., 2011). Based on this formula, along with the effect of ultracentrifugation force and density of MVs, larger particles would sediment more effectively in the same conditions. Also, according to Scott et al. (2005), materials with higher densities (for example higher concentration of MVs) have additional instability after sedimentation, which cause pelletted MVs to detach and return into supernatant. This could be a reason for lower efficiency and smaller average size of MVs/MPS observed in plasma and serum (Scott et al., 2005; Sustar et al., 2011). According to the formula, there are many other factors that could affect sedimentation efficacy such as difference between density of MVs and fluid, centrifugal force (g), temperature, type of rotor (fixed angle versus swing out) and time; further studies are required to assess each factor along with their synergism to improve efficacy of ultracentrifugation protocol. As mentioned above, another factor that should be taken into account is the great likelihood of MVs/MPs fusion, based on natural stickiness of MVs/MPs, which could be influenced by their different derived media and its buffer characteristics such as salt concentration, and ionic contents (Balaj et al., 2011; Jayachandran et al., 2012). Follow-up studies exploring the differences in MVs concentration and size over a range of RCFs (e.g., 100K, 150K, 200K $\times$ g), various ultracentrifugation time spans, and different rotors and subsequently different k factors, investigating the stability of vesicles isolated at those conditions could be of great importance.

In conclusion, by comparing concentration and size of MVs in different biofluids, we determined that viscosity of biofluids could significantly affect sedimentation efficiency. Also, this study revealed that the size of MVs in more viscous biofluids significantly increase after ultracentrifugation. Considering MVs and their extensive diagnostic and therapeutic potential, more systematic research studies regarding the standardization of isolation

protocols and identification of effective factors for sedimentation efficiency are necessary.

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Alternative methods for characterization of extracellular vesicles

Fatemeh Momen-Heravi[1†], Leonora Balaj[2†], Sara Alian[3], John Tigges[4], Vasilis Toxavidis[4], Maria Ericsson[5], Robert J. Distel[1,6], Alexander R. Ivanov[7], Johan Skog[8] and Winston Patrick Kuo[1,9]*

[1] *Harvard Catalyst Laboratory for Innovative Translational Technologies, Harvard Medical School, Boston, MA, USA*
[2] *Department of Neurology and Radiology, Massachusetts General Hospital, Boston, MA, USA*
[3] *Biopolymers Facility, Harvard Medical School, Boston, MA, USA*
[4] *Harvard Stem Cell Institute/BIDMC Flow Cytometry Core Facility, Harvard Medical School, Boston, MA, USA*
[5] *Department of Cell Biology, Harvard Medical School, Boston, MA, USA*
[6] *Translational Research Laboratory, Dana-Farber Cancer Institute, Boston, MA, USA*
[7] *Barnett Institute of Chemical and Biological Analysis, Northeastern University, Boston, MA, USA*
[8] *Exosome Diagnostics Inc., New York, NY, USA*
[9] *Department of Developmental Biology, Harvard School of Dental Medicine, Boston, MA, USA*

Edited by:
Claudia Verderio, Consiglio Nazionale delle Ricerche, Italy

Reviewed by:
Douglas Taylor, University of Louisville, USA
Ian L. Sargent, University of Oxford, UK

****Correspondence:***
Winston Patrick Kuo, Harvard Catalyst Laboratory for Innovative Translational Technologies, Harvard Medical School, 4 Blackfan Circle, Room 144, Boston, MA 02115, USA.
e-mail: wkuo@catalyst.harvard.edu

[†]Fatemeh Momen-Heravi and Leonora Balaj have contributed equally to this work.

Extracellular vesicles (ECVs) are nano-sized vesicles released by all cells *in vitro* as well as *in vivo*. Their role has been implicated mainly in cell–cell communication, but also in disease biomarkers and more recently in gene delivery. They represent a snapshot of the cell status at the moment of release and carry bioreactive macromolecules such as nucleic acids, proteins, and lipids. A major limitation in this emerging new field is the availability/awareness of techniques to isolate and properly characterize ECVs. The lack of gold standards makes comparing different studies very difficult and may potentially hinder some ECVs-specific evidence. Characterization of ECVs has also recently seen many advances with the use of Nanoparticle Tracking Analysis, flow cytometry, cryo-electron microscopy instruments, and proteomic technologies. In this review, we discuss the latest developments in translational technologies involving characterization methods including the facts in their support and the challenges they face.

Keywords: characterization, concentration, methods, exosome, extracellular vesicles, microvesicles, size

INTRODUCTION

Release of membrane vesicles from the plasma membrane is a physiological process known to occur in cell cycle activation and growth without affecting cell viability, and it is a process widely observed both *in vitro* and *in vivo* (Cocucci et al., 2009; Thery et al., 2009). Extracellular vesicles (ECVs) are generated during a process called microvascularization either at the plasma membrane (microvesicles) or within endosomal structures (exosomes) and are comprised of a very heterogeneous population of vesicles ranging in size and content. Their sizes vary from 20 nm in diameter and have been reported up to 900 nm, the former comprising the more homogenous population of exosomes released from multivesicular bodies (MVBs) and the latter, commonly referred to as MVs, shedding from the plasma membrane (Thery et al., 2009). In this mini-review, we will refer to all types of shed vesicles under the common term of ECVs.

Extracellular vesicles' content varies from cell to cell and it has been shown to reflect the content and surface markers of the cell from which they originate (Skog et al., 2008; Balaj et al., 2011). These ECVs can also be taken up by neighboring or distant cells where they release their cargo which can affect the cell's status (Cocucci et al., 2009; Camussi et al., 2010). It has been shown that ECVs can affect immunoresponses, promote tumor invasiveness, and metastasis, can confer resistance to drugs, and promote endothelial cell migration, invasion, and neovascularization acting as carriers of angiogenic stimuli (Lee et al., 2011). Also, since they carry cell-specific signatures, assessment of ECV' content may be used for diagnostic purposes for early diagnosis of different cancers, including melanoma, ovarian cancer, kidney, and brain tumors (Meng et al., 2005; Skog et al., 2008; Lima et al., 2009; Grange et al., 2011).

Along with physiological signal mediators, ECVs appear as potential new tools for clinical diagnostics and may be useful in novel treatment modalities (Lima et al., 2009; Chen et al., 2012). Several groups are currently looking at ECVs as potential carriers of therapeutic drugs or molecules that would down-regulate toxic proteins or elicit an anti-tumor immune response when encapsulating specific siRNAs or adeno-associated viral vectors (Alvarez-Erviti et al., 2011; Maguire et al., 2012).

Although this branch of science is growing very fast, it is hampered by limitations in isolation and purification technologies as well as the ability to measure ECV size, concentration, and molecular content (Momen-Heravi et al., 2012). There is an urgent need for more reliable and reproducible extracellular vesicle characterization methods so downstream studies in ECVs genomics, proteomics, and lipidomics can be more standardized and efficient. In this review, we provide a brief overview of some recently used methods for ECV measurement and characterization for

sizing and assessing their concentration while emphasizing on novel cutting-edge technologies.

CHARACTERIZATION OF EXTRACELLULAR VESICLES

Analysis of ECV subpopulations is highly interesting, but has turned out to be a major challenge due to their small size and none of the techniques available today can reliably distinguish them at the single particle level. This analysis would reveal information about ECV size, concentration, charge, subcellular origin, formation process, content, as well as their potential function. In this mini-review we discuss some new mainstream technologies including flow cytometry, scattering and fluorescence flow cytometry, impedance-based flow cytometry, transmission electron microscopy (TEM) and scanning electron microscopy (SEM), cryo-electron microscopy (Cryo-EM) and single particle analysis, Nanoparticle Tracking Analysis (NTA), qNano, and large-scale molecular profiling.

FLOW CYTOMETRY

One method for high-throughput multi-parametric analysis and quantitation of ECVs is flow cytometry. This technology is designed to scan and sort at a rate of thousands of single cells or particles per second (van der Pol et al., 2010). Flow cytometry is widely used to detect origin, size, and morphology of circulating ECVs (Kim et al., 2002; Hunter et al., 2008; Kesimer et al., 2009; Mobarrez et al., 2010; Orozco and Lewis, 2010; Zwicker et al., 2012). Through hydrodynamic focusing, the suspended cells flow through a compressed chamber to the interrogation point, where the sample encounters the laser. The emitted scatter and fluorescence is then captured and measured by detectors facing forward and perpendicular to the laser. The intensity of detected light is reported as forward light scatter (FLS) and side light scatter (SLS). The quantity of light scattered forward is proportional to the diameter while SLS denotes morphology and inner anatomy of ECVs (Kim et al., 2002; van der Pol et al., 2010). In tandem, fluorescent light emitted from labeled ECVs travels perpendicular to the laser, as in SLS, and optics guide the wavelengths to detectors that record the intensities. Compatible dyes with discrete emission peaks can be used to detect multiple fluorescences from a single laser. Filters provide the necessary parameters to capture the appropriate range of emission peaks enabling the identification of heterogeneous populations. In an effort to guide and control data collection, flow cytometry employs automated and user configured thresholds which set points of reference for FLS that must be surpassed for data collection. It appears in the future by reducing flow chamber dimensions, optimizing the flow chamber geometry, reducing the flow velocity, the next generation of flow cytometry instruments will be capable of measuring ECVs with high sensitivity.

SCATTERING AND FLUORESCENCE FLOW CYTOMETRY

Scattering flow cytometry requires bead calibration with polystyrene/latex microspheres of known size and count, to permit quantitation and delineation of heterogeneous ECVs. The detection limit is greater and/or equal to 300 nm and as such, scatter detection alone is an inefficient method for analyzing smaller vesicles (Hein et al., 2008). Fluorescence flow cytometry is more sensitive due to emitted fluorescence intensity being higher than light scatter intensity for the MP size range of less than 300 nm (van der Pol et al., 2010). Fluorescence-activated cell sorting (FACS) enables ECVs to be characterized on the basis of the spectral properties of the fluorescence signal enabling morphological classification and specific sorting (Perez-Pujol et al., 2007).

The limitation of flow cytometry is its ability to sort small ECVs below 130 nm. Zwicker et al. (2012) suggest a bead-based gating strategy to identify the lower sensitivity of size-related forward scatter for ECV measurements (Robert et al., 2009). Improvements in standardization of vesicle measurements have been reported by Lacroix et al. (2010) on behalf of the International Society of Thrombosis and Haemostatic (ISTH). Using Megamix beads, this study determined that instrumentation with wide-angle FLS produced consistent measurements of vesicles (Chandler et al., 2011; Yuana et al., 2011). van der Pol et al. (2012) also used the Megamix bead gating strategy to standardize the relationship between scatter and ECVs' diameter. Notably, they concluded flow cytometers can indeed detect smaller ECVs in the range of exosomes by swarm detection, the capture of smaller ECVs grouped together and characterized as a single event (van der Pol et al., 2012). Comparison of newer instruments in Chandler et al. (2011) show the Apogee A40 calibrated with 0.4 μm polystyrene beads for 1.0 μm micro particles (MPs) can detect higher numbers of MPs and platelets compared to Megamix gating use.

Heterogeneous ECVs stained with fluorescently labeled antibodies can be identified and sorted by fluorescence flow cytometry. Non-specific binding and unbound dye can impede accurate analysis of labeled ECVs, especially smaller vesicles like exosomes (Hoen et al., 2012). Hoen et al. (2012) reported successful antibody-mediated detection of phenotypically heterogeneous exosomes using fluorescence threshold triggering. Their labeling method and optimization of the Becton Dickinson Influx flow cytometer (Becton Dickinson, Brussels, Belgium) eliminated noise signals and permitted comparison of vesicle subsets within the whole vesicle population, as well as detection of fluorescent vesicles down to 100 nm in diameter (Hoen et al., 2012). Mobarrez et al. (2010) found that measuring the intensity of the markers bound to platelet-derived ECVs and then translating those intensities to Molecules of Equivalent Soluble Fluorochrome (MESF) values increased reproducibility and permitted comparison of results obtained from different instruments. Inaccuracies and instrument variability in measuring the absolute number of particles per volume unit is eradicated through use of MESF values to generate a standard curve based on beads with predefined fluorescence labeling (Mobarrez et al., 2010).

IMPEDANCE-BASED FLOW CYTOMETRY

The displaced solute increases the impedance across the circuit by generating a voltage spike proportional to the volume of the ECV. The lower detection limit of impedance-based flow cytometry is 300 nm. Note that aperture size indicates or dictates the instruments sensitivity to ECV size (Jy et al., 2010). Using different channel diameters, two or more impedance-based flow cytometers are recommended to encompass the submicron range (van der Pol et al., 2010). Zwicker et al. (2012) used the Cell Lab Quanta SC (Beckman Coulter) with an aperture diameter of 40 μm for optimal sizing, characterization, and concentration of

ECVs. They affirm impedance-based ECV sizing lower limits are commonly 2% of the aperture's diameter (Zwicker et al., 2012). Impedance-based cytometry enhances the sensitivity in comparison with standard flow cytometers, but the limiting size range excludes a small fraction of ECVs (<300 nm; van der Pol et al., 2010; Zwicker et al., 2012).

Pre-analysis, some suggestions such as calibration of polystyrene beads and optimization of antibody concentrations are recommended to standardize the analysis (Zwicker et al., 2012). The technology cannot provide sourcing based on surface markers, morphological, or biocompositional data of ECVs unless combined with fluorescence and scattering flow cytometry (van der Pol et al., 2010). Limitations in resolution will cause smaller particles to go undetected but newer instruments such as Gallios (Beckman Coulter) and BD-Influx (Becton Dickinson) are equipped with more sensitive detectors that can enable for more accurate discrimination of particle populations down to 100 nm in diameter (Lacroix et al., 2010). Orozco and Lewis found that basing their threshold on the number of background "noise"/events per second when double filtered (0.2 μm) phosphate buffered saline (PBS) was passed through the Gallios instrument (Beckman Coulter) was effective (Orozco and Lewis). This assay will probably be further explored in the future and may shed light into the ECVs subpopulation subtypes quantitatively and qualitatively.

TRANSMISSION ELECTRON MICROSCOPY AND SCANNING ELECTRON MICROSCOPY

There are two types of electron microscopes, the TEM and the SEM. TEM has similarities to light microscopes, transmitting a beam of electrons through a thin specimen and then focusing the electrons to create an image on a screen or on film. TEM is the most commonly used and has the highest resolution. SEM, on the other hand, scans a fine beam of electrons onto a specimen and collects the electrons scattered on the surface. Although SEM resolution is less than TEM, it confers detailed three-dimensional (3D) images of surfaces. Because the wavelength of electrons is more than three orders of magnitude shorter than the wavelength of visible light, the resolution of TEM can be lower than 1 nm (Pisitkun et al., 2004; van der Pol et al., 2012). Since TEM is performed in a vacuum, biomaterials require fixation and dehydration, which reduces their size and changes their morphology. ECVs usually appear 20–100 nm in size and cup-shaped when visualized by TEM. Employing immuno-gold labeling could lead to biochemical information regarding ECVs' surface (van der Pol et al., 2012; **Figure 1**). Although TEM has been used extensively for detection of ECVs (Baran et al., 2010; Miranda et al., 2010; Waldenstrom et al., 2012), this method only provides semi-quantitative information on ECVs. Furthermore, sample dehydration and vacuum procedures required in Electron Microscopy (EM) might affect the characteristics of ECVs. The measurement time is in the order of hours.

CRYO-ELECTRON MICROSCOPY AND SINGLE PARTICLE ANALYSIS

Cryo-electron microscopy is a form of EM where samples are analyzed at temperatures below −100°C and has been successfully applied to ECV analysis. The advantage of this technique is that samples are analyzed in frozen conditions without being stained or fixed. This technique has been used for the study of ECVs isolated from urine and revealed repetitive "mushroom-shaped" features on the surface of ECVs (Conde-Vancells et al., 2010).

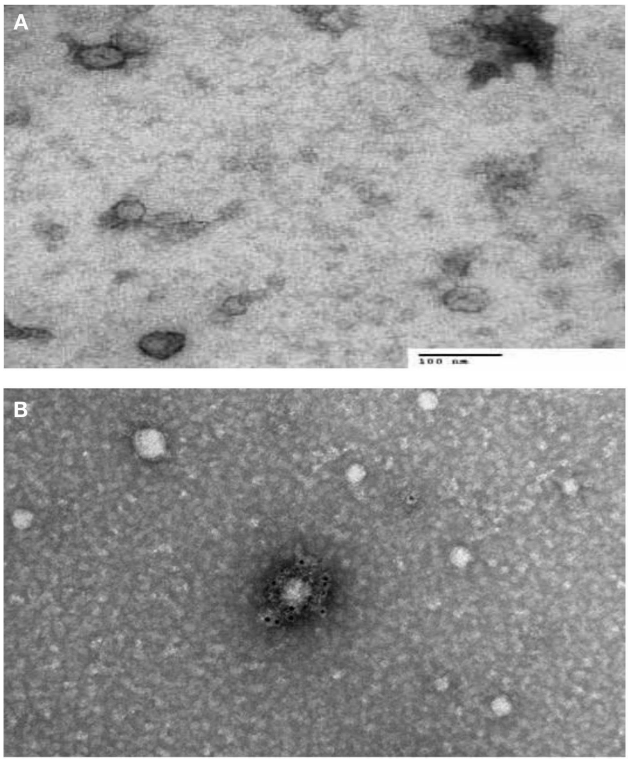

FIGURE 1 | Transmission electron microscopy (TEM) characterization of human serum derived extracellular vesicles (ECVs). (A) ECVs were negatively stained with 2% uracyl acetate after removing the extra moisture. Cup-shaped structures, with 30–100 nm size were identified as being exosome/microvesicles. **(B)** ECVs isolated from human serum expressing CD63 Transmembrane protein which is believed to be exosome/microvesicles marker. ECVs were immuno-gold labeled with rabbit polyclonal Abs against CD63.

Usually categorized as one of the techniques of cryo-EM, single particle EM reconstruction has recently become a popular tool to get the 3D structure of proteins and viruses. This method has advantages in comparison with X-ray crystallography including no need to crystallize the proteins and no need for large amounts of protein samples (in range of microliters; Liu and Wang, 2011). Despite single particle EM has the ability to map the 3D structure of samples at 1 nm resolution, it works better for more symmetrical structures. The techniques has the capability of distinguishing different molecular orientations and digitalizing it. Employing two-dimensional (2-D) alignment and classification methods, homogenous molecules in the same view are grouped into their respective classes. In each view, their averages increase the signal of the molecule's 2-D shapes. Afterward, software orders the structures with the proper relative orientation (Euler angles) and generates the 3D images based on combining 2-D digitalized micrographs. Liu and Wang (2011) described procuring a 3D reconstruction of yeast exosome complex using negative staining

EM and single particle EM. This technique will need to be further explored in the future of ECV characterization.

NANOPARTICLE TRACKING ANALYSIS

A recently developed technique that allows sizing and counting of ECVs is the NTA (Dragovic et al., 2011; **Figure 2**). It utilizes a laser light scattering microscope, charge-coupled device camera (CCD), and proprietary analytical software. A laser beam hits the ECVs and their Brownian motion is then determined by a highly sensitive CCD camera and the mean velocity of each particle is calculated with image processing software. ECVs from 30 to 1000 nm in diameter at a concentration range of 10^8–10^9 can be counted with relatively high sensitivity. The NTA software is then able to identify and track individual ECVs moving under Brownian motion and relates the movement to a particle size based on the following formula derived from the Stokes–Einstein Equation (Filipe et al., 2010):

$$\overline{(x,y)^2} = \frac{2k_B T}{3r_h \pi \eta}$$

where k_B, is the Boltzmann constant and $\overline{(x,y)^2}$is the mean-squared speed of a particle at a temperature T, in a medium of viscosity η, with a hydrodynamic radius of r_h.

The Nanosight technology allows detection of ECV subpopulations by using antibody-mediated fluorescent labels that specifically bind to the antibodies of interest on the surface of ECVs (Dragovic et al., 2011). This feature enables users to detect, analyze, and count only the specific nanoparticles to which the fluorescently labeled antibody are bound, with background non-specific particulates being excluded through the use of appropriate optical filters.

qNANO (IZON)

The qNano is a relatively new technology that allows detection of a ECVs passing through a nanopore by way of a single-molecule electrophoresis. Branton et al. (2008) introduced nanopores as a promising approach for studying biophysics at the single-molecule level. The technology is based on the Coulter principle at the nano scale, and operates by detecting transient changes in the ionic current generated by the transport of the target particles through a size tunable nanopore in a polyurethane membrane (Garza-Licudine et al., 2010). The qNano instrument consists of a nanopore formed by needle perforation on a polyurethane membrane that is stretched mechanically to permit real-time manipulation of nanopore size. A transmembrane voltage is generated and as particles travel across the nanopore the altered ionic current is captured. Furthermore, data is presented by particles transitory blockage of the pore establishing measurable change in the elasticity of the channel. Fixed geometry pores are typically useful for detecting a limited size range or type of particle. qNano provides quantitative analysis of particle samples spanning from 70 nm to 10 μm in diameter and concentrations from 10^5 to 10^{12} ml^{-1}. Furthermore, real-time monitoring of ionic current flow across the pore at different aperture settings enables one to tune the detection and discrimination of individual nanoparticles populations in mixed multimodal suspensions. Despite the individual particle-by-particle readout, the lower limit of detection for ECVs is in the range of 100 nm (**Figure 3**). As the technology evolves, we believe this aspect will improve over time.

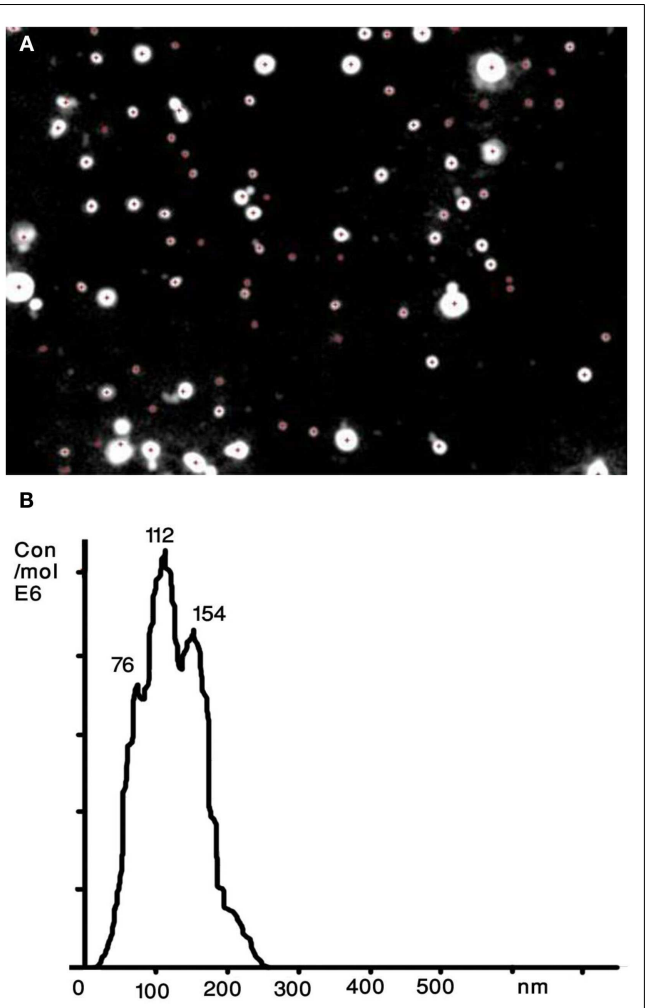

FIGURE 2 | Nanoparticle tracking analysis (NTA) of human serum derived extracellular vesicles (ECVs). (A) The image presents particles moving under Brownian motion. **(B)** The NTA software then rapidly generates a distribution graph on a particle-by-particle basis and a count (in terms of absolute number concentration) of the vesicles.

RAMAN SPECTROSCOPY

Raman Spectroscopy is a spectroscopic method, based on inelastic scattering of monochromatic light (mostly laser light). It is used to study vibrational, rotational, and other low-frequency transitions in a system (Puppels et al., 1990). Photons interact with molecular vibrations, photons, or other excitations in the system, leading to a slight up- or down shift of their energy. The shift in energy provides information about the vibrational transitions in the molecules (Puppels et al., 1990; van der Pol et al., 2010). Given the makeup of ECVs, their chemical composition could be distinguished by RS with the advantage that ECVs do not have to be pre-processed or labeled. RS is a quantitative technique and the signal strength is linearly

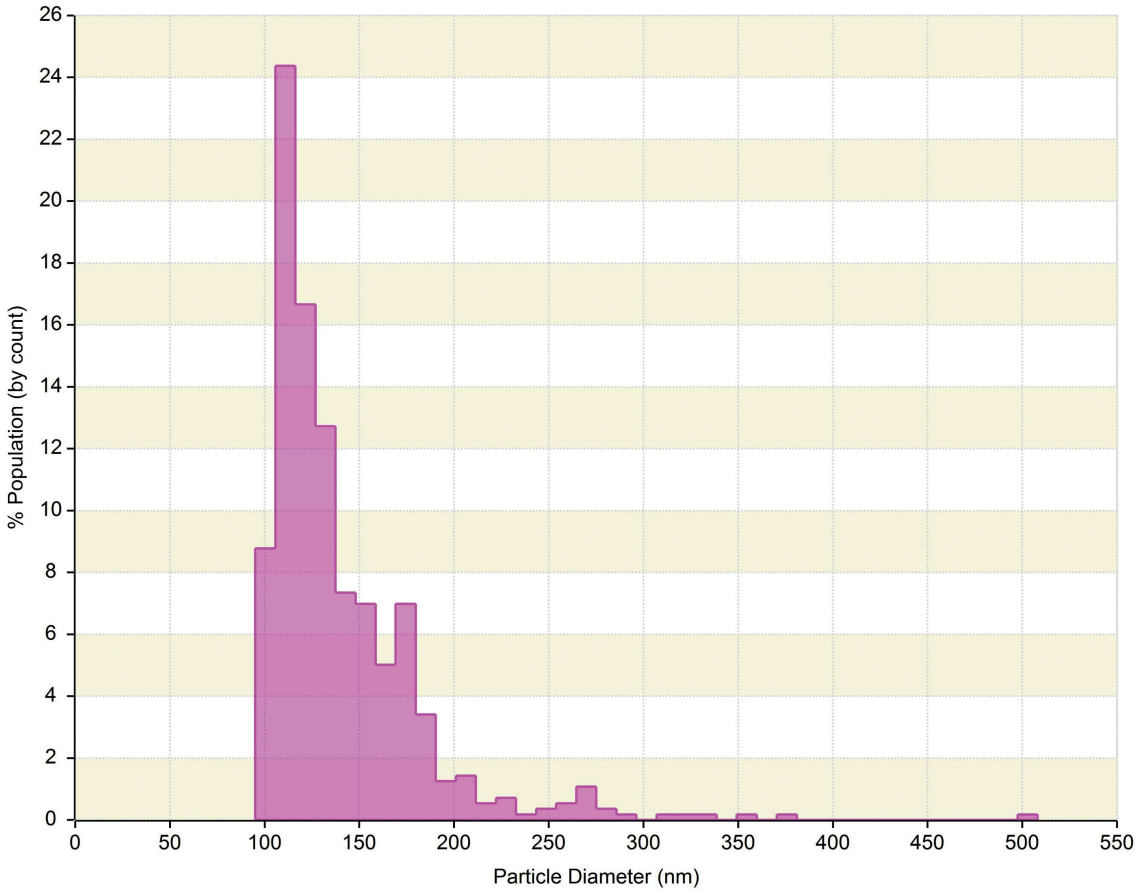

FIGURE 3 | qNano generated data of human serum derived extracellular vesicles (ECVs). Plot depicts particle size diameter vs. percentage (%) of population. The concentration was reported as 1.4×10^{10} particles ml^{-1} with mode of 120 nm.

proportional to composition of the ECVs. The measurement time is in the order of few hours. RS can also be coupled with TEM, NTA, and dynamic light scattering devices to correlate detailed biochemical information with the relative size distribution and morphology.

LARGE-SCALE MOLECULAR PROFILING "OMIC" TECHNOLOGIES IN COMPOSITIONAL CHARACTERIZATION OF ECVs

Shedding a nuclear fragments of cellular membrane, ECVs, is an integral part of physiological homeostasis and communication of various cells of the organism. Alterations in vesicle concentrations and molecular compositions have been associated with diseases and physiological states, indicating their diagnostic potential (Simak and Gelderman, 2006). Emerging "omic" approaches for in-depth molecular profiling seem attractive for revealing MV-related diagnostic and prognostic biomarkers as well as for understanding biogenesis and signaling of cells and ECVs. Recent advances in "omic" technologies could play an important role in order to elucidate the roles of ECVs studying their molecular composition. Several recent reports have effectively utilized proteomic, metabolomic, and microarray profiling techniques to address specific questions through molecular characterization of ECVs isolated from various physiological fluids and cell cultures (Mayr et al., 2009; Didangelos et al., 2012).

Proteomic technologies allows for both unbiased discovery-driven and targeted large-scale protein profiling. Moreover, MV constituents revealed by proteomics techniques can be used in antibody-based enrichment, detection, and characterization by the above discussed methodologies. During the past several years 2-D gel- and mass spectrometry (MS)-based proteomics has been successfully applied to MV research, leading to the identification of novel signaling and secreted proteins that may have important physiological roles (Garcia et al., 2005; Smalley et al., 2008; Dean et al., 2009; Parguina et al., 2012; Shai et al., 2012).

The traditional 2-D gel electrophoresis technique utilizes in-gel isoelectrofocusing followed by SDS polyacrylamide gel electrophoresis to separate individual proteins that can be visualized by fluorescent or visible staining, quantified by optical density readouts, digested with proteolytic enzymes, and identified by MS-based proteomics. As an example, 2-D gel analysis followed by MS-based protein identification demonstrated

that significantly higher levels of phosphatidylserine-bearing ECVs originated mostly from oxidatively damaged platelets and RBCs can be successfully linked β-thalassemia/hemoglobin (Eβ-thal/HbE) disorder (Chaichompoo et al., 2012). Another recent report shows that platelets shed EVCs in different amounts and of different protein composition depending on the stimulus (Shai et al., 2012).

The field of MS-based proteomics has substantially advanced over the last decade due to revolutionary changes in technology, sample preparation, separation platforms, and bioinformatics. Current proteomic technologies are capable of low attomole detection and therefore more efficient in analysis of small sample amounts. The conventional MS-based proteomic profiling uses up-front single or multidimensional separation of proteins or protein digests followed by on-the-fly structural characterization by single stage and tandem MS. The most common separation technique used in proteomic analysis of ECVs prior to liquid phase chromatography coupled to MS is 1-D SDS gel electrophoresis. The main advantages of this technique is its simplicity and relative efficiency in analysis of hydrophobic and membrane proteins that are expected to be enriched in ECVs. Also, 1-D PAGE effectively delipidates lipid-rich ECVs, that can be beneficial for downstream MS analysis. Rapid progress in high accuracy high resolution MS enabled reliable quantitative proteomic analysis and profiling of post-translational modifications. A recent study focused on the physiological erythrocyte aging process; they applied MS-based proteomic profiling to support a hypothesis stating vesiculation of damaged and degraded membrane patches of erythrocytes may serve to postpone the premature removal of functional cells (Bosman et al., 2012). This study demonstrated a selective accumulation of ubiquitinylated proteins or peptides as well as several other post-translational modifications in ECVs derived from aging RBCs that can lead to the subsequent recognition and fast removal of ECVs by the immune system (Bosman et al., 2012). MS-based profiling allows one to reliably assess the baseline of intra- and inter-individual variability in ECV composition prior to any effort for biomarker detections (Rubin et al., 2010; Bastos-Amador et al., 2012).

New fields of large-scale metabolomic, lipidomic, and peptide/protein array profiling techniques are emerging following the recent wake of the genomic and proteomic revolutions (Griffiths et al., 2011). These new "omic" technologies are expected to also be very instrumental in providing complementary information about structural features of ECVs and in development of novel diagnostic, prognostic, and therapeutic approaches.

CONCLUSION

In conclusion, a combination of the different methods described above can provide information on the different characteristics of ECVs. These methods should be further assessed and validated by comparing measurement results, so that precise, reliable, and fast extraction methods and measurements could eventually be translatable from the bench to the clinic. As the area of ECVs shift to the clinical arena, the characterization step will need to be standardized to ensure a more precise and sensitive measurement. This may include combining complementary characterization methodologies.

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