Dual Perspectives

Dual Perspectives Companion Paper: NeuroEVs: Characterizing Extracellular Vesicles Generated in the Neural Domain, by Christie D. Fowler

Extracellular Vesicles and Neurodegenerative Diseases

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Extracellular vesicles (EVs) include exosomes and microvesicles and have been shown to have roles in the CNS ranging from the removal of unwanted biomolecules to intercellular communication to the spread of pathogenic proteins associated with neurodegenerative diseases. EVs carry protein, lipid, and genetic cargo, and research over more than a decade has shown that they contain the misfolded forms of proteins associated with Alzheimer's, Parkinson's, and the prion diseases. Altered genetic cargo, usually in the form of miRNAs, have also been identified in EVs patients with these diseases, suggesting that EVs may be a source of disease biomarkers. Whether EVs play a key role in the pathogenesis of neurological diseases remains to be firmly established because most current research is performed using cell culture and transgenic animal models. If EVs are identified as a key pathological contributor to neurological conditions, they will form a novel target for therapeutic intervention. This Dual Perspectives article will discuss the current understanding of the role of EVs in neurological diseases and raise some of the limitations of our current understandings of this field.

Key words: **extracellular vesicles; exosomes; neurodegenerative disease; Alzheimer's disease; prion disease; cell biology**

Introduction

Extracellular vesicles (EVs) are small, membranous particles released by cells in a number of different ways. The collective term of EV was coined to describe the many different cell-derived membranous particles that had been given names that reflect their method of biogenesis, functional characteristics, or morphology. The most widely studied EVs are exosomes, which are derived from endosomes, and microvesicles, which form from outward budding of the plasma membrane.

The term exosome was used originally to describe extracellular vesicles released by multivesicular endosomes from differentiating reticulocytes (Harding et al., 1983; Pan and Johnstone, 1983). Much work has centered on studying the cellular biology of the biogenesis of exosomes, and this term is now preferred to be used for vesicles with a defined endosomal origin. This can be achieved by using certain protein markers of the endosomal biogenesis pathway. In-depth proteomic studies have demonstrated that, even within exosomes, there exists heterogeneity in protein cargo that can identify subclassifications of exosomes (Kowal et al., 2016). Exosomes are ~100 nm in diameter, but distinct vesicles of smaller size have been identified and termed exomeres (Zhang et al., 2018).

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The term microvesicle is used to describe extracellular vesicles that form from outward budding of the plasma membrane. Other terms for microvesicles include microparticles and budding vesicles. Some of the protein cargo of microvesicles overlaps with those of exosomes, which makes definitive identification difficult. But the size of microvesicles is more heterogeneous with diameters ranging from 100 nm to 1 μm.

Isolating highly purified preparations of different types of EVs is challenging, and it is clear, even within each class, that there is heterogeneity. Nomenclature therefore remains problematic for the field. The International Society for Extracellular Vesicles has recently published guidelines for EV research, which proposes a nomenclature based on the physicochemical properties and cellular origin of the vesicles (Théry et al., 2018).

Over the last three decades, EVs have been shown to have multiple functions, first being described, in the case of exosomes, as a mechanism for removal of the transferrin receptor during reticulocyte maturation (Harding et al., 1983; Pan and Johnstone, 1983). In recent years, the identification of protein and genetic cargos associated with EVs has led to recognition of their roles in intercellular communication and signaling. In addition, much research has identified a role for EVs in disease processes, either in contributing or responding to pathogenic mechanisms, or as a source of biomarkers for disease. For example, exosomes have been found to contribute to diverse biological processes, such as angiogenesis, inflammation, morphogen transportation, and programmed cell death (Colombo et al., 2014). Significant areas of EV research focus on their roles in disease settings, with cancer being the most prominent; EVs released by tumors have been shown to have the ability to regulate distant cellular environments to initiate premetastatic niche formation (Hood et al.,

2011; Peinado et al., 2012; Costa-Silva et al., 2015), a mechanism that demonstrates the possibility that EVs can spread disease information throughout the body (Hoshino et al., 2015).

With the ability of EVs to transport cargo packaged by the originating cell, their role in the pathogenesis of neurological conditions, particularly neurodegenerative diseases associated with misfolded proteins, has become a growing area of interest. Neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease, Creutzfeldt–Jakob disease, and amyotrophic lateral sclerosis, share a common mechanism in which distinct proteins become misfolded and deposited in specific regions during the pathogenic process. Another common feature of these disorders is that these misfolded proteins "spread" to defined brain regions, suggesting the disease process involves intercellular movement of these proteins (Braak et al., 2003). EVs can carry the many different proteins associated with neurodegenerative diseases. The pathological significance of this is currently under investigation. In particular, the translation of findings from *in vitro* systems, such as cell culture models, to *in vivo* settings, particularly in human disease, requires further validation.

Initial studies (Fevrier et al., 2004; Vella et al., 2007) showed the prion protein, in both its normal (PrP^{C}) and disease-associated, transmissible (PrP^{Sc}) conformations, is efficiently transported with EVs and can transmit the "prion" conformation when these vesicles are injected into susceptible animals or in cells in culture. Modification of the release of EVs from cell cultures, either by coinfection with a virus (Leblanc et al., 2006) or using chemicals that increase or decrease the release of these PrP^{Sc}-containing vesicles, has demonstrated levels of prion infectivity relate to the levels of EVs released (Trajkovic et al., 2008; Guo et al., 2015). Evidence that transmissible prion activity is present in EVs isolated from blood in a rodent model of prion disease (Saá et al., 2014; Cervenakova et al., 2016) provides further support for EV associated prion transfer.

For many years, the disease-associated prion protein (PrP^{Sc}) involved in prion disease was the only known transmissible protein for the spread of disease (Prusiner, 1982), but recent evidence using both animal and cellular models has shown that other neurodegenerative proteins may also be transmissible (Aguzzi and Rajendran, 2009; Prusiner et al., 2015). This includes α-synuclein in Parkinson's disease and tau and Aβ in Alzheimer's disease. For example, in a rodent model of Alzheimer's disease, it was shown that the spread of tau occurred by the release of exosomes containing this protein and that depleting microglia reduced the propagation of tau. As in the studies on modulating EV release and prion propagation above, inhibiting EV release was shown to reduce tau propagation in both cell culture and a mouse model (Asai et al., 2015). For Aβ, it has been shown that neurotoxic, oligomeric forms of this protein are associated with EVs isolated from brain tissue, and that these vesicles can mediate interneuronal propagation of this protein. These Aβ-containing vesicles were also shown to be neurotoxic to primary cultured neurons indicating, at least *in vitro*, functional activity associated with the vesicle harboring this pathogenic protein (Sardar Sinha et al., 2018). Together, these data point to a potential common mechanism of propagation of disease-associated proteins associated with neurodegenerative conditions, whether this is a primary driver of disease pathogenesis still remains to be determined.

Another way in which EVs provide insights into the pathological processes involved in neurological conditions is their use as sources of biomarkers. Because EVs can be isolated from blood (serum and plasma), CSF, urine, and other biofluids, opportunities exist to determine whether EV-derived biomarkers, be they protein, genetic, or lipid, can report on neural conditions. One of the first examples is in the mRNA/miRNA signature associated with glioblastoma-derived EVs, which could be detected in the periphery (Skog et al., 2008). Since then, several published studies have reported EV, associated protein, and genetic biomarkers for a number of neurological diseases, which will be discussed below.

Role of the blood–brain barrier (BBB)

The question of whether EVs cross the BBB in either direction is also one that needs to be considered when studying neuronally derived vesicles, especially in the periphery. While several studies suggest that EVs are transferred across the BBB into the periphery, mechanistically this is not well understood. EVs released from cancer cells have been shown to destroy the BBB through the action of microRNA-181c, which acts on pathways leading to actin mislocalization (Tominaga et al., 2015). This suggests that, in cases of disease, the breakdown or increased "leakiness" of the BBB allows the transfer of EVs from the CNS into the periphery. It is known that breakdown of the BBB occurs in many neurodegenerative diseases, usually as a result of inflammation, which provides another potential mechanism for EV transfer to the periphery. Transfer of EVs from the periphery into the brain has been demonstrated. For example, vesicles from hematopoietic cells can be transferred to Purkinje cells in the brain, resulting in a modification of gene expression in these cells and thus suggesting functional significance (Ridder et al., 2014). Transfer of EVs across the BBB has also been achieved through the use of engineered vesicles containing a surface protein that enables the transfer (Alvarez-Erviti et al., 2011). Understanding at the mechanistic level how EVs traverse the BBB in both directions might provide promise for the design of therapeutic vesicles targeting the CNS, in addition to perhaps increasing the sensitivity of biomarkers for neurological conditions using peripheral biofluids as a source of material.

Extrapolation from cell culture and *in vivo* systems

The majority of studies examining the role of EVs in the nervous system have used the immortalized or primary cell cultures. This has enabled the study of distinct classes of EVs originating from different CNS cell types, including neurons, astrocytes, microglia, and oligodendrocytes. Primary cortical neurons and astrocytes release exosomes, and this release is regulated by depolarization. Exosomes from these cultures contain proteins, such as the prion protein, L1 cell adhesion molecule, and some subunits of glutamate receptors (Fauré et al., 2006). Oligodendrocytes also release exosomes, which contain myelin and a number of proteins associated with protection against cell stress, suggesting a role in providing axonal support against injury (Krämer-Albers et al., 2007). The functional effects of oligodendroglial exosomes also extend to protection from the effects of oxidative stress through the actions of vesicle-associated proteins catalase and SOD1 (Fröhlich et al., 2014). Further evidence for a supportive role of exosomes in neuronal health comes from studies on cultured astrocyte-derived EVs that contain the protein synapsin-I, which is known to promote neuronal survival (Wang et al., 2011). While these, and other, studies have generated important data, the extrapolation of this to the *in vivo* situation forms a current gap in knowledge and an area of current investigation.

Advances in the study of EVs from CSF has allowed some correlative work to be performed to demonstrate the *in vivo* relevance of EVs in the CNS (Vella et al., 2008; Street et al., 2012). More recently, techniques developed to isolate EVs from brain

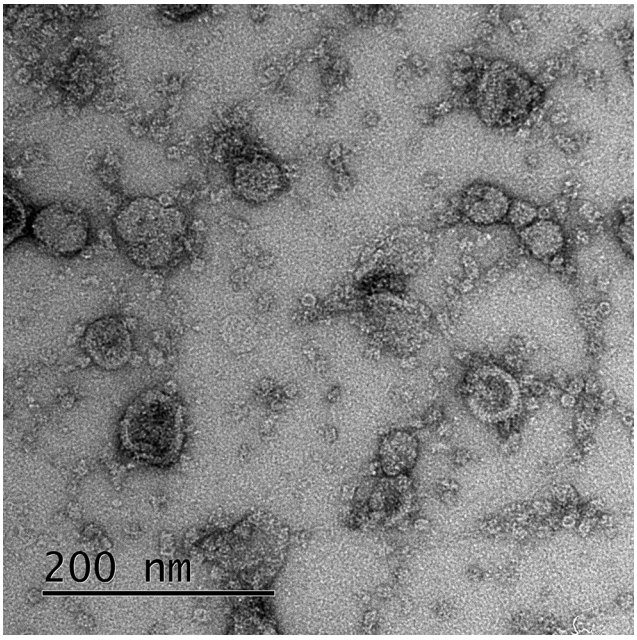

Figure 1. Electron micrograph demonstrating the isolation of extracellular vesicles from frozen human brain tissue. These were isolated using the protocol published by Vella et al. (2017).

tissue have given more *in vivo* evidence for their significance in the CNS. These methods rely on the gentle disruption of brain tissue and ultracentrifugation on density media and have demonstrated the presence of key proteins associated with neurodegenerative diseases (Perez-Gonzalez et al., 2012; Vella et al., 2017). This provides some *in vivo* evidence that the proteins seen in cell culture systems are indeed associated with EVs and, as such, are biologically relevant. Functional studies remain essential in demonstrating a clear role for EVs in the CNS and their contributions to or changes seen as a result of pathological consequences associated with disease.

Isolation of neurally derived EVs

To define the role that EVs play in neurodegenerative diseases, there are some methodological challenges that need to be overcome. Isolation of EVs can be achieved using a variety of approaches depending on the source material (e.g., choice of biofluid) and the downstream assays to be performed (Fig. 1). The use of differential ultracentrifugation has been primarily used when studying samples, such as conditioned cell-culture media, usually due to the larger volumes of material available for processing. With biomarker studies, clinical samples are usually available only in limited quantities; sometimes this is suitable for techniques using centrifugation or the use of size-exclusion chromatography. Isolating neural-specific EVs is especially challenging, especially with respect to using them for biomarker discovery, as it is not currently known how many EVs cross the BBB, and access to the primary tissue is not amenable to routine testing. Analysis of EVs isolated from CSF is possible and has been used in biomarker discovery studies for a number of neurological diseases (Vella et al., 2008; Lusardi et al., 2017; Saugstad et al., 2017).

Protocols exist to isolate neutrally derived EVs from peripheral blood using a pulldown procedure involving an antibody against the neural cell adhesion molecules NCAM or L1CAM (CD171) after precipitation of EVs from plasma or serum using a commercially available reagent (Fiandaca et al., 2015). Although NCAM and L1CAM are expressed predominantly in neuronal cells, they also have significant expression (according to the Human Protein Atlas; www.proteinatlas.org) outside the CNS, which may mean that the EVs pulled down with these antibodies are not solely derived from neural tissues. This requires further validation to determine other potential sources of EVs containing these proteins and to identify the proportion of these that are indeed of neural origin. Nevertheless, using ELISA-based testing against several proteins associated with Alzheimer's disease in these isolated EV preparations has shown diagnostic utility with the ability to discriminate disease from controls based on levels of proteins, such as Aβ and tau (Fiandaca et al., 2015; Goetzl et al., 2015, 2016a,b, 2018). Another study compared levels of full-length tau protein in CSF-derived exosomes and compared these with those found in the periphery using a modification of the above method (Guix et al., 2018). The modification was used to prevent cross contamination with tau from a component used in the isolation method (thromboplastin D). Using this approach, the authors found that only a small fraction of the tau associated with neurally derived exosomes isolated from plasma was full-length tau: most tau peptides were fragmented species.

Astrocyte-derived exosomes have also been isolated with a similar approach, using an antibody against an astrocyte-specific antigen, and these were shown to have different levels of Alzheimer's-disease-associated proteins, such as BACE-1 and gamma-secretase (Goetzl et al., 2016a). This demonstrates a potentially important approach to isolating neurally derived EVs in the periphery from specific neural cell types, in this case from glia and astrocytes. Combining data from EVs isolated from primary neuronal cell types, tissues, CSF, and through the use of these pull-down approaches in peripheral blood will provide an opportunity to further define the origin and potential function of neurally derived EVs in addition to confirming the utility of EVs as sources of biomarkers for neurological diseases.

EVs as sources of biomarkers for neurological diseases

The identification of EVs as carriers of pathological proteins associated with neurodegenerative diseases provides, at the moment, indirect support for the hypothesis that they play a role in the pathogenesis of these conditions. To date, *in vivo* analyses using biofluid- or tissue-derived EVs have demonstrated low levels of these proteins, which in some cases is at odds with what is observed in cell culture or transgenic mouse models, perhaps a reflection of expression-level differences. However, while low amounts of these pathological proteins are present, this does not preclude them from having a role in disease pathogenesis, particularly in the case of protein aggregation disorders (e.g., Alzheimer's, Parkinson's, and prion diseases) wherein very low levels of "seed" aggregates are required to initiate the aggregation process. That being said, the use of EV-protein-based biomarkers may be technically challenging given the low levels of proteins identified, although the use of neural EVs from blood, as discussed above, has provided some encouraging results.

The genetic and lipid cargo of EVs also offer suitable targets for neurologic disease-based biomarkers that might be detected in the periphery. Studies using CSF and blood have identified panels of EV-related miRNA panels that show expression differences between control and diseased individuals (Cheng et al., 2015; Saugstad et al., 2017). While there has been some overlap in the signatures detected for some of these disease conditions, lack of standardized procedures for isolation and analysis of the EVs (which also include standardized procedures for sample collec-

tion and biobanking) can present challenges in comparing studies. The quality of biomarker discovery is also heavily dependent on the cohorts used, both in terms of size and quality of the specimens and supporting data, which also present challenges in comparison between studies.

EVs and interneuronal communication

The role that EVs play in intercellular communication throughout the nervous system is beginning to be unraveled. The findings that all cell types of the CNS release EVs and that functions ascribed to these include maintaining neuronal homeostasis and neuroprotective properties suggest that they may be important regulators of neuronal health. The physiological role of EVs in the brain is likely to be multifactorial; and perhaps, as illustrated by the commonality of misfolded protein association with EVs in a number of neurodegenerative diseases, perturbation of protein packaging into EVs could be a pathogenic process in these disorders.

Therapeutic approaches involving EVs in the nervous system

A current area of interest in the EV field is the use of these vesicles as potential therapeutic agents. This might be particularly useful for the treatment of neurological conditions, in which crossing the BBB is an important hurdle to overcome. Two potential approaches have been tested: the first is using EVs loaded with a therapeutic cargo, and the second involves pharmacological modification of the release of EVs potentially containing pathogenic forms of neurodegenerative disease-associated proteins.

It is not currently known whether the number of EVs is upregulated or downregulated in neurological diseases; understanding which neuronal subtypes release disease-associated EVs would be important. However, one study using a transgenic mouse model of Alzheimer's disease has shown that reducing the levels of EV release using a neutral sphingomyelinase inhibitor (GW4869) altered neuropathology in the animals, suggesting that reducing EV release might have some therapeutic benefit (Dinkins et al., 2014). Of course, reducing total EV numbers may have undesirable side effects, which means that specificity (e.g., targeting distinct pathways or neuronal cell types) may be required.

In conclusion, with growing evidence that EVs play roles in the removal of unwanted material and intercellular communication, their role in the brain and CNS (in both healthy and disease conditions) has come under increasing attention. The findings that EVs can potentially propagate the misfolded proteins associated with neurodegenerative disorders (e.g., prion disease) could underlie a common pathogenic mechanism in these conditions that share the spread of misfolded proteins. However, to date, most evidence for this has come from cell culture and transgenic mouse models; defining the role in human disease remains to be formally demonstrated. The cargo carried by extracellular vesicles, either in the CNS or periphery, has shown promise for potentially diagnosing human neurological diseases, by measuring either protein or genetic content (e.g., miRNA expression). Standardization of methodologies and testing across different cohorts remain key challenges in realizing the potential of EVs in the diagnosis of neurological diseases.

Understanding how EV cargos are packaged, whether actively or through specific targeting of cargo to be loaded, will also aid in determining the role of these vesicles in interneuronal communication. How neuronal EVs are transported to and from the periphery also requires further understanding, the key role of the BBB in mediating this is very important. Mechanistic, rather than correlative, studies will be required to determine these factors and also to answer the question of whether in cases of disease EVs are causative or result from the disease process.

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