The regulation of exosome function in the CNS: implications for neurodegeneration

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Summary

Exosomes are nanovesicles, generally 50 to 90 nm in diameter, that correspond to the intraluminal vesicles of the endosomal multivesicular bodies and are secreted upon fusion of multivesicular bodies with the plasma membrane. Their molecular content is highly selected and includes not only specific proteins and lipids, but also RNA species, such as messenger RNAs (mRNAs) and microRNAs (miRNAs), which are delivered and active in target cells. As they are released in body fluids, exosomes can shuttle molecules for long distances. In the CNS they have been shown to regulate neuronal development and regeneration, and to modulate synaptic functions. In neurodegenerative diseases, they have an important role in propagating neurotoxic misfolded protein from one cell to another and, as recent data show, possibly other molecules contributing to neurotoxicity. Some exosomal lipids such as gangliosides GM1 and GM3 enhance the aggregation of alpha-synuclein, and RNA exosomal cargo is also altered during pathologies such as Alzheimer’s disease, prion diseases and amyotrophic lateral sclerosis.

The aim of this review is to focus on the regulation of CNS exosomal function and highlight pathways that might have a role in the neurodegenerative process. The identification of the novel exosomal molecules involved in neurodegenerative diseases could provide important insights into the pathogenesis and contribute to the finding of novel diagnostic biomarkers and therapeutic approaches.

Key words: exosomes; CNS; miRNAs; lipid; prion; amyloid; Alzheimer; ALS; Parkinson; glia

Introduction

In recent years, knowledge of the biology and functions of extracellular vesicles, released by most cell types, has increased considerably, with the production of a significant amount of published data in a relatively short time. It is now clear that the released vesicles are involved not only in physiological functions such as cell-to-cell communication, but also in the pathogenesis of relevant diseases, including tumours and neurodegenerative conditions (1–3). Recent data indicate that neurotoxic misfolded proteins involved in neurodegenerative diseases are shuttled across the central nervous system (CNS) via exosomes, contributing to the spread and propagation of amyloidogenesis. In the case of prion diseases the presence of amyloid-asso-
associated vesicles in body fluids might also have a significant implication in the secondary transmission of the disease [4].

As the association of neurotoxic proteins with exosomes has been covered by many exhaustive reviews [5–7], the current manuscript will also focus on other pathways that might be deregulated during neurodegeneration as a result of an altered neural homeostasis and shuttle molecules across the CNS via extracellular vesicles. While it is yet not entirely clear which factors are involved in the triggering of the protein misfolding process, other molecules, including lipids and small RNAs, might contribute to the pathogenesis and progression of neurodegenerative conditions. One hypothesis is that these non-amyloid neurotoxic signals might also spread from one neuron to another via secreted vesicles, enhancing or critically contributing to disease progression. Their identification could improve the molecular understanding of these diseases and might provide useful biomarkers for diagnosis and prognosis.

The regulation of exosome functions in the CNS

It is now acknowledged that extracellular vesicles are mainly divided into two categories depending on their cellular origin [8]. Ectosomes are microvesicles derived from the budding process of the plasma membrane and have a diameter ranging from 100 to 350 nm. They are characterised by a very fast timing of release and the ability to associate with specific proteins, depending on cell type, such as the purified complement protein C1q [9]. Exosomes represent a specific subtype of secreted nanovesicles (generally 50–90 nm in diameter but also larger) that are formed in internal endosomal compartments and correspond to the intraluminal vesicles of the endosomal multivesicular bodies. They are released as exosomes into the extracellular environment upon fusion of multivesicular bodies with the plasma membrane [10]. Because of their variety of functions within the CNS and involvement in neurodegeneration, this review will largely focus on these vesicles.

Overall, data suggest a rather complex functional scenario for exosomes, substantially different from that first indicated by Raposo et al., which described exosomes as antigen-presenting vesicles [11]. Indeed it is now known that they are involved in various important processes, including immune regulation, cancer progression, stem cell regulation, gamete functions, neuronal development and regeneration, and the modulation of synaptic functions [3, 12]. They are, therefore, important intercellular communicators shuttling molecular and genetic information from one cell to another. In 2007 a milestone publication by Valadi and colleagues revealed the presence of RNA species in human and mouse mast cell exosomes that are delivered to, and functional in, target cells [13]. These RNAs have been called exosomal shuttle RNAs (esRNAs) and include messenger RNA (mRNA) encoding up to 1 300 genes and various microRNA (miRNA) species.

Exosomal functions are regulated at many levels (fig. 1), most importantly in the specificity of their cargo. The exosomal lipid environment, for instance, is unique and crucially associated with exosomal functions. Interestingly, there was estimated to be an 8.4-fold enrichment of lipids per mg of proteins in exosomes compared with cells [14]. Exosomal lipids are characterised by the presence of less phosphatidylcholine than in plasma membrane, which is more saturated, and by a very high concentration of raft components, cholesterol, sphingomyelin and ceramide. Ceramides in particular have an established role in the formation of the vesicles [15]. Indeed, impairment in their formation by inhibition of neutral sphingomyelinase (nSMase) reduces vesicle formation in multivesicular bodies [15]. Exosomal lipids are also likely to regulate exosomal sorting of small RNAs and proteins. nSMase regulates exosomal miRNA secretion in T cells and in tumour-promoting angiogenesis [16, 17]; in the nervous system, inhibition of the same pathway showed a role of the same lipids in the formation of exosomes and in the specific packaging of the cellular prion protein (PrPSc), but not of its disease-associated form PrPSc [18].

In multivesicular bodies, phosphatidylinositol regulates the recruiting of the endosomal sorting complex required for transport (ESCRT), via the zinc-finger domain of the Vpr27 ESCRT-0 subunit [19]. ESCRT largely controls the uptake of ubiquitinated proteins into the intraluminal vesicles of multivesicular bodies for lysosomal degradation [20], but recent evidence suggests that exosomal protein sorting also depends on some of its components. The P-type ATPase PARK9, which is largely expressed in the CNS, controls formation of exosomes in mouse primary cortical neurons and α-synuclein release via the ESCRT machinery [21, 22]. Other examples include the exosomal transferrin receptor sorting in reticulocytes, which is linked to binding to ESCRT-associated proteins [23]. Alx, an ESCRT-associated protein, regulates the uptake of exosomal syneclins on tumour cell lines, in a process crucial for multivesicular body and exosome biogenesis [24]. Interestingly, syneclins are involved in the selective exosomal uptake of proteins such as CD63 but not CD9, CD81 and flotillin-1 [25].

Finally, a recent report, that will be further discussed below, indicates that certain exosomal lipids (gangliosides GM1 and GM3) can promote aggregation of neuronal amyloid proteins such as α-synuclein [26], suggesting that exosomes are not just passive shuttles of molecules but that, via their lipid content and acidic environment [27], they actively prime some of their components with possibly important implications related to the spreading and neurotoxicity of misfolded proteins. In this context a relevant work by Fang and co-workers [28] demonstrated that in Jurkat-T cells, an exosomal sorting pathway selects proteins on the basis of high-order oligomerisation and plasma membrane association. It is unclear if this mechanism is effective in vivo, but nevertheless, these data could have crucial implications for the understanding of CNS amyloid diseases: by default, CNS cells could divert misfolded proteins to an extracellular propagation pathway whose lipid components are effective primers for neurotoxicity [28].

Protein specific uptake on exosomes was also demonstrated by the evidence that Nedd4 family-interacting protein 1 (Ndfip1), which is present in exosomes of primary neurons, controls the sorting into exosomes of Nedd family
proteins. Interestingly, these proteins are known to be associated with neuronal survival after brain injury [29]. Faure et al. [30], who first described neuronal exosomal vesicles release, showed that the protein content was not random as it contains L1 cell adhesion molecule, the GPI-anchored prion protein and the GluR2/3, but not the NR1 subunits of glutamate receptors. Also, proteins with specific post-translational modifications are preferentially sorted into extracellular vesicles. These include small ubiquitin-related modifications (SUMOs), phosphorylation and glycosylation (reviewed in [31]). Abnormal phosphorylation could also promote the uptake of amyloid proteins into exosomes, with implications for neurodegeneration [32]. A SUMO of the protein heterogeneous nuclear ribonucleoprotein A2B1 controls the binding of this protein to miRNAs and, therefore, miRNA loading into exosomes [33]. Exosomes are indeed enriched with a subset of miRNAs, as shown in several cell lines, not necessarily of the same repertoire of parent cell [17]. Specific exosomal miRNAs include miR-150, miR-124-3p, and miR-451 and miR-320 [34, 35].

Exosomal function regulation is not only controlled by a sound selection of the molecular content. There is evidence that both spatial and temporal exosomal secretion are tightly regulated and linked to their extracellular fate (fig. 1). In neurons, for example, the secretion of exosomes is polarised. Multivesicular bodies that are located in the proximity of the presynaptic membrane release exosomes that are implicated in the regulation of synaptic functions [36]. Korkut et al. demonstrated that presynaptic exosomal release of synaptotagmin-4 controls retrograde signals implicated in activity-dependent synaptic growth [37]. Polarised release of exosomes and unidirectional miRNA shuffling has also been extensively demonstrated in immune synapses between T cells and antigen-presenting cells [17, 38]. Also, the timing of exosomal release in the nervous system is tightly controlled, and in cortical neurons is regulated by calcium influx and by glutamatergic synaptic activity [39] and potassium chloride depolarisation [30]. In human neuroblasts small RNA sorting into exosomes and their release is temporarily linked with potassium chloride depolarisation [40].

Another level of regulation of exosomes is on the specificity of cellular targets (fig. 1), which are not random, by mechanisms that are still, to date, unclear. In terms of target selectivity, Chivet et al. [41] recently showed that in the nervous system GFP-CD63-tagged exosomes produced by neuroblastoma cells indiscriminately bind hippocampal glia and neuronal cells, whereas exosomes isolated from stimulated cortical neurons bind only neurons. These data demonstrate that the delivery of exosomes to target cells is not random but rather has a possible selective mechanism. Overall, state-of-the-art data show that exosomes are strongly involved in cell-to-cell communication and the ability to shuttle selected molecules to precise and distant cellular targets defines them as a highly organised physiological system extending cell functions to long distances. Some of the identified CNS exosomal pathways of intercellular communication are described in following section.

**Exosomes in neuron-to-glia communication**

Glia include different cellular subpopulations, which carry out specialised functions that go far beyond the mere structural, passive role of neuron supporters. Among these, their essential and active involvement in neural homeostasis maintenance, signal transduction and cellular cross-talk have been validated [42–44]. In this scenario, which implies a close cooperation between neurons and glia, exosomes might fulfil the role of CNS intercellular stakeholders, by favouring protective and/or pathogenic states. The molecular mechanisms underlying the exosome-mediated exchange of information show that glial exosomes exert an influence on neuron physiology by displaying a direct, significant impact on firing rate (the average number of action potentials per unit time) increase [45]. Neurons internalise exosomes of oligodendroglial origin at both axonal and dendritic levels. Glial exosomes are released in response to glutamate neurotransmitter neuronal exosomes signalling by Ca²⁺ entry through oligodendroglial N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. It was shown that they have a neuroprotective role after oxidative stress and starvation [45, 46]. Interestingly, astrocyte exosomes also release heat shock proteins (Hsps) with a possible neuroprotective role for neurons under stress condition [47, 48]. Specifically, hyperthermia increases the release of Hsp:c70 into the extracellular environment via extracellular signal-regulated kinase 1/2 (ERK1/2) and phosphatidylinositol-3 kinase pathways [48]. Synapsin 1 is another neuroprotective molecule released by astrocytes on exosomes, which promotes neurite outgrowth on hippocampal neurons and survival on cortical neurons under stress conditions [49].

Treatment of primary oligodendrocytes with a calcium ionophore increases the release of exosomal vesicles, which may be indicative of the existence of a fine calcium-dependent process [50, 51]. In addition, a study conducted on microglia found evidence of calcium-dependent signalling in the regulation of exosome release through stimulation mediated by the serotoninergic receptor 5-HT₄ [50].
All this experimental evidence suggests the presence of an elegant cargo system from glia to neurons mediated by exosomal vesicles.

Molecular and proteomic analyses of oligodendroglial exosomal content prove the involvement of exosomes in myelin biogenesis [52] and in the building of myelin architecture by showing the presence of typical myelin membrane components, such as myelin proteolipid protein (PLP), 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP), myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG) [44, 51]. Exosomes take a direct part in remyelination process through the differentiation of oligodendrocyte progenitor cells (OPC) via delivery of active molecular content. Specifically, miR-219, a small noncoding RNA, significantly deficient in patient brains with multiple sclerosis lesions [53], promotes the entry of neural stem cells into the OPC lineage via exosomes, operating via direct inhibition of some regulators of protein differentiation [54–56].

Another exciting study explored a new pathway mediated by excitatory amino-acid transporters (EAAT) and glutamate transporter 1 (GLT1) receptor. Astrocyte exosomes contain EAAT1, which is increased under stress conditions via a protein kinase C pathway [57]. Interestingly, cellular EAAT2 levels are increased by exosomal miR-124a of neuronal origin [58]. This exosomal miRNA is impaired and reduced in rodent models of amyotrophic lateral sclerosis [58] and in the cerebrospinal fluid (CSF) of patients with amyotrophic lateral sclerosis (Properzi et al., unpublished data), suggesting that reduced levels of cellular EAATs on astrocytes might have a role in CNS pathological conditions.

Furthermore, could the so-called “Trojan exosomes hypothesis” [59], which relates to the principle of exosomes as cargo of pathogenic and misfolded proteins [60–64], find a place also in glia to neuron cross-talk?

In Alzheimer’s disease soluble forms of Aβ, i.e. low molecular weight precursors of amyloid fibrils, are the most neurotoxic species [65–68]. Recent evidence shows that microglia promote the formation of these neurotoxic Aβ oligomers via extracellular microvesicles [69], by changing the equilibrium between soluble and aggregate Aβ species [70] and by favouring the uptake of the soluble ones [71]. In line with this, levels of CSF microvesicles of myeloid origin, which are markers of microglial activation, significantly increase in parallel with the degree of hippocampal atrophy in Alzheimer’s disease patients and with the white matter tracts damage in mild cognitive impairment patients [72], suggesting the involvement of these small vesicles of glial origin in neural impairment. [73]. Glial vesicle activity, in clearance or in genesis of abnormal protein species, may depend on the presence or absence of specific cofactors, such as glycosphingolipids (GSLs) [74, 75], nSMase2 [15, 76] or specific neurotransmission signals that could influence the fate of exosomal content and pathogenic spread to neighbouring cells. Glial exosomes may represent the filler to plug at least some of the gaps in information about neural signal processing and maintenance, abnormal relocation of pathological conformers and shipping of aberrant genetic contents. Indeed, the analysis of glial-exosome content and related pathways will not only help us to understand human neurodegenerative states caused by a failure in glial-neuron interaction, but also to consider new targets for drug therapies, engaging a new scientific challenge.

**CNS exosomal pathways and neurodegeneration**

Apart from mediating normal brain function such as neuronal development, repair, and synaptic function, it is clear that extracellular vesicles / exosomes also contribute to the development of disease states. Exosomes contain a variety of proteins, lipids, miRNA, mRNA, collectively termed as “cargo” content and delivered to surrounding cells or carried to distant cells. Owing to their active cargo content, exosomes can mediate various signalling functions [77] and cause dysfunction in brain disorders. This is particularly relevant in neurodegenerative diseases such as transmissible spongiform encephalopathies, also known as “prion diseases”, Alzheimer’s and Parkinson’s diseases. All these diseases have a common molecular and cellular mechanism, which involves protein misfolding and aggregation, and the formation of inclusion bodies in selected brain areas. Exosomes have been reported to sequester and spread “toxic” (pathogenic) proteins such as prion [64, 78], α-synuclein [62], amyloid precursor protein (APP) [60], and phosphorylated tau [32]. The pathogenic forms of these proteins are associated with extracellular vesicles such as microvesicles and exosomes, which preferentially load higher oligomerisation state proteins [28], providing new evidence of their implication in the processing and intercellular spread of these misfolded proteins. The hypothesis evolved first in the context of the interneuronal spread of prion diseases such as bovine spongiform encephalopathy, the variant form of Creutzfeldt-Jakob disease (vCJD) and scrapie. During prion infection, the extracellular vesicles released by cells in vitro contain both the physiological cellular prion protein, PrPSc, and the abnormal infectious form, PrPRes [64]. The extracellular vesicles containing the protease-K resistant PrPSc are infectious [64, 79], indicating that exosomes have a central role in the spread of prion proteins throughout the host. Although prions have long been thought to be the only transmissible cause of neurodegenerative disease, the idea is now emerging that other key misfolded proteins involved in proteinopathies such as Alzheimer’s disease, Parkinson’s disease and amyotrophic lateral sclerosis have the potential to induce the misfolding of cellular proteins by a “prion like” mechanism [5, 7].

Amyloids involved in Alzheimer’s disease, such as Aβ, are released via exosomes and can act as seed for plaque formation [60, 80]. Additionally, the α- and β-secretases have been identified in exosomes, indicating that cleavage of APP could occur within these vesicles [81]. Although exosomes containing Aβ may play a role in the pathogenesis of Alzheimer’s disease, studies have also demonstrated that exosomes could have beneficial effects against the pathology and functional effects of Aβ [74, 75, 82, 83]. Similar to prions and Aβ, both tau and α-synuclein are also found in extracellular vesicles [7, 32, 84–86]. Mounting evidence strongly suggests that accumulation of abnormal tau is mediated through the spread of seeds of the protein from cell
to cell and points to the involvement of extracellular tau species as the main agent in the interneuronal propagation of neurofibrillary lesions and spreading of tau toxicity throughout different brain regions in these disorders [87]. Tau has been reported to be secreted unconventionally in naked form or associated with exosomes [32] and/or other membrane vesicles [88]. Exosomal tau secretion has been suggested to account for the elevated cerebrospinal fluid CSF tau levels typically observed in early Alzheimer’s disease and to be involved in disease-associated tau misprocessing [32]. Alpha-synuclein oligomers are present in the exosomal fractions from both neuronal and nonneuronal cells. Exosome-associated α-synuclein oligomers are more prone to being taken up by cells than exosome-free oligomers, and confer more cytotoxicity when compared with the increase in caspase activation [89]. It has also been hypothesised that lysosome dysfunction can accelerate exosomal α-synuclein release and propagation to neighbouring cells, with associated increase in protein inclusion formation [84]. Recently it has also been suggested that exosomes provide catalytic environments for nucleation of α-synuclein aggregation [26]. Some hypotheses for the role of exosomes in secondary aggregation of toxic proteins are now emerging and consider not only the enrichment/increased concentration of these proteins in a limited volume or the exosomal luminal physicochemical conditions (acidic pH), but also the lipid composition or content of other cofactors. Indeed, the role of lipids in neurodegenerative disorders is now emerging. Lipid-driven membrane organisation allows the segregation of membrane-associated components into lipid rafts, dynamic platforms with signal transduction, protein processing and membrane turnover functions. A number of events essential for the correct functioning of the nervous system occur in lipid rafts and depend on lipid raft organisation. Alterations of lipid composition lead to alteration of lipid raft organisation and are often associated with neurodegenerative diseases. Moreover the amyloidogenic processing of proteins involved in the pathogenesis of major nervous system diseases, including Alzheimer’s disease, Parkinson’s disease and transmissible spongiform encephalopathies, requires lipid raft-dependent compartmentalisation at the membrane level, and altered lipid composition of the rafts may promote or facilitate the formation of the pathological forms of the proteins [90]. The exosome membrane contains lipid rafts enriched in cholesterol, sphingomyelin and ganglioside GM2 and GM3, which are believed to participate in vesicle structure and function. PrPSc is tethered to the plasma membrane by a GPI anchor, and the conversion of PrPSc to PrPSc has been suggested to occur in lipid raft regions [91]. The presence of lipid rafts in exosomes could also be important for the transmission of PrPSc. It seems possible that the generation of new PrPSc during infection requires the insertion of PrPSc into lipid rafts [92]. Exosomes containing PrPSc may then be able to insert their PrPSc cargo into the membrane of recipient cells upon contact. Another function of the lipid raft nature of exosomes may be to stabilise a particular infectious form of PrPSc. The in-vitro generation of infectious PrPSc from bacterially expressed recombinant PrPSc has been shown to require the presence of lipid cofactor [93]. Moreover synthetic pri-

ons with a high level of infectivity have been produced in vitro, showing that various major classes of host-encoded cofactor molecules such as lipids and small RNA molecules are required to form and maintain the specific conformation of infectious prions [94].

In Alzheimer’s disease, the regulation of β site APP cleaving enzyme 1 (BACE1) activity is determined by its access to APP, which is in turn lipid-dependent and involves lipid raft formation. Moreover γ-secretase activity is regulated by membrane levels of cholesterol and sphingomyelin [95], and lipids such as ganglioside GM1 modulate the pathogenic potential of Aβ by affecting its propensity to aggregate [96]. Considering the lipid raft nature of exosomes and that exosomes of Alzheimer’s disease patients contain both APP and BACE 1, it is tentative to speculate their active role in Aβ generation and aggregation.

Recently, it has been reported that exosomes are able to accelerate α-synuclein aggregation and that the lipid content in exosomes is sufficient for this catalytic effect to arise [26]. In addition, the authors prepared vesicles from pure lipids (starting from exosomal lipids) most of which were found to inhibit α-synuclein aggregation with the exception of vesicles containing GM2 and GM3 (truncated version of GM1) that instead accelerated the process. These observations provide strong evidence for the in-vivo involvement of exosomes in the spread of multiple neurodegenerative diseases. The prion-like mechanisms of spread and how exosomes / exosomal lipids facilitate this, will be of strong interest to future studies. In line with this view, levels of tau, and Aβ1-42 in extracts of neurally derived blood exosomes, were found to be significantly higher 10 years before clinical onset of Alzheimer’s disease [97].

Levels of autolysosomal proteins are also deregulated in blood exosomes of patients with Alzheimer’s disease up 10 years before clinical onset. Particularly levels of cathepsin D, lysosome-associated membrane protein, and ubiquitinylated proteins were significantly higher and of heat-shock protein 70 significantly lower [98]. In addition to the protein and lipid cargo content, exosomes contain nucleic acids, in particular miRNA and mRNA as reported in the ExoCarta database [99]. The mRNA and miRNA present in exosomes can also be transferred to recipient cells imparting a biological effect on them. Profiling the level and composition of esRNAs in particular diseased state will be helpful for diagnosis and for better understand pathogenesis. Despite the increasing body of evidence implicating miRNA expression in a number of neurodegenerative diseases and exosomes involvement in some way with these pathologies, very little is known of the potential role of esRNA in pathogenesis and diagnosis of neurodegenerative disorders. Exosomal miRNA profiling of the serum of Alzheimer’s disease patients at early stages of the pathology revealed a specific signature of 16 Alzheimer’s disease-specific deregulated miRNAs [100]. The majority of them has been shown to be implicated in Alzheimer’s disease pathogenesis, such as tau phosphorylation, α-secretase and APP levels of expression, or to interfere with neuroprotective mechanisms such as the transforming growth factor-β pathway.
A distinct exosomal miRNA signature in prion-infected neuronal cells was recently reported. Pathway analysis revealed several gene interactors of the prion protein including BACE1, SP1, p53, AGO1 and AGO2. The same study reported mRNA fragments in exosomes from prion-infected neuronal cells and further analyses are needed to disclose their eventual role in neurodegenerative pathologies [86].

Micro RNA expression levels are largely changed during neurodegenerative diseases, suggesting their direct role in neurodegeneration and their potential use as biomarkers of these disorders (table 1 and table 2). Some studies of the regulation of miRNAs (as listed in table 1) give apparently contrasting results, maybe as a result of the stage of disease progression and/or the nature of the starting material. The analysis of exosomal content could be helpful to extrapolate new tracking information about the selected potential diagnostic markers.

Moreover, although an effect of other exosomal cargos on recipient cells cannot be completely excluded, miRNA are now widely considered the key functional elements. Their functions can be classified into two types. One is the conventional function of regulatory control over the expression levels of target genes inside the cell where they are produced. This function is important in sporadic Alzheimer’s disease where aberrant regulation of miRNA-dependent gene expression is associated with Aβ production, neurofibrillary tangle formation, and neurodegeneration. MicroRNAs 106a, 502c, -106b and 17-5p, for example, could downregulate APP gene expression [101, 102]. Decreased miR-29a and miR-29b-1 in Alzheimer’s disease patients correlated with increased BACE1 expression. BACE1 is a target for both miRNAs and the rate-limiting enzyme for Aβ production, indicating correlative evidence for a mechanism in Alzheimer’s disease. Similarly, a number of miRNAs has been identified in Alzheimer’s disease patients regulating important target genes such as PSEN1 and PSEN2 in transfected neurons [103, 104].

In Parkinson’s disease, few miRNAs have been recognised to be associated with the dopaminergic phenotype: miR-133b is upregulated in sporadic Parkinson’s disease patients [105] and it regulates the PITX3 gene expression, a key factor in the development of the dopaminergic neuronal phenotype in mice. miR-7 was shown to suppress α-synuclein in human neuroblastoma cells and may be implicated in Parkinson’s disease pathogenesis [106]. Another example is miR-205, which regulates LRKK2 R1441G mutant in transfected neurons [107].

The second function is a novel mechanism identified in some miRNAs studied as exosomal miRNAs rather than intracellular. Exosomal miR-21 and miR-29a, in addition to the classic function of targeting mRNA, were discovered to have the capacity to act as ligands that bind to toll-like receptors and activate immune cells [108]. This study uncovered an entirely new function of miRNAs that needed further investigation. It is, however, tentative to speculate the role of miRNAs according to this latter function in prion diseases. As described above [94], small single-chained RNA may have a role as ligand enhancing or maintaining prion infectivity. In the same direction, a recent study, searching for a putative PrPSc-associated factor contribut-

The role of exosomes in amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease caused by motor neuron loss, characterised by progressive paralysis and motor neuron death. There are two forms of the disease: a sporadic form (sALS), with unknown aetiology, and a familial form (fALS) due to genetic defects. To date, only a limited number of genes, including C9orf72, SOD1, TARDBP and FUS, are responsible for a considerable proportion of fALS cases, but the pathological mechanism is still not clear. Around 20% of the fALS cases are caused by mutations in superoxide dismutase 1 (SOD1), but the relationship between mutated SOD1 and the disease remains not clarified [110].

To date, it is postulated that mutant SOD1 is responsible for the cytotoxic activity associated with the neurodegeneration. Interestingly, accumulation of misfolded oligomers or protein inclusions containing SOD1 or TDP-43 and FUS is commonly observed in patients with sALS. The pathological role and mechanism of formation of these protein aggregates are not known to date.

In agreement with observations in the prion diseases and with the recent prion-like hypothesis, Gomes and colleagues [61] have shown, in both in-vitro and in-vivo studies, that wild-type and sSOD1 mutants were present in the supernatant medium from NSC-34 cells stably expressing mutant SOD1 and that this protein was associated with exosomes that could, in a sense, represent a way to transfer cytosolic proteins like SOD1 between different cells. They explained the presence of the wild-type cytosolic proteins in the extracellular environment as a cellular mechanism of protection against the physiological production of reactive oxygen intermediates that could affect the plasma-membrane surface. Furthermore, they hypothesised that the release of mutant SOD1 in the extracellular media via exosomes could be acting as an inflammation trigger. In agreement, Urishitani et al. [111] have shown that, while wild-type SOD1 inhibits microglial activation in vitro, extracellular SOD1 mutants cause gliosis and neuron death. These data could confirm the hypothesis that the release of SOD1 by the cell could be associated with the inflammatory response.

Recent work by Grad et al., [112] showed for the first time that aggregates of human wild-type SOD1 (HuWTsOD1) can propagate to naïve cells via two mechanisms: (1) dying cells release protein aggregates, which are taken up by macrophages; (2) the aggregates could be secreted from living cells by means of exosomes (data observed both in HEK293 cell cultures via conditioned media and cultured mouse primary spinal cord cells transgenically expressing human SOD1). In addition, it was shown that misfolded and protease-sensitive HuWTsOD1 comprises up to 4% of total SOD1 in the spinal cords of sALS patients, sug-
gesting that the misfolded protein propagation could under-
lie the molecular pathogenesis of sALS [112].
Another indication of the fact that ALS can be classified as a prion-like disease comes from Nonaka et al. [63], who showed that TDP-43 aggregates may also be transferred from cell to cell, at least partly via exosomes [63].

Aggregates of misfolded proteins are definitely not the only molecules that take part in the disease process. Events such as neuroinflammation, mitochondrial dysfunction and oxidative stress, glutamate excitotoxicity, and defective RNA processing could be part of a complex network of systems that contribute to the altered course of the disease and might be linked to exosomes [113]. For instance, epi-

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<td>miR-135a-5p, miR-298-5p, miR-669-3p</td>
<td>AD</td>
<td>Down</td>
<td>Mouse brain [143]</td>
</tr>
<tr>
<td>miR-138</td>
<td>AD</td>
<td>Up</td>
<td>Human serum [125]</td>
</tr>
<tr>
<td>miR-146a</td>
<td>AD</td>
<td>Up/down</td>
<td>Human CSF [131, 138]</td>
</tr>
<tr>
<td>miR-153</td>
<td>AD</td>
<td>Down</td>
<td>Mouse brain [144]</td>
</tr>
<tr>
<td>miR-181</td>
<td>AD</td>
<td>Up/down</td>
<td>Mouse brain [145] / human, mouse brain [146]</td>
</tr>
<tr>
<td>miR-181b</td>
<td>AD</td>
<td>Up</td>
<td>Human BMGs [134]</td>
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<tr>
<td>miR-200, miR-331-3p</td>
<td>AD</td>
<td>Up</td>
<td>Mouse brain [137]</td>
</tr>
<tr>
<td>miR-206</td>
<td>AD</td>
<td>Up</td>
<td>Mouse brain [147, 148], human brain [149]</td>
</tr>
<tr>
<td>miR-219</td>
<td>AD</td>
<td>Down</td>
<td>Human brain [149]</td>
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<tr>
<td>miR-590-3p</td>
<td>AD</td>
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<td>Human blood cells [150]</td>
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<tr>
<td>miR-16-2-3p, miR-26a-23p, miR-30a</td>
<td>PD</td>
<td>Up</td>
<td>Human blood [151]</td>
</tr>
<tr>
<td>miR-30c, miR-148b</td>
<td>PD</td>
<td>Down</td>
<td>Human serum [152]</td>
</tr>
<tr>
<td>miR-34b/c</td>
<td>PD</td>
<td>Down</td>
<td>Human brain [153]</td>
</tr>
<tr>
<td>miR-64, miR-65</td>
<td>PD</td>
<td>Down</td>
<td>Caenorhabditis. elegans [154]</td>
</tr>
<tr>
<td>miR-133b</td>
<td>PD</td>
<td>Down</td>
<td>Human brain [156]</td>
</tr>
<tr>
<td>miR-311-5p</td>
<td>PD</td>
<td>Up</td>
<td>Human plasma [156]</td>
</tr>
<tr>
<td>miR-450b-3p/ miR-1826</td>
<td>PD</td>
<td>Up</td>
<td>Human plasma [157]</td>
</tr>
<tr>
<td>let-7b</td>
<td>TSEs</td>
<td>Up</td>
<td>Murine neuronal cells [89], brain [158]</td>
</tr>
<tr>
<td>let-7i, miR-128a, miR-21, miR-222, miR-29b, miR-424*</td>
<td>TSEs</td>
<td>Up</td>
<td>Murine neuronal cells [86]</td>
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<tr>
<td>miR-124a-3p, miR-132-3p, miR-29a-3p, miR-16-5p, miR-26a-5p, miR-140-5p</td>
<td>TSEs</td>
<td>Up</td>
<td>Murine brain [158]</td>
</tr>
<tr>
<td>miR-148*</td>
<td>TSEs</td>
<td>Down</td>
<td>Murine neuronal cells [86]</td>
</tr>
<tr>
<td>miR-148a</td>
<td>TSEs</td>
<td>Up</td>
<td>Murine brain [158, 160], microglial cell lines [158]</td>
</tr>
<tr>
<td>miR-146a-5p</td>
<td>TSEs</td>
<td>Up</td>
<td>Murine brain [161]</td>
</tr>
<tr>
<td>miR-150-5p, miR-26b-5p miR-410-3p</td>
<td>TSEs</td>
<td>Up</td>
<td>Murine brain [161]</td>
</tr>
<tr>
<td>miR-320, miR-328, miR-126, miR-139-5p</td>
<td>TSEs</td>
<td>Up</td>
<td>Murine brain [156]</td>
</tr>
<tr>
<td>miR-336-3p, miR-337</td>
<td>TSEs</td>
<td>Down</td>
<td>Murine brain [156]</td>
</tr>
<tr>
<td>miR-342-3p</td>
<td>TSEs</td>
<td>Up</td>
<td>Murine [158], monkey and human brain [162]</td>
</tr>
<tr>
<td>miR-351, miR-542-5p</td>
<td>TSEs</td>
<td>Up</td>
<td>Murine n2a [163]</td>
</tr>
<tr>
<td>miR-494</td>
<td>TSEs</td>
<td>Up</td>
<td>Human brain [162]</td>
</tr>
</tbody>
</table>

AD = Alzheimer’s disease; BMCs = peripheral blood mononuclear cells; CSF = cerebral spinal fluid; miRNA = microRNA; PD = Parkinson’s disease; TSE = transmissible spongiform encephalopathy

* Exosomal miRNAs
genetic phenomena are linked to various diseases, and include RNA editing and the action of noncoding RNAs such as miRNAs. The role that miRNAs play in ALS is also becoming more prominent, and indeed they are potential candidates for the diagnosis and prognosis of the disease (table 2) [114]. In neuroinflammatory diseases such as ALS, miRNAs, by regulating gene expression, affect the immune system, whose dysfunction contributes to these pathologies [115].

As limited information exists on miRNAs deregulation in ALS, we will summarise the recent work, and present some results obtained by our research group. Several studies have examined miRNAs in ALS models and in patient biopsy samples. Recent work by Freischmidt and colleagues [116] indicated that in patients with sALS, the expression of certain miRNAs was altered in CSF and serum (miR-132-5p, -132-3p, -143-3p, miR-143-5p, and -574-5p). The miRNAs had previously been shown to bind to TDP-43 in vitro [116].

In G93A-SOD1 transgenic mice, used as the mouse model of ALS, it was seen that the expression of miR-206, which plays a role in skeletal muscle development, increases significantly in synaptic regions of muscle fibres of the mouse model of ALS. It was postulated that miR-206 is capable of sensing damage or loss of motor neurons and promoting the regeneration of functional synapses between individual neurons in the muscles, to attenuate muscle injury. Data showed that the disease develops faster in miR-206 knock-out mice and the survival rate of miR-206 knockout mice is lower [117]. The levels of this miRNA are also increased systemically in both mice and humans, confirming the potential of this miRNA as ALS biomarker [118].

Important results that strengthen the role of miRNAs in the pathogenesis of ALS and confirm them as possible candidates for diagnostic markers, are described by Parisi and colleagues [115], who performed a comparative screening of miRNAs in resting and activated SOD1-G93A microglia and identified selected miRNAs (miR-22, miR-155, miR-206, miR-125b and miR-146b) to be used as novel tools for further dissecting and controlling mechanisms. These results support the idea that ALS is a neuroinflammatory disease, as they identified specific miRNAs that operate by modulating ALS-linked inflammatory genes and suggested their deregulation as pathogenetic mechanisms of the disease [115].

In this scenario, what is the role of exosomes? It is well established that miRNAs transfer from a donor cell into a recipient cell via exosomes and microparticles. In order to communicate also between the brain and distant organs via biological fluids, exosomes may play an important role as carriers of miRNA across the endothelial cellular layers of the blood brain barrier, releasing their contents into the circulation [35]. For this reason, miRNAs found to be highly abundant in the brain have also been detected in human biological fluids such as plasma, urine and CSF [3].

On the basis of this idea, our research group has recently carried out the task of screening for exosomal miRNAs isolated from CSF of sALS patients, in order to identify in one or more of them some early ALS biomarkers. In our preliminary study (data not shown) we have identified 16 miRNAs significantly deregulated in the CSF of 15 sALS patients when compared with 15 relevant neurological controls. In particular, we found a specific miRNA, previously observed to be upregulated in myotonic dystrophy type-2 in biopsies of skeletal muscle [119], deregulated in about 30% of sALS patients and in no control.

MicroRNA-124 has been described as associated with ALS; in detail it is associated with microglia activation [120] and a new neuron-to-astrocyte communication pathway [58]. In the healthy adult CNS, microglia are normally quiescent cells, but they are activated in areas of trauma and infection in the aging human brain as well as in neurodegenerative and neuroinflammatory diseases. As miR-124 has emerged as a key regulator of microglia quiescence in the CNS, Ponomarev et al. [120] showed both in vivo (mouse brain

<p>| Table 2: Deregulated miRNAs in amyotrophic lateral sclerosis (ALS) |
|-----------------|---------|-------------------------------|</p>
<table>
<thead>
<tr>
<th><strong>MicroRNA</strong></th>
<th><strong>Disease</strong></th>
<th><strong>Up-/down-regulated</strong></th>
<th><strong>Tissue</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>let-7b</td>
<td>ALS</td>
<td>Down</td>
<td>Human serum [116]</td>
</tr>
<tr>
<td>miR-9</td>
<td>ALS</td>
<td>Up/down</td>
<td>Murine spinal cord [164], murine brain [165] / human iPSC-derived neuron [166]</td>
</tr>
<tr>
<td>miR-19a, miR-19b</td>
<td>ALS</td>
<td>Up</td>
<td>Murine brain [165]</td>
</tr>
<tr>
<td>miR-22, miR-125b, miR-146b</td>
<td>ALS</td>
<td>Up</td>
<td>Murine microglia [115]</td>
</tr>
<tr>
<td>miR-23a, miR-29b, miR-455</td>
<td>ALS</td>
<td>Up</td>
<td>Human skeletal muscle [167]</td>
</tr>
<tr>
<td>miR-29a</td>
<td>ALS</td>
<td>Up</td>
<td>Murine spinal cord [168]</td>
</tr>
<tr>
<td>miR-124a*</td>
<td>ALS</td>
<td>Up/down</td>
<td>Murine brain [165] / murine spinal cord [58]</td>
</tr>
<tr>
<td>miR-132-5p, miR-132-3p, miR-143-3p</td>
<td>ALS</td>
<td>Down</td>
<td>Human CSF and serum [116]</td>
</tr>
<tr>
<td>miR-143-5p</td>
<td>ALS</td>
<td>Up/down</td>
<td>Human CSF and serum [116]</td>
</tr>
<tr>
<td>miR-574-5p</td>
<td>ALS</td>
<td>Up</td>
<td>Human CSF [116]</td>
</tr>
<tr>
<td>miR-155</td>
<td>ALS</td>
<td>Up</td>
<td>Murine and human brain [169], Rodent and human spinal cord [170]</td>
</tr>
<tr>
<td>miR-206</td>
<td>ALS</td>
<td>Up</td>
<td>Murine brain [117], serum [171] muscle and plasma, human serum [116], skeletal muscle [167]</td>
</tr>
<tr>
<td>miR-219</td>
<td>ALS</td>
<td>Down</td>
<td>Murine brain [165]</td>
</tr>
<tr>
<td>miR-338-3p</td>
<td>ALS</td>
<td>Up</td>
<td>Human blood leucocytes, CSF and spinal cord [172]</td>
</tr>
<tr>
<td>miR-373, miR-506, miR-518a-5p, miR-518c, miR-551a, miR-890</td>
<td>ALS</td>
<td>Up</td>
<td>Human spinal cord [114]</td>
</tr>
<tr>
<td>miR-451, miR-1275, miR-328, miR-838, miR-149, miR-865</td>
<td>ALS</td>
<td>Down</td>
<td>Human blood leucocytes [173]</td>
</tr>
</tbody>
</table>

CSF = cerebrospinal fluid; iPSC = induced pluripotent stem cell; miRNA = micro RNA
* Exosomal miRNAs
and spinal cord during experimental autoimmune encephalomyelitis) and in vitro that high levels of miR-124 are linked to quiescent microglia and down-regulation causes the activation of microglia [120]. These results are in line with some of our preliminary data showing down-regulation of miR-124 in the CSF of patients. Interestingly, peripheral administration of miR-124 helps in ameliorating the experimental diseases [120].

Morel and colleagues [58] showed that exosomes isolated from cultured primary neuron conditioned medium can be directly internalised into astrocytes, increasing miR-124a and GLT1 protein levels. MicroRNA R-124a is selectively reduced in the spinal cord tissue of endstage SOD1 G93A mice, the mouse model of ALS. Subsequent exogenous delivery of miR-124a in vivo through stereotaxic injection significantly prevents further pathological loss of GLT1 proteins, as determined by GLT1 immunoreactivity in SOD1 G93A mice. Importantly, intranuclear injections of specific antisense RNA against miR-124a into adult mice dramatically reduces GLT1 protein expression and glutamate uptake levels, possibly contributing to ALS glutamate neurotoxicity, which has been extensively reported by many publications [58]. Overall these results could pave the way to the finding of novel exosome associated biomarkers related to inflammatory pathways and ALS.

Conclusions

In the CNS, exosomal functions are regulated not only by a tight selection of the vesicle cargo but also by their intracellular localisation and timing of release, which can be controlled by depolarisation events and Ca2+ influx, and by cellular target specificity. All these fine regulatory mechanisms are likely to be altered during the neurodegenerative process. It is well established that misfolded proteins involved in neurodegenerative diseases are shuttled via nanovesicles, contributing to the spreading of neurotoxicity. Nevertheless other molecules and pathways could have a role in neurotoxicity and be transported via nanovesicles to other cells. As an example, exosomes that are shuttled between glia and neurons in physiological conditions have a role in supporting and maintaining neuronal homeostasis. One mediator of this function is exosomal miR-124a, which during neurodegenerative conditions such as ALS is downregulated in both spinal cord and CSF. The identification of exosomal pathways and molecules other than amyloid involved in neurodegenerative diseases could provide not only important insights in the pathogenesis of these diseases but, as nanovesicles are released in biological fluids, also novel diagnostic and prognostic biomarkers and therapeutic approaches.

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References


Figure 1
Regulation of exosomal function in the central nervous system. Black circles represent exosomes whose functions can be regulated in (A) intracellular cargo sorting; (B) space-time regulation of secretion; (C) cellular target specificity.
esRNA = exosomal shuttle RNA