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Interplay Between Exosomes, microRNAs and Toll-Like Receptors in Brain Disorders

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Abstract Extracellular vesicles (EVs), including exosomes, microvesicles and apoptotic bodies, participate in intercellular communication, and particularly, in paracrine and endocrine signalling. The EVs and their specific contents have been considered hallmarks of different diseases. It has been recently discovered that EVs can co-transport nucleic acids such as DNAs, ribosomal RNAs, circular RNAs (circRNAs), long noncoding RNAs (lnRNAs) and microRNAs (miRNAs). miRNAs are important regulators of gene expression at the post-transcriptional level, although they may also play other roles. Recent evidence supports the hypothesis that miRNAs can activate Toll-like receptors (TLRs) under certain circumstances. TLRs belong to a multigene family of immune system receptors and have been recently described in the nervous system. In the immune system, TLRs are important for the recognition of the invading microorganisms, whereas in the nervous system, they recognise endogenous ligands released by undifferentiated or necrotic/injured cells. In the neuronal disease field, TLRs activity has been associated with amyotrophic lateral sclerosis (ALS), stroke, Alzheimer's and Parkinson's disease. Herein, we reviewed the current knowledge of the relationship between miRNA release by EVs and the inflammation signalling triggered by TLRs in neighbouring cells or during long-distance cell-to-cell communication. We highlight novel aspects of this communication mechanism, offering a valuable insight into such pathways in health and disease.

Keywords Extracellular vesicles · EVs · Diseases · Parkinson's disease · Alzheimer's disease · ALS · Stroke · Long-distance communication · miRNAs · TLR ligands · TLR signalling · Neurodegeneration · Apoptosis · Central nervous system · CNS

Introduction

To defend themselves against injuries or diseases, organisms provide ordered responses. For maintaining homeostasis, cells should be in constant communication. Three different ways of cellular communication are widely used in the nervous system. The best-known method is signal transmission via chemical synapses, initiated by the release of neurotransmitters. The second mechanism, attracting increasing attention in the recent years, is the cell coupling provided by gap junction channels [1–4]. The third form of communication is paracrine signalling, which encompasses several distinct mechanisms [5, 6]. Recent evidence suggests that the extracellular vesicles (EVs), including exosomes, microvesicles and apoptotic bodies, could be the fourth form of communication, ensuring short- and long-range exchange of information [7–10].

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EVs and Transport of miRNAs

EVs are small lipid-membrane microvesicles (30–100 nm in diameter), found in prokaryotic and eukaryotic cells [11]. These vesicles originate from different cellular compartments such as membranes or endosomes, and are secreted into the extracellular medium [10, 12, 13]. The endosomes containing EVs move along microtubules to fuse with the plasma membrane and then release their microvesicles [14].

In the central nervous system (CNS), neurons, microglia, astrocytes and oligodendrocytes secrete microvesicles into the extracellular environment. Exosomes have been isolated from primary cultured neurons in vitro [15, 16].

EVs with different sizes, contents and from different sources can freely move through extracellular medium and are frequently found in diverse corporal fluids. EVs have been detected in the blood [17], urine [18], sweat [19], interstitial liquid, lung fluid [20], semen [21], colostrum [22] and saliva [23]. Notably, EV contents in the blood of cancer patients have been used as an indicator of metastasis [7]. The encapsulation of molecules in EVs enhances the protection against degradation and dilution in the extracellular space, allowing long-distance delivery through the bloodstream or interstitial fluid [17].

Interaction of EVs with target cells under physiological conditions is not well understood. Most of the empirical evidence has arisen from in vitro studies. According to recent data, EV functions may be executed in three distinct modes of action: (i) internalisation by target cells and cargo retrieval, (ii) binding to the cell surface and triggering second messenger pathways and (iii) releasing the components into the extracellular matrix [24].

Neuronal EVs are predominantly distributed within the somatodendritic compartment, where they are 50 times more abundant than in the axons [25]. It is well known that EVs can transport proteins and lipids [26]. It has been recently discovered that EVs could shuttle noncoding nucleic acids such as genomic DNAs [27], ribosomal RNAs (rRNAs), circular RNAs (circRNAs), long noncoding RNAs (lnRNAs) and microRNAs (miRNAs) [28].

Several research groups have shown a close relationship between apoptosis process and the release of exosomes-containing miRNA [29, 30]. Studies about adipose tissue-derived from MSCs characterised the mRNA and miRNA cargo of EVs. Factors involved in functions associated with alternative splicing, apoptosis, and chromosome organization were found in released EVs. Furthermore, four miRNAs that target transcription factors, as well as genes that participate in several cellular pathways, including apoptosis and proteolysis were also described [31].

It was recently proposed that some of the miRNAs are expressed at higher levels in the exosomes than in the cells. In fact, around 30 % of released miRNAs do not reflect the pool of miRNAs in the source cell, suggesting that miRNA is not distributed randomly and particular sequences are selected to occupy a specific cellular microenvironment [32, 33].

Control of miRNA Specificity: New Players on the Block

miRNAs are small noncoding RNAs of approximately 18–21 nucleotides. They are important post-transcriptional regulators of gene expression, acting at the level of mRNA, usually promoting its destabilization or decreasing the translation rate [34–36]. These short oligonucleotides are evolutionarily well conserved and are involved in many aspects of the biology of metazoans, from viral infection and replication [37] to cell proliferation, differentiation [38] and apoptosis [39]. The number of miRNAs encoded in the genomes varies from a few to around a thousand in mammals [40, 41]. Computational predictions and genome-wide identification of miRNA targets estimate that each miRNA regulates hundreds of different mRNAs, suggesting that approximately 50 % of the human transcriptome is subject to miRNA regulation [42, 43]. Most miRNAs are processed from longer hairpin transcripts by the consecutive actions of the RNase III-like enzymes Drosha and Dicer [44]. One strand of the hairpin duplex is loaded into an Argonaute-family protein to form the core of miRNA-induced silencing complexes (RISCs). RISCs silence the expression of target genes, predominantly at the post-transcriptional level [43–45].

The specificity of miRNAs towards mRNAs depends on the concentrations of both molecule types [46]. The copy number of a particular miRNA depends not only on the biosynthesis level, but also on the balance of stability and degradation. Some recent studies have described the participation of an atypical RNA polymerase PAPD4 and exoribonuclease XRN2 [47–50] in miRNA stability and degradation, respectively.

It has been suggested that miRNAs move between cells of the same organism via gap junction channels [51–53], exosomes [32, 54], apoptotic bodies [55] and in the synaptic cleft, coupled to the enzyme Argonaute 2 [56]. Migrating miRNAs are apparently stable and retain their activity in the target cells [57]. Figure 1 reviews the general mechanism of miRNA formation, maturation and uptake into exosomes.

Defective biogenesis or function of miRNAs have been identified under various physiological and pathological conditions, e.g., in neurodegeneration and autoimmunity disorders [58]. Several miRNAs are considered to belong to a newly defined class of mediators of inflammation [59, 60]. A correlation between miRNA-146a levels and the regulation of Toll-like and interleukin-1 receptor signalling and the consequent impact on immunity has been reported; it supports this hypothesis [61, 62].

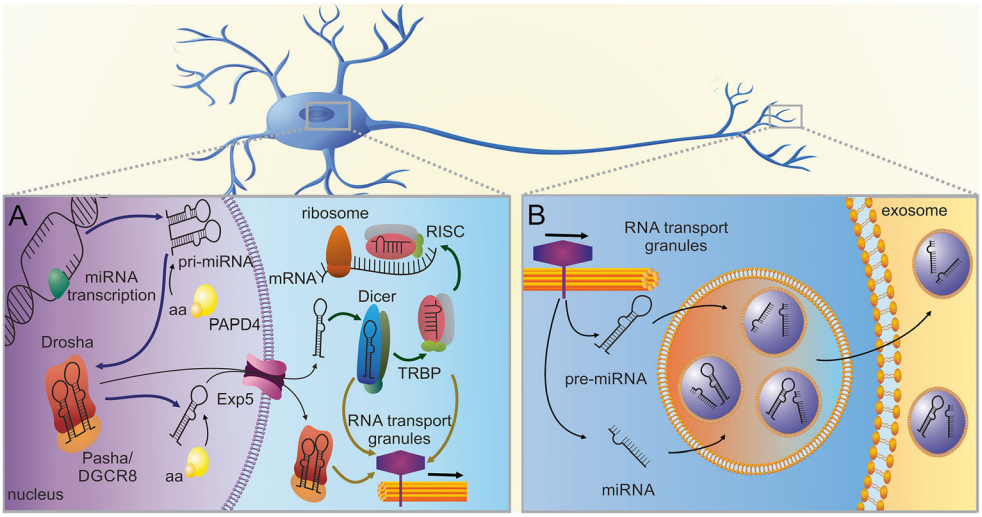

Fig. 1 microRNA (miRNA) biogenesis pathway and exosome uptake. **a** miRNAs are generated when primary miRNAs (pri-miRNA) are transcribed by RNA polymerase II and cleaved by microprocessor (*blue arrows*), a multi-protein complex formed by Drosha and Pasha/DGCR8. This process generates a hairpin structure with approximately 70 nucleotides, known as pre-miRNA. Within neuronal nuclei, pri- and pre-miRNA may be stabilized by 3′-terminal adenylation performed by PAPD4. Exportin 5 transports both pri- and pre-miRNAs to the cytoplasm. In the cytoplasm of the neuronal soma, pre-miRNA is cleaved by Dicer, producing an RNA duplex whose strands are separated, and one of them is incorporated into the RNA-induced silencing complex (RISC, *green arrows*). **b** Alternatively, pri-miRNAs and miRNA processing proteins, such as Drosha and DGCR8/Pasha, may be assembled with proteins of RNA transport granules. These molecules are then transported to specific neuronal compartments, where mature or precursor miRNAs are enveloped in vesicles or exosomes to be released elsewhere

New Insights Into TLR Pathways and Their Activation

Several roles of TLRs have recently been postulated. These receptors are classified as type I membrane-glycoproteins, mediating adaptive immune responses in the defence against pathogens [63–65]. The *Toll* gene was first described in *Drosophila melanogaster* [66]. Since then, 13 members of the TLR family have been described in mice and 11 in humans [67, 68]. As illustrated in Fig. 2, TLRs1-2, TLRs4-6 and TLRs11-13 proteins are localized on the cell surface, whereas TLR3 and TLRs 7–9 accumulate in the endosome or lysosome compartments and in the endoplasmic reticulum (ER) as shown in Fig. 3 [69]. Several cell types related to the immune system express TLRs, such as B-lymphocytes [70], mast cells [71], natural killer cells [72], T-lymphocytes [73], macrophages, monocytes, neutrophils [74], basophils and epithelial [75] and endothelial cells [76].

During the last decade, these receptors were found in different neural cells. Protein profiles for TLRs 3, 4, 7 and 9 were documented in human neuronal cells [77, 78], whereas TLRs 2–4, 6–8 and 11–13 were detected in murine neurons [78–81]. TLR2 protein has been detected only in human oligodendrocytes [82]; however, expression of TLRs 2–4 has been reported in murine oligodendrocytes [83–85]. Human astrocytes show TLR3- and TLR4-specific protein expression [86, 87], whereas TLRs2–5 and TLR9 have been detected in murine astrocytes [85, 88, 89]. Human microglia expresses TLR1–4 proteins [86, 87, 90], and murine microglia expresses TLR2, 4 and TLR9 proteins [83, 91, 92].

The extracellular domain of TLRs contains leucine-rich repeat motif that recognises conserved pathogen-associated molecular patterns (PAMPs) of a broad spectrum of infectious agents such as bacteria, viruses, yeasts, fungi and parasites [63]. TLR1 and TLR6 form heterodimers with TLR2, which can discriminate between triacylated and diacylated lipoproteins. TLR2 and TLR4 also form oligomers which interact with microbial motifs like peptidoglycan (PGN), lipoproteins and lipopolysaccharide (LPS) [93]. TLR5 is known for sensing flagella of motile bacterial species. TLRs 3 and 7–9 recognise intracellular pathogen-derived nucleic acid motifs, double-stranded RNA (dsRNA), single-stranded RNA (ssRNA) and DNA delivered to the intracellular compartments after the uptake of viruses, other pathogens or infected cells [94]. TLR9 recognises non-methylated CpG motifs of bacterial and viral DNA; TLR11 respond to pathogenic bacteria such as uropathogenic *E. coli*, as well as a profilin-like protein from the parasite *T. gondii*. However, respective PAMPs for TLR10, 12 and 13 are still unknown [95–97].

Apart from PAMP detection, recently reported evidence has disclosed that another class of molecules may trigger TLRs. TLRs in the CNS are activated by endogenous ligands released by necrotic cells in injured or stressed tissues [98, 99]. Some of these released molecules act as pro-inflammatory factors, and are also known as damage-associated molecular patterns (DAMPs). β-defensin 2, heat shock protein (HSP)

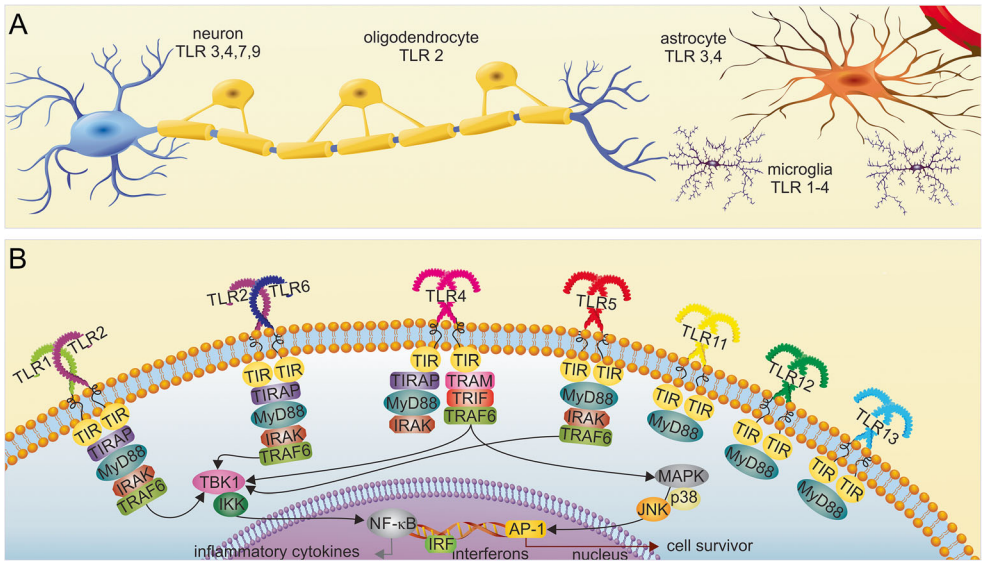

Fig. 2 Neural cell types and their Toll-like receptor (TLR) expression. **a** Different nervous system resident cells express Toll-like receptors. Protein profile for TLR 3, 4, 7 and 9 has been documented in different neural phenotypes from humans, whereas protein profile for TLRs 2–4, 6–8 and 11–13 has been reported for murine neurons. In human oligodendrocytes, only TLR2 protein is detected; however, TLRs 2–4 are found in murine oligodendrocytes. Human astrocytes show TLRs 3–4 protein accumulation, whereas TLRs 2–5 and 9 are detected in murine astrocytes. Human microglia contains TLR1–4, whereas murine microglia has a specific TLR2, TLR4 and TLR9 protein profile. **b** In the cellular membrane, TLR1/TLR2 and TLR2/TLR6 form oligomers and are associated with adapter proteins containing Toll-interleukin-1 receptor (*TIR*) domain. TLRs activate protein adapters such as TIR-domain-containing adapter protein (*TIRAP*), myeloid-differentiation primary response gene 88 (*MyD88*) and, consequently, interleukin-1 receptor-associated kinase (*IRAK*). TRLs also activate TNF receptor-associated factor (*TRAF*)-6 adapters, leading to the activation of TRAF-family-member-associated nuclear factor-KB (NF-κβ) activator (TANK)-binding kinase-1 (*TBK-1*) and Iκ-B kinase (IKK), ending with the activation of NF-κβ and release of cytokines. TLR4 forms oligomers with another TLR4 and is associated with TIRAP, MyD88 and IRAK proteins or translocating chain-associated membrane protein (*TRAM*), TIR-domain-containing adapter-inducing interferon-β (*TRIF*) and TRAF6, to activate the NF-κβ pathway or the map kinase (*MAPK*) pathway via p38 and c-Jun N-terminal kinase (*JNK*), leading to activation of neuroprotective transcription factors (*AP-1*). TLRs 5, 11, 12, and 13 form homo-oligomers. Their specific signalling pathways have not been determined

60, HSP70, high-mobility group protein B1 (HMGB1), oxygen radicals and urate crystals are considered DAMPs for associated TLR1/2 and/or TLR2/6, the TLR proteins that form oligomers [100]. The ssRNA acts as DAMP for TLR3 [101]. Similarly, β-defensin 2, HSP60, HSP70, HSP72, HMGB1, fibrinogen/fibrin, surfactant protein, minimally modified LDL (cholesterol) and pancreatic elastase activate TLR4. An RNA-immune complex was identified as DAMP for TLR7 and 8, whereas CpG chromatin-IgG complexes, the DNA immune complexes, are possible ligands for TLR9 [101–104]. The association of TLRs with their specific PAMPs or DAMPs leads to receptor activation and initiation of the cascade of intracellular signalling, culminating with NF-κβ activation and changes in gene expression.

Several adapter proteins containing Toll-interleukin-1 (TIR) domain associate with TLRs when activated. Most of the TLRs are coupled with myeloid-differentiation factor 88 (MyD88), similar to MyD88 adapter. TLR3 is an exception; it is the only TLR coupled with an adapter-inducing IFNβ of the TIR domain (TRIF) [105, 106]. The binding of these proteins triggers the signalling cascade that leads to activation of nuclear factor kappaβ (with NF-κβ). As a result, genes encoding pro-inflammatory tumour cytokines, such as tumour necrosis factor (TNF), interleukin 1 (IL-1), IL-6, IL-8, IL-12 and chemokines, are overexpressed. Although cytokine production is critical for host defence, it can also lead to irreversible tissue damage [107].

Some new data suggest that miRNAs regulate the TLR-signalling pathway at several steps, including the regulation of TLR mRNA expression, direct activation of the receptor, binding to TLR or TLR-specific signalling pathway components and TLR-induced transcription factors and functional cytokines [97, 101–104, 108].

Since miRNAs are short single-stranded RNA molecules, they can mimic viral RNA, and consequently, bind directly to TLRs. It has been reported that in the immune system, the natural killer cells (NK) can detect miRNAs via TLR1 activation [109, 110]. Specific miRNA sequences in miR-122 and miR-15b have been identified as ligands of TLR1 that can activate the transcription factor NF-κβ. The adapter proteins interleukin-1 receptor-associated kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6) are important components of the myeloid-differentiation primary response gene (MYD88)-dependent pathway. MYD88 is an adapter protein used by almost all TLRs (except TLR3) to activate NF-κβ.

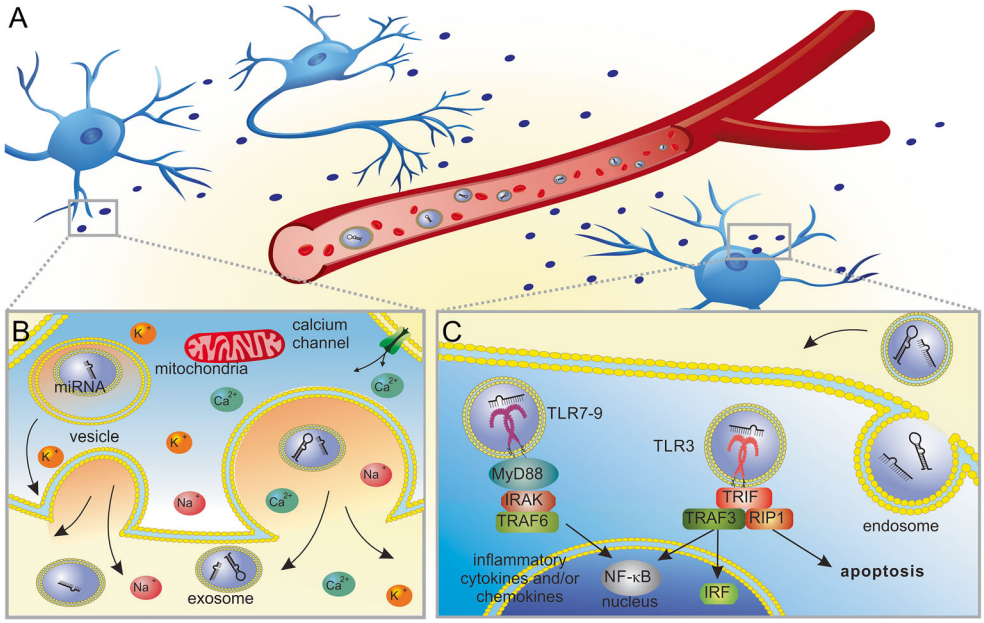

Fig. 3 Long-distance cell–cell communication: microRNAs (miRNAs) and Toll-like receptors (TLRs). **a** Neurons and glial cells can release exosomes to the extracellular space. These exosomes could shuttle proteins and miRNAs for long distances via the blood vessels or act in the neighbouring cells. **b** In both types of cells, miRNAs are previously enveloped in exosomes or vesicles in order to be released. **c** When the vesicles fuse with the cell membrane, their content binds to the endosome TLRs. TLR3, TLR7, TLR8 and TLR9 oligomerise with the same receptors. TLRs 7–9 couple with myeloid-differentiation primary response gene 88 (*MyD88*), which activates interleukin-1 receptor-associated kinase (*IRAK*) and TNF receptor-associated factor (*TRAF6*). These processes culminate in the activation of the nuclear factor-κβ (*NF-κβ*). The TLR3 is coupled with TIR-domain-containing adapter-inducing interferon-β (*TRIF*), which activates TRAF3 and receptor-interacting serine-threonine kinase RIP1 protein, leading to apoptosis. Our hypothesis is that these TLRs could recognise mature miRNAs, triggering inflammatory signalling under various conditions, including neurogenesis and diseases

IRAK1 and TRAF6 are also targets of miR-146. Taganov et al. have suggested that miR-146 downregulates the signalling pathway MyD88/NF-κβ after microbial infection [61, 111]. miR-155 controls the expression of inhibitor of NF-κβ kinase subunits beta (IKKβ) and epsilon (IKK), reducing NF-κβ activity [112].

However, it has been recently discovered that TLRs 7–9 recognise specific miRNAs as agonists in the CNS. For example, miRNA let-7 is an abundant regulator of gene expression, highly expressed in microglia cells and in neurons, which interacts with TLRs [29]. miRNA-21 and 29a have been also described as agonists of TLRs 7–8 in rat and human macrophages. The binding of these miRNAs to TLRs induces the secretion of TNF-α and IL-6, leading to the activation of NF-κβ signalling and secretion of pro-inflammatory cytokines [113]. Besides secretion of cytokines, the regulation of TLR signalling by miRNAs occurs at different levels. Various molecules involved in the TLR pathway are targeted, such as TLR-signalling molecules, TLR-induced transcription factors, regulators of the TLR-signalling pathway and the expression of TLRs themselves [97, 114].

Considering the role of TLRs and assuming that exosomes carry miRNAs, we can hypothesise that miRNAs are signalling molecules with important functions in NS diseases (Fig. 3) [115].

miRNAs Activating TLRs in Neurological Diseases

Neurological diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), are characterised by neuronal cell loss. These diseases are expected to become more common due to extended life expectancy. Despite significant research efforts, the primary causes of neurodegeneration remain largely unknown. It has been recognised that these disorders emerge as a result of different genetic programming and environmental influences [116].

miRNAs have been associated with pathological alterations during the course of many neurological diseases, including AD, PD, ALS and stroke, suggesting that miRNAs may be a contributing factor in neurodegeneration [116]. It has been recently reported that miRNA levels are altered in the blood of AD, PD, ALS and stroke patients. These small RNAs may be used as biomarkers to enable an early diagnosis and identify new therapeutic targets [117].

It is not clear whether inflammation in the CNS contributes to the progress of neurological diseases. However, increasing evidence highlights the participation of TLR-dependent pathways in neuronal diseases [118]. Neuroinflammation is observed as consequences of trauma, infections, tumours and neurodegenerative diseases and involves microglia, pericytes and reactive astrocytes as well as T-lymphocytes,

macrophages and dendritic cells crossing the brain-blood barrier, which is damaged in the inflamed brain (reviewed in [119]). Innate immunity providing an onset of the inflammatory response involves the actions of TLRs and the liberation of pro-inflammatory cytokines. Short neuroinflammatory responses are considered to be neuroprotective and may contribute neuronal development; however, when persisting they result in neurodegeneration [120]. In this regard, crucial functions may be attributed to endogenous miRNAs as ligands of TLR-promotion of neuroinflammation, as these are responsible for fine-tuning activity levels of TLRs and subsequent kinetics of innate immune response.

In agreement with the hypothesis of a chronic neuroinflammatory process, the involvement of TLR activation has been documented in AD [29, 121], PD [121, 122], ALS [123] and stroke [124]. The analysis of EVs is now an increasingly popular topic in the field of neurodegeneration; these vesicles may transport pathogenic proteins such as alpha-synuclein (α-syn) and amyloid precursor protein (APP) that are involved in PD and AD, respectively.

Alzheimer's Disease

AD is the most common cause of dementia in the modern world [125]. The main characteristics of the disease are the accumulation of extracellular senile plaques (composed of amyloid-β peptide, Aβ), intracellular neurofibrillary tangles (NFTs) containing hyperphosphorylated tau protein, activated microglia, astrocytes and degenerating neurons [126].

Several appraisals of AD pathogenesis have revealed that the catabolism of APP occurs in the endosome; the pathogenic proteins, such as Aβ and tau, are secreted from the exosomes into the extracellular space [127–129]. TLR2, 4 and 9 are overexpressed in an animal model of AD [130]. These receptors could be activated by Aβ as they mediate the microglial inflammatory response and are associated with Aβ-plaque clearance from the brain [131–133].

Studies using blood samples from AD patients have identified 60 miRNAs differentially expressed in these patients in comparison with healthy individuals [134, 135]. miR-191 has a regulatory role in cellular processes such as cell proliferation, differentiation, apoptosis and migration; it targets important transcription factors, chromatin remodellers and cell cycle-associated genes [136]. It is likely that this miRNA is a key player in the initiation and progression of several diseases.

Type III RNase Dicer enzyme is responsible for the maturation of miRNA. Aberrant expression or malfunction of this regulator in adult forebrain impairs the expression of several miRNAs, ultimately causing pathological hyperphosphorylation of NFT-forming tau protein, leading to neuronal death [137].

The levels of miRNA let-7 are enhanced in AD patients. It has been suggested that let-7 activates the RNA-sensing TLR7, and thus, induces neurodegeneration in these patients [29]. The results of experiments with TLR7-KO mice have shown that these mice are resistant to neurodegenerative factors [29]. It is not clear how the let-7 miRNA reaches the endosome TLR7 receptor in the CNS. However, studies of the metastatic gastric cancer have revealed that let-7 miRNA is secreted into the extracellular environment via exosomal transport [133].

Inflammation has been held responsible for many neurological diseases as it increases cell damage and causes neuronal death. Further studies of the receptors associated with these processes and molecules triggering the inflammation are necessary to understand these serious disorders.

Parkinson's Disease

PD is characterised by a selective degeneration of dopaminergic neurons in the *substantia nigra pars compacta* (SNpc) with various symptoms affecting the motor system such as tremor, stiffness, bradykinesia and postural instability [138]. The cellular hallmark of PD is the accumulation of proteinaceous intracellular inclusions termed Lewy bodies (LB), primarily composed of fibrillar alpha-synuclein (α-syn) and ubiquitinated proteins, in the surviving neurons [139].

The aggregation of α-syn activates microglia, increasing dopaminergic neurotoxicity [140, 141]. However, the precise molecular mechanism of the process is still unclear. Increased secretion of exosomes is one mechanism for α-syn action. These activated exosomes express a high level of major histocompatibility complex (MHC) II and TNF-α, which then promote apoptosis in the recipient cells [142]. α-syn can also be encapsulated in exosomes released by neuroblastoma and cause neuronal cell death [129].

Some cancer studies report that protein-transporting exosomes can also transport miRNAs [117]. The levels of miR-205, miR-184 and let-7 are correlated with the expression of α-syn and leucine-rich repeat kinase2 (LRRK2), coded by the two main genes associated with PD [143]. A recent report has also indicated that let-7 represses the expression of α-syn and is downregulated in PD models [144]. Increasing evidence suggests the existence of a close relationship between PD and TLRs. It has been recently shown that extracellular α-syn increases the expression of TLR1, TLR2, TLR3 and TLR7 [145, 146].

Recent studies have described TLR2 as an endogenous receptor for α-syn that is released from damaged neurons, responsible for microglial activation observed in PD [121]. However, TLR4-KO mice are less vulnerable to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) intoxication than wild-type mice. After MPTP administration, these TLR4-

KO animals also have fewer ionised calcium-binding adaptor molecules 1 (Iba1)$^{+}$ and MHC II^{+} activated microglial cells and lower levels of microglia/macrophage-specific calcium-binding protein. These results suggest that the TLR4 pathway is involved in PD [108].

The available experimental evidence points to a close relationship between EVs release, miRNA and TLR signalling in PD. However, further studies in this area should be conducted to clarify the specific roles of these molecules in this disease.

Amyotrophic Lateral Sclerosis

ALS is a chronic neurodegenerative disease, characterised by progressive loss of motor neurons, leading to muscle atrophy, paralysis and death usually within 3 to 5 years after diagnosis [147]. Several studies have demonstrated the involvement of non-neuronal cells in ALS pathogenesis, including microglia and astrocytes, increasing the release of superoxide dismutase 1 (SOD1), nitrate and nitrite [148].

SOD1 is secreted via exosomes from mouse motor neuron-like (NSC-34) cells overexpressing the wild-type and a mutant enzyme, used as in vitro model for ALS [149]. It has been demonstrated that exosomes cargo may include several different classes of molecules [32]. As we have previously mentioned, in addition to SOD1, exosomes may transport miRNAs. Several miRNAs such as miR-146b, miR-29b, let-7a/b, miR-27b, miR-21, miR-210 and miR-155 have their expression upregulated in ALS [150, 151]. Furthermore, the levels of miR-9 are enhanced in this disease in the ventral horn of the spinal cord, the locus of neurodegeneration [152]. Among those miRNAs, miR-155, miR-146b and miR-125b are typical components of the innate immune system, and most of them converge in NFKB-mediated immune cell response [151].

The aetiology and pathogenesis of ALS still remain unclear, although available evidence suggests that inflammation plays a critical role in this process [153]. Studies of high expression of SOD1 in mice have shown elevated levels of TLR1, 2, 7 and 9 [123]. TLR2 and TLR4 gene expression levels are upregulated in ALS patients. TLR2 is predominantly detected in the microglia, whereas the TLR4 is strongly expressed in astrocytes. The activation of TLRs may contribute to the progression of inflammation and can explain the resultant motor neuron injury in ALS [154]. A study using combined inhibitory antibodies against TLR2 and TLR4 has shown significant microglial suppression [155].

An effective therapy for this disease is still undiscovered. However, the results showing that in ALS patients both, neuronal and non-neuronal cells, release EVs concomitantly with the activation of TLRs add to our knowledge of ALS and immune responses.

Stroke

Stroke is one of the most common causes of adult disability, and its prevalence augments with ageing population, despite the advances in prevention and acute interventions [156]. Stroke injury mechanisms include the excitotoxicity, mitochondrial dysfunction, oxidative stress [157] and inflammation [158].

Molecular chaperones and some members of the Bcl-2 family (apoptosis regulatory proteins) that protect mitochondrial function have been suggested as miRNA targets [157]. miRNA expression following stroke and other types of hypoxia-ischemia/reperfusion injuries varies regionally and temporally. The regional distribution of miR-181 and miR-121 differs depending on the distribution of blood flow [157].

Altered expression of several miRNAs (miR-140, miR-145 and miR-331) has been reported 3 days after ischemia/reperfusion; a progressive increase in the levels of miRNAs has been observed 3 h following reperfusion [159]. miR-200b, miR200c and miR-429 are elevated after 3 h of reperfusion in a model of ischemic preconditioning [160]. In a rat model of stroke, the levels of miR-290 [161], miR-10a, miR-182, miR-200b and miR-298 [162] increase in the blood and brain 24 h after ischemia/reperfusion; increased plasma levels of miR-124 are observed 6 h after reperfusion [163]. The level of miR-210, known as the major hypoxia-inducible miRNA or hypoxamir [164], is positively correlated with improved prognosis in stroke patients [165].

miRNAs are differentially expressed in the blood of patients with acute ischemic stroke; the levels of miR-122, miR-148a, let-7i, miR-19a, miR-320d and miR-4429 decrease, whereas miR-363 and miR-487b levels increase. These miRNAs are predicted to regulate several genes in pathways previously identified by gene expression analyses, including TLR signalling and NF-κβ signalling [158]. Several of these miRNAs have a known biological function. miRNA let-7 regulates TLR signalling in monocytes and modulates the differentiation of dendritic cells [166]. miR-122 regulates the expression of peroxiredoxin 2, a DAMP involved in immune activation after stroke [167]. miR-148 fine-tunes the immune response by altering cytokine production (IL6, TNF-a, IL-12, TNFSF7) [162, 168], although their biological effects in neuronal cells are unknown.

Studies focusing on stroke therapies with multipotent mesenchymal stromal cells (MSCs) have reported that these cells can release exosomes-containing miR-133b. These exosomes are transferred to the adjacent astrocytes and neurons, where they regulate gene expression, with subsequent benefits for neurites remodelling and functional recovery after stroke [169]. However, several studies have indicated the participation of TLRs in stroke [170, 171]. TLR9 gene expression is upregulated in ischemia-neuronal damage and may play a critical role in the induction of inflammatory response and

apoptosis [172, 173]. Studies using TLR7 and TLR9 preconditioned with unmethylated cytosine-phosphate-guanine rich oligonucleotide (CpG) have shown some neuroprotective effects [174].

Other reports reveal a significant increase in TLR8 gene expression 6 h post-ischemia. The levels of pro-inflammatory cytokines such as IL-6 and IL-1β also change along with TLR8 levels. Treatment with a TLR8 agonist activates pro-apoptotic c-Jun N-terminal kinases (JNK) and increases neuronal cell death after stroke [80].

TLR2 and TLR4 gene expression is also upregulated under the stress or damage conditions such as ischemia or hypoxia [172, 173]. These oligomerised receptors can detect dangerous proteins like HSP and low molecular weight hyaluronan. HMGB1 and fibrin/fibrinogen are predominantly detected by TLR4 [175]. Studies using LPS for preconditioning have found that it re-programmes the cellular response (through activation of its receptor TLR4), possibly reflecting the endogenous processes that protect the brain against additional injury [176]. Following a cerebral focal ischemia injury, TLR2- and TLR4-KO mice have smaller infarcts than wild-type animals [177, 178]. miR-19b negatively regulates inflammation in humans and activates the expression of TLR2 and TLR4, promoting the inflammatory response in ischemic stroke [24, 25]. In neonatal hypoxic-ischemic (HI) mice brain, the activation of TLR3 can increase susceptibility to injury [124]. It is now widely accepted that miRNAs activate TLRs in the immune system. However, more studies are needed to determine the mechanisms of their action in the neuronal cells. We also need to confirm the relationship between EVs and the transport of these miRNAs in stroke.

Conclusions and Future Directions

In cancer research, EVs have been considered important biomarkers for the detection of metastases. The information transfer by EVs may constitute a novel mechanism of intercellular shuttling of molecules related to apoptosis. It is possible that EVs have similar roles in different systems, especially in the nervous system. The recent discovery of the ability of exosomes-containing miRNAs to reach TLRs in the endosomes of surrounding cells offers a new insight into various regulation mechanisms employed under physiological condition and in disease.

Investigation of the possible relationships between exosomes, miRNAs and TLRs in the nervous systems is still in its infancy. However, we can hypothesise that miRNAs entering the cells via exosomes may regulate the activation of TLRs. Furthermore, TLR tolerance, a hyporesponsive state of the receptor, characterised by reprogramming of TLR-mediated signal transduction [179], may achieved by intracellular delivery of miRNA using exosomes. Positive effects based on TLR tolerance have been observed in an animal model of stroke. If this hypothesis is confirmed, it will provide a new insight into the regulation of TLRs and new therapeutic strategies for CNS inflammation-related diseases.

A recent study has demonstrated an effective delivery of functional siRNA into mouse brain by systemic injection of exosomes [180]. Systemic exosome administration could be an alternative way to deliver the active components of cell-based therapy to the CNS [181]. Further detailed investigation of cellular communications mediated by EVs holds great promise for drug delivery and interference-RNA applications.

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