Symposium

Extracellular Vesicle-Mediated Neuron–Glia Communications in the Central Nervous System

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Communication between neurons and glia significantly influences the development maturation, plasticity, and disease progressions of the nervous system. As a new signaling modality, extracellular vesicles display a diverse role for robust functional regulation of neurons through their protein and nucleic acid cargoes. This review highlights recent breakthroughs in the research of signaling mechanisms between glia and neurons mediated by extracellular vesicles that are important for neural development, axonal maintenance, synaptic functions, and disease progression in the mammalian nervous system. We will discuss the biological roles of extracellular vesicles released from neurons, astroglia, microglia, and oligodendroglia in the nervous system and their implications in neurodegenerative disorders.

Key words: **Alzheimer's disease; astrocytes; axonal integrity; extracellular vesicles; intercellular communication; microglia; multivesicular bodies; neurodegenerative disorders; oligodendrocytes; synaptic transmission; tauopathy**

Introduction

The mammalian central nervous system (CNS) is composed of highly heterogeneous neurons and glial cells. The molecular understanding of complex signaling between neurons and glial cells through the secretion of molecules and cell surface contacts is of crucial importance for understanding the development of the CNS, its functions, and the pathogenesis of CNS diseases. In the past decade, extracellular vesicle-mediated communication between neurons and glia has gained attention but our understanding of the transferred cargo, which includes all types of biomolecules or even organelles, is limited. Recent breakthroughs in genetic tools and multiomic databases advanced our understanding of the molecular composition of extracellular vesicles (EVs) and their roles in neuron–glia communication. Cross talk between cells within the CNS via EV exchange has been shown to be critically involved in essential biological functions including brain development, neural circuitry maturation, and innate immune response (for review see Kramer-Albers and Hill, 2016; Schnatz et al., 2021). This communication is in part mediated by EV cargo contents and occurs in an omnidirectional manner, where all cell types communicate with and influence one another (Fig. 1).

EVs were commonly defined by the size of vesicles as exosomes (30–150 nm in diameter) originating from intracellular multivesicular bodies (MVBs), microvesicles (MVs, 100 nm to 1 μm in diameter) originating from the plasma membrane, and apoptotic bodies (0.8–5 μm in diameter) originating from apoptotic cells (Colombo et al., 2014; DeLeo and Ikezu, 2018). Since the size does not correctly reflect the origin of EVs and EVs below 100 nm can also originate from the plasma membrane, EVs are generally categorized as small (<150 nm) or large EVs (>150 nm), as recently indicated in the guideline Minimal information for studies of extracellular vesicles 2023 (MISEV2023) from the International Society for Extracellular Vesicles (ISEV; Welsh et al., 2024). The diverse EV cargo molecules include lipids, nucleic acids, and proteins. The composition of these EV cargo molecules is different depending on the cell type (Nazarenko et al., 2010), the state of the cell (Eldh et al., 2010; Carayon et al., 2011; de Jong et al., 2012), and the age (Pusic et al., 2014), making them an attractive reference for the condition of the secreting cells, tissues, and individuals.

The biogenesis of smaller EVs including exosomes and microvesicles are mainly regulated by two pathways: the endosomal sorting complexes required for transport (ESCRT) machinery and ceramide-dependent biogenesis. The ESCRT machinery mediates budding processes away from the cytoplasm in a stepwise manner from ESCRT-0 to III (Hurley, 2010; van Niel et al., 2018) and hence the formation of intraluminal vesicles (ILVs) as well as shedding of microvesicles at the plasma membrane. In terms of ceramide-dependent biogenesis, Trajkovic et al. first showed biogenesis of small EVs derived from an oligodendrocyte

Received June 19, 2024; revised July 17, 2024; accepted July 24, 2024.

Author contributions: T.I., Y.Y., C.V., and E.-M.K.-A. wrote the paper.

Figures were created using Biorender.com. We thank Martina Gabrielli for support with Figure 3. T.I. is supported by National Institutes of Health (NIH) grants R01AG066429, R01AG067763, RF1AG082704, and RF1AG054199. Y.Y. is supported by NIH grants R01NS125490, R01AG078728, and R01NS118747. E.-M.K.-A. is supported by Deutsche Forschungsgemeinschaft (DFG) grants KR 3668/1-2, KR 3668/2-2, and the Leibniz ScienceCampus NanoBrain. C.V. is supported by Horizon 2020 Framework Program of the European Union (grant agreement no. 874721/PREMSTEM) and PRIN 2022-3CHCY. Illustrations were created using BioRender.

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The authors declare no competing financial interests.

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https://doi.org/10.1523/JNEUROSCI.1170-24.2024

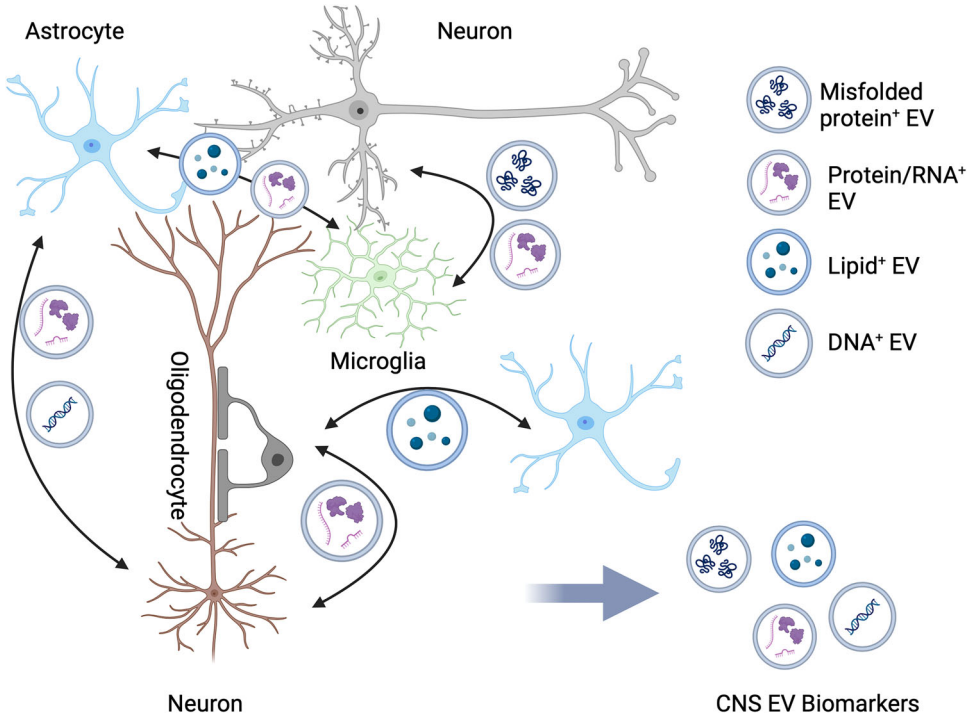

Figure 1. Intercellular communication of CNS cells via EVs. EVs carry multiple species of molecules, including lipids, nucleic acids (DNA and RNA) and proteins including misfolded proteins. EV-mediated transfer of proteins and nucleic acids has been shown to occur between neurons and astroglia, microglia and oligodendrocytes, and misfolded proteins between neurons and microglia to neurons. Arrows indicate distinct EV-mediated signaling pathways between CNS cells. The CNS EV cargo is representative of the donor cell and may be detected as biomarker if carried to peripheral biofluids, assisting decoding of disease states and mechanisms.

cell line is dependent on neutral sphingomyelinase 2 (nSMase2), as its chemical inhibition reduced the secretion of exosomes derived from MVBs (Trajkovic et al., 2008). nSMase2 also controls constitutive EV budding from the plasma membrane (Menck et al., 2017). The trafficking of MVBs and release of small EVs by fusion of MVBs with the plasma membrane is largely mediated by Rab27a and Rab27b (Ostrowski et al., 2010). Targeting of either molecule is effective in suppression of EV secretion from CNS cells, including neurons (Iguchi et al., 2016; Song et al., 2019) and astrocytes (Neckles et al., 2019). In oligodendrocytes, MVB fusion and exosome release are dependent on Rab35 (Hsu et al., 2010, Mukherjee et al., 2020). Large EVs budding outward from the plasma membrane also use the ESCRT complex (Nabhan et al., 2012). In addition, their release is regulated by the small GTPase ARF6 (Sedgwick et al., 2015). Apoptotic bodies represent a subclass of microvesicles, which mainly fall into the category of large EVs. Their biogenesis involves apoptotic signaling and activation of Rho-associated coiled-coil–containing protein kinase 1 (Rock1; Coleman et al., 2001).

More recent studies indicate that EV biogenesis and the generated EV subtypes are even more diverse. EVs can be derived from mitochondria (D'Acunzo et al., 2021), secretory lysosomes, and amphisomes (Buratta et al., 2020; Ganesan and Cai, 2021) or even contain complete organelles such as functional mitochondria (Peruzzotti-Jametti et al., 2021). Intriguingly, the machinery regulating autophagic flux can regulate EV cargo loading and secretion (Leidal et al., 2020). These findings indicate that EV biogenesis and release is linked to the autophagic system.

Although the basic machinery of EV biogenesis is ubiquitous, some factors regulating EV release may be tissue dependent and specific to the nervous system (e.g., Rab35 regulation of EV release in oligodendrocytes; Hsu et al., 2010; Verweij et al., 2022). Using genome-wide shRNA library screening of murine microglial tdTomato-CD63 reporter cell line, *Sepp1*, *Mcfd2*, and *Sdc1* were identified as the regulators of ATP stimulation-induced EV secretion from microglia (Ruan et al., 2022). *Sepp1* encodes selenoprotein P and is exported from the liver in exosomes via its interaction with apolipoprotein E (ApoE; Jin et al., 2020). Since ApoE is a known marker of disease-associated/neurodegenerative microglia (Krasemann et al., 2017), which enhance EV secretion (Clayton et al., 2021), Sepp1 may regulate ApoE-containing EV secretion from activated microglia. The application of a spectrum of similar technical tools will be important in the future to determine the involvement of EVs in intercellular communication in the CNS network.

The internalization mechanisms of EVs include direct fusion to the plasma membrane (Bonsergent et al., 2021), clathrin-dependent or clathrin-independent endocytosis, and micropinocytosis (Fitzner et al., 2011; Verdera et al., 2017). Internalized EVs colocalize with endosomes and lysosomes, and their content release is dependent on the pH (Bonsergent et al., 2021). The efficiency and the route of EV uptake are dependent on the target cell type and most likely molecules on the surface of EVs and the target cell, regulating targeting selectivity. For example, EVs from oligodendrocytes are known to transfer to microglia by macropinocytosis (Fitzner et al., 2011) and to neurons by clathrin-dependent endocytosis (Frühbeis et al., 2013); however, the molecules regulating selectivity are unknown. The route of uptake may also affect the fate of the EV cargo in target cells. In which cells EV cargo can undergo endosomal escape and how this is regulated is still a matter of debate (van Niel et al.,

2022). Much work is needed for the comprehensive understanding of the route of EV internalization and the fate of the cargo in CNS cells.

Molecular and Biological Characterization of Extracellular Vesicles from Neuronal Cells

Protein composition in CNS EVs

Among all the cargo molecules, proteins can define the unique molecular composition of the EV cargo and are critical for their specificity in host cell destination and signaling. A number of studies performed proteomic profiling of EVs enriched from brain tissues (Koul et al., 2020; Vassileff et al., 2020; Muraoka et al., 2020b,c, 2021a,b; Huang et al., 2022; You et al., 2022), cerebrospinal fluid (Chiasserini et al., 2014; Bastos et al., 2017; Guha et al., 2019; Muraoka et al., 2019; Pieragostino et al., 2019; Muraoka et al., 2020a,b), and neuronal cells (You et al., 2020; You et al., 2022). Brain-derived EVs are isolated from unfixed frozen brain tissue using mild enzymatic digestion and discontinuous sucrose gradient ultracentrifugation to remove soluble protein fractions (Vella et al., 2017). Brain-derived EVs are not expected to represent pure EVs but aim at enriching EVs from their original source and allow to compare different CNS states such as healthy and diseased (Su et al., 2021). Proteomic profiling of brain-derived EVs identified EV-specific markers as well as specific markers of neurons, oligodendrocytes, activated astrocytes (You et al., 2022), and microglia (Muraoka et al., 2021b). Astrocytic activation was represented prominently in AD brain-derived EVs, with ITGB1 being identified as EV marker correlating with AD pathology and cognitive dysfunction (You et al., 2022).

Human AD brain-derived EVs are enriched for p-tau and can propagate tau pathology after intrahippocampal injection into wild-type mice (Ruan et al., 2021). In line, elevation of p-tau, antioxidant peroxiredoxins PRDX1 and PRDX6 in AD brain-derived EVs was observed (Huang et al., 2022). In human chronic traumatic encephalopathy (CTE) brain-derived EVs, there is enrichment of pT181 tau (Muraoka et al., 2021a), which in combination with SNAP-25, PLXNA4, and UBA1, can distinguish CTE cases from control cases with >93% accuracy (Muraoka et al., 2021a). In human amyotrophic lateral sclerosis (ALS) brain-derived EVs, several RNA-binding proteins are upregulated, which may be associated with stress granule formation (Vassileff et al., 2020). Proximity-dependent biotinylation proteomics strategy identified the involvement of microtubule-associated protein 1 light chain 3 beta (LC3)-conjugation machinery for the packaging of RNA-binding proteins and noncoding RNAs for their loading to EVs and neutral sphingomyelinase 2 for EV biogenesis (Leidal et al., 2020), suggesting the LC3-dependent EV cargo loading and secretion mechanism. A more recent study reports the plasma EV 3R/4R tau and TDP-43 as diagnostic biomarkers in frontotemporal dementia and ALS (Chatterjee et al., 2024), promoting the broad applicability of plasma EV-based biomarkers for the diagnosis of neurodegenerative disorders.

CNS cell type-specific EV markers

A number of studies have used presumptive protein markers of neurons, astrocytes, microglia, and oligodendrocytes to identify and enrich CNS cell type-specific EVs in human biospecimens such as plasma and cerebrospinal fluids (Sandau et al., 2024), although none of them was identified by unbiased screening. Frequently, CD171/L1CAM was used for affinity purification of neuron-derived EVs from human biofluids (Goetzl et al., 2015) although conflicting evidence was reported (Norman et al., 2021), claiming that L1CAM in the plasma or cerebrospinal fluid is mostly detected in the soluble fraction. However, a more recent study validates the neuronal origin of plasma L1CAM$^+$ EVs, by flow cytometry as well as proteomic profiling of L1CAM$^+$ EVs (Nogueras-Ortiz et al., 2024). CNS cell type-specific markers for EVs were also identified from induced pluripotent stem cells (iPSCs) differentiated to neurons, astrocytes, microglia, and oligodendrocytes (You et al., 2022). Among those candidates, ATPase Na+/K+ transporting subunit α (ATP1A3) was identified as a neuronal EV marker, which was extensively validated in EVs isolated from human brain tissues, plasma, and cerebrospinal fluid samples (You et al., 2023). For astrocyte-derived EVs, GLAST is the most well-documented EV marker (Goetzl et al., 2016), although also expressed in neural progenitors (Regan et al., 2007). Previously used microglial markers including CD11b, CD45, and HLA-DR are commonly found in other myeloid cells. Nevertheless, EVs isolated from human brain samples by CD11b affinity capture contained the microglial markers TMEM119, purinergic receptor P2Y12 (P2RY12), and triggering receptor expressed on myeloid cells 2 (TREM2; Cohn et al., 2021). Oligodendrocyte-derived EV markers include MOG (Dutta et al., 2021), a major component of myelin sheets specific to mature oligodendrocytes. EVs isolated from primary mouse oligodendrocytes contained four myelin proteins: PLP1, 2′,3′-cyclic nucleotide 3′ phosphodiesterase phosphodiesterase (CNP), MBP, and MOG (Kramer-Albers et al., 2007). Transthyretin (TTR) has been used as a marker for choroid plexus-derived EVs (Balusu et al., 2016). Additional studies are warranted to assess the expression of these cell type-specific protein markers in biospecimens from healthy and neurologically impaired living donors, as disease states can alter the expression of some of these markers.

Biological functions of neuron-derived EV signaling to neurons and glia

Neurons release EVs upon neuronal firing (Faure et al., 2006) and glutaminergic signaling in vitro (Lachenal et al., 2011). EVs released from neurons are enriched in glutamate receptor subtypes, AMPA receptors, microtubule-associated protein 1B (MAP1B), and neurite-associated miRNAs such as miR-124 (Yang et al., 2017), suggesting a role of neuron-derived EVs as vehicles to export glutamate receptors and miRNAs which can modulate neuronal excitability and neurotransmitter release (Faure et al., 2006; Budnik et al., 2016). These neuron-derived EVs are taken up by other neurons and glial cells and can modulate the function of recipient cells in multiple ways. For example, neuronal EVs containing miRNAs (Let7c and miR-21) interact with toll-like receptor 7 (Tlr7) and restrict dendritic growth in a cell autonomous manner (Liu et al., 2015). Moreover, BDNF-treated neurons release EVs that increase the formation of excitatory synapses in hippocampal neurons and increase synchronous neuronal activity (Antoniou et al., 2023).

Neuron-derived EVs also suppress microglial activation and enhance their survival in vitro, suggesting their role in microglial homeostasis (Peng et al., 2021). This study showed that treatment of primary cultured rat microglia with neuron-derived EVs suppresses the gene expression of proinflammatory molecules induced by stimulation with lipopolysaccharide.

Neurons also release EVs containing activity-regulated cytoskeleton-associated protein (ARC) essential for long-lasting information storage. ARC contains group-specific antigen (Gag) molecular property and forms a capsid-like structure that contains

Arc mRNA, which is released in EVs and transferred to recipient neurons (Pastuzyn et al., 2018). Neurons receiving *Arc* mRNA via EVs can translate ARC an activity-dependent manner. Drosophila Arc1 (dArc) also forms capsid-like structure and transfers dArc mRNA-containing cargo in EVs across synaptic boutons (Ashley et al., 2018). A more recent study shows that dorsal root ganglion neurons transfer Arc-containing EVs to skin, regulating local inflammatory response and vasodilation (de la Pena et al., 2021).

Neuron-derived EVs can also modulate functions of astrocytes and microglia. For example, transfer of neuron-derived EVs containing miRNAs, particularly miR-124a, to astrocytes increase expression of excitatory amino acid transporter 2, a marker for mature astrocytes (Morel et al., 2013). Neuronal EVs also facilitate synaptic pruning by microglia via upregulation of complement factors (Bahrini et al., 2015).

Biological functions of glia-derived EV signaling to neurons

Astrocytes and microglia enhance maturation of neurons and vice versa via EVs (Delpech et al., 2019). Astrocytic EVs enhance neuronal differentiation and firing, suggesting their role in neural development (Chaudhuri et al., 2020; You et al., 2020). Proteomic profiling of human astrocyte-derived EVs revealed that integrins enriched in EVs after IL-1β stimulation of astrocytes were responsible for increased neuronal EV uptake (You et al., 2020). The neuritogenic role of astrocyte-derived EVs is supported by their enrichment in the synaptic molecule, synapsin-1, enhancing neurite outgrowth in vitro (Wang et al., 2011). Microglial EVs also modulate neurogenesis and synapse stability. Indeed, inflammatory microglia shed EVs containing miR-146a-5p and suppress neurogenesis via targeting *Klf4* expression (Fan et al., 2022) and impair dendritic spine stability via targeting postsynaptic neuroligin1 (Nlg1; Prada et al., 2018).

Astroglia to Neuron EV Signaling in CNS Development and Neurodegeneration

Role of astrocyte-derived EVs in development

Astroglia, the most abundant glial cells in the mammalian CNS, play many active roles in regulating neuronal and synaptic development and functions, as well as maintaining CNS homeostasis (Allen and Barres, 2009). Astroglial secretion represents one of the primary modes of action for astroglia to impact neuronal functions. During early postnatal development, developing astroglia secrete soluble proteins such as thrombospondins 1 and 2 (Tsp1/2; Christopherson et al., 2005), Hevin (Kucukdereli et al., 2011), Glypicans (Allen et al., 2012), Chordin-like 1 (Blanco-Suarez et al., 2018), and Neurocan (Irala et al., 2024) to promote excitatory and inhibitory synapse formation and stimulate glutamatergic activity. In adult CNS, mature astroglia also secrete small molecule signals, like D-serine or TGF-β and cholesterol to actively influence synaptic transmission (Allen and Eroglu, 2017).

In recent years, EVs especially $CD63^+$ exosomes have been increasingly shown to impact neuronal development and functions. Astroglia-derived EVs (ADEVs) isolated from ATP stimulated astrocytes using differential ultracentrifugation of astrocyte conditioned medium (ACM) significantly enhance neuronal excitability and dendritic arborization (Chaudhuri et al., 2018). In contrast, ADEVs, from astrocytes treated with IL-1β and TNF-α, reduce dendritic growth and complexity and furthermore, decrease neuronal spike rates and burst activity via delivery of miR-125a-5p and miR-16-5p (Chaudhuri et al., 2018). Proteomic analysis of secreted small EVs from astrocytes, neurons, and C6 glioma cells also identified extracellular matrix protein fibulin-2 as an astrocyte small EVs cargo to promote synapse formation in a TGF-β-dependent manner (Patel and Weaver, 2021). By employing size-exclusion chromatography (SEC)-based EV isolation, astroglial exosomes were recently shown to stimulate developmental axon growth of cortical layer V pyramidal neurons through the cell adhesion molecule hepatic and glial cell adhesion molecule (HepaCAM; Jin et al., 2023). The stimulating effect is axon specific with a primary action on axon growth cones but not affecting dendritic arborization, length, and synaptogenesis. Consistent with these observations, SEC-isolated astroglial small EVs are minimally associated with known astroglia-derived soluble proteins that regulate synaptogenesis (Jin et al., 2023). In contrast, secreted extracellular matrix and lipoproteins have been observed to be copelleted together with EVs, when isolated by differential ultracentrifugation (Li et al., 2017; Sluijter et al., 2018). Astrocyte-secreted synaptogenic proteins were also observed with ultracentrifugation-isolated astrocyte exosomes (Jin et al., 2023). Thus, it is noteworthy to distinguish the function of astrocyte-secreted proteins versus exosomes (or other vesicles) and their effects on neurons. In addition to exosomes or small EVs secreted from astrocytes, astrocytes also directly shed microvesicles from the plasma membrane in a glycerophosphodiester phosphodiesterase 3 (GDE3)-dependent manner by regulating actin dynamics (Levy-Myers et al., 2023). Interestingly, MVs released from wild-type but not GDE3-deficient astrocytes are able to restore miniature excitatory postsynaptic current (mEPSC) amplitudes in GDE3-deficient hippocampal slices through modulation of mGluR1/5 signaling. As GDE3 is expressed in astrocytes but not in neurons, this GDE3-dependent release of astrocyte EVs is a unique pathway for astrocytes to modulate neuronal synaptic signaling (Levy-Myers et al., 2023; Fig. 2). These studies clearly suggest that astrocyte-secreted extracellular vesicles represent a distinct and unique class of secreted signals from astrocytes to modulate neuronal development and synaptic signaling. Such EV-mediated signals are protected through encapsulation, potentially mobile for long distance, and elicit actions through either internal miRNA or surface protein cargoes.

Astrocyte-derived EVs and neurodegeneration

In addition to the important modulating roles of astrocytes in CNS development and homeostasis, astrocytes often become reactive with drastically altered gene expression and morphology in neurodegenerative conditions, partially because of elevated cytokines or expression of disease-causing mutant proteins (Liddelow et al., 2017; Escartin et al., 2021). Reactive astrocytes secrete several factors that affect neuronal survival, including cytokines, ROS, ATP/adenosine, saturated lipids, and excessive polyphosphate (Vargas and Johnson, 2010; Ng et al., 2015; Guttenplan et al., 2021; Arredondo et al., 2022). EVs secreted from reactive astrocytes have also been implicated in disease pathogenesis in various neurodegenerative disease models. This is particularly of interest to amyotrophic lateral sclerosis (ALS) pathogenesis, as noncell autonomous glial mechanisms are well established to contribute to motor neuron death in ALS (Philips and Rothstein, 2014). Early studies showed that expression of mutant superoxide dismutase 1 (SOD1) in astrocytes proteins inhibits conventional secretory pathways and promotes exosome secretion (Basso et al., 2013). Secreted astrocyte exosomes also contain mutant SOD1 to be transferred to spinal neurons and induce toxicity to motor neurons (Basso et al., 2013). Additional studies further showed that misfolded SOD1 and astrocyte marker GLAST were both detected in CNS-derived EVs from SOD1G93A mice and human SOD1 familial ALS

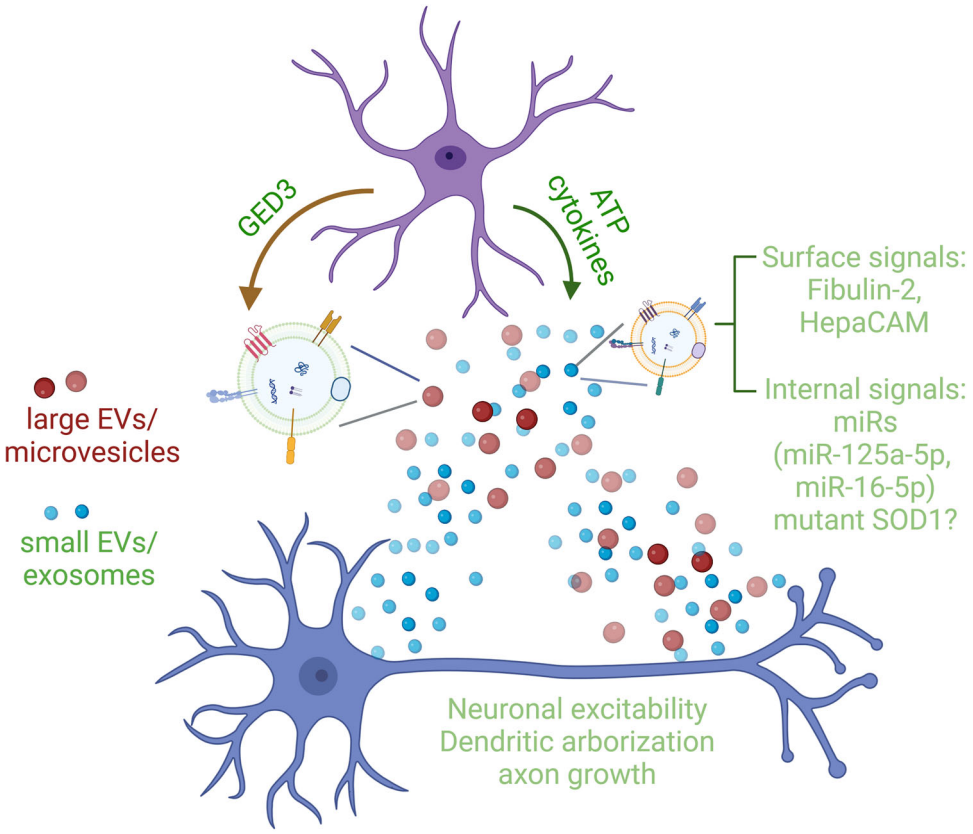

Figure 2. Extracellular vesicle (EV)-mediated signaling between astroglia and neurons. Astroglia can secrete both large EVs (microvesicles) and small EVs (exosomes) to significantly modulate neuronal development and activity, including excitability, dendritic arborization, and axon growth. Several signals, including ATP, cytokines, and glycerophosphodiester phosphodiesterase 3 (GDE3), have been shown to regulate EV secretion from astroglia, though the exact location of release (soma vs process) remains unclear.

samples (Silverman et al., 2019). These studies begin to suggest a notion that astrocyte EVs may serve as an alternative pathway, in addition to gap junctions, to propagate protein aggregates and facilitate disease spreading in ALS (Kim et al., 2022). However, in these studies EVs were isolated using differential ultracentrifugation, which may copellet misfolded SOD1 aggregates together with EVs. Indeed, we found that mutant SOD1G93A protein was not detected in SEC-isolated astrocyte small EVs in vitro and was very minimally associated with astrocyte EVs in spinal cords of diseased SOD1G93A mice by using cell type-specific hCD63-GFP floxed mice (Jin S, et al., unpublished observations). Whether ADEVs are associated with other typical protein aggregates associated with neurodegenerative diseases remains less understood.

In vivo analysis of astrocyte-derived EVs

While accumulating evidence suggests that secreted EVs represent an exciting new mechanism in mediating astroglia to neuron signaling, majority of these studies were limited to cell culture models. Astrocytes undergo dramatic postnatal developmental maturation to acquire unique morphological and molecular features which are lost in cultured astrocytes (Freeman, 2010). Thus, in vivo studies to understand roles of astrocyte-secreted exosomes are essential. Recent generation of several Cre-dependent cell type-specific exosome reporter mouse tools (Men et al., 2019; Neckles et al., 2019; Sheller-Miller et al., 2019) will significantly facilitate in vivo examination of astrocyte exosome secretion and their functions in physiological and pathological conditions. Indeed, a recent study showed that astrocyte exosomes are more abundantly localized outside of astrocytes during the first postnatal week when astrocyte morphology remains primitive with only limited processes (Jin et al., 2023). In addition, as proinflammatory cytokines are often elevated in neurodegenerative diseases and such cytokines significantly impact astrocyte exosome secretion, cargo composition, and internalization to neurons (Chaudhuri et al., 2018; You et al., 2020), the contribution of cytokine-altered astrocyte exosomes in the pathogenesis of neurodegenerative diseases will also be interesting to investigate in the future.

Microglial EVs Travelling Anterogradely Inside and Outside Axons: Implication in Aβ/tau Signaling across the Synapse

Synaptic dysfunction propagation and tau spreading mediated by microglial EVs

Microglia have long been known to actively contribute to the pathogenesis of neurodegenerative diseases (Gao et al., 2023) such as AD, starting from the first disease stages (Welikovitch et al., 2020). Excessive uptake of misfolded Aβ and tau proteins, abnormally build up in AD, leads to disease-associated transcriptional changes in microglia (Keren-Shaul et al., 2017), excessive complement-mediated synaptic pruning (Hong et al., 2016), autophagy impairment (Pomilio et al., 2020), and augmented release of EVs carrying Aβ and tau proteins (Agosta et al., 2014; Joshi et al., 2014; Clayton et al., 2021). The latter can cause synaptic dysfunction and/or act as amyloid seeds, favoring formation of Aβ/tau aggregates, along AD-vulnerable brain circuits. The Verderio group recently showed that large microglial EVs

carrying Aβ (Aβ-EVs) induce and propagate synaptic dysfunction along the entorhinal→hippocampal circuit (Falcicchia et al., 2023), a key site for memory formation (Eichenbaum et al., 2007), recapitulating first synaptic alterations that correlate with cognitive decline in AD (Selkoe, 2002). A single injection of large Aβ-EVs into the entorhinal cortex (EC), a region regarded as the origin of AD neuropathological spread, impaired long-term potentiation (LTP), a form of synaptic plasticity implicated in learning and memory, first in the vicinity of the injection site, and at a later time-point (24 h) in the dentate gyrus of the hippocampus (Gabrielli et al., 2022). Importantly, spreading of LTP impairment along the circuit was accompanied by persistent network hyperexcitability in the cortex and in the hippocampus. This was accompanied by progressive memory deficits, first affecting associative memory, that depend on the EC, and subsequently involving nonassociative memory that requires hippocampal functionality, as evidenced by chronic electroencephalographic recordings and behavioral tasks (Falcicchia et al., 2023). Similarly to Aβ-EVs, small EVs encapsulating tau (tau-EVs) were recently shown to distribute pathologic tau seeds and accelerate propagation of tau aggregates along the same circuit in the mouse brain (Asai et al., 2015; Clayton et al., 2021). Super-resolution confocal microscopy and the use of a microglia-specific lentiviral EV reporter system expressing mEmerald-CD9 (mE-CD9) fusion protein allowed quantification of microglial mE-CD9+ EVs release in the EC, revealing augmented EV production from reactive microglia surrounding Aβ plaques (Clayton et al., 2021). In this process the danger signal ATP acts a key driver (Abdullah et al., 2024): microglia shed tau-EVs in response to stimulation of P2X purinoceptor 7 (P2RX7) by excessive ATP in the extracellular environment, leaking from the damaged tissue. In support of this notion, inhibition of EV secretion by a P2RX7 antagonist ameliorates tau pathology and cognitive impairment in a tau mouse model (Ruan et al., 2020; Abdullah et al., 2024; Fig. 3). While indicating that microglial EVs carrying Aβ/tau proteins contribute to AD onset and pathological progression, these recent studies raise the questions of how microglial EVs spread early synaptic dysfunction and tau lesions across connected brain regions.

Large Aβ-EVs move at the axon surface

In the case of large microglial Aβ-EVs, carrying Aβ on their surface (Joshi et al., 2014; Gabrielli et al., 2022), the spreading of Aβ-related synaptic dysfunction can be mediated by the extracellular motion of Aβ-EVs along axonal projections (Gabrielli et al., 2022). By imaging in vitro the interaction of individual large Aβ-EVs with axons, Gabrielli et al. showed that Aβ-EVs, too big to be internalized into thin axons, move outside neurons toward the axon tip. The EV motion can be impaired by annexin-V, a molecule that cloaks phosphatidylserine (PS) externalized on the EV surface and may anchor EVs to sites of the neuron plasma that have externalized PS (G. D'Arrigo, personal communication), such as synapses (Scott-Hewitt et al., 2020). Importantly, when Aβ-EV motility was inhibited, no propagation of LTP deficit, network aberrant activity, and memory impairment occurred along the entorhinal→hippocampal circuit following Aβ-EV injection into the EC (Gabrielli et al., 2022; Falcicchia et al., 2023), implicating Aβ-EV motion along the perforant path, the connecting route between the EC and the hippocampus, in the spreading of Aβ-related synaptic dysfunction. What is/are the mechanism(s) mediating microglial Aβ-EVs motion at the axon surface? Our hypothesis is that at least a fraction of Aβ-EVs bind to neuronal receptors which drift on the cell surface transported by neuronal actin cytoskeletal rearrangements, as previously shown for astrocyte-derived EVs (D'Arrigo et al., 2021) and adenoviral particles (Burckhardt et al., 2011). It is worth noting that by moving extracellularly microglial Aβ-EVs, which expose Aβ on their surface, synaptic dysfunction may propagate via an extracellular signaling pathway that is triggered by Aβ interacting with its various neuronal receptors (Jarosz-Griffiths et al., 2016) without the need of Aβ delivery into neurons.

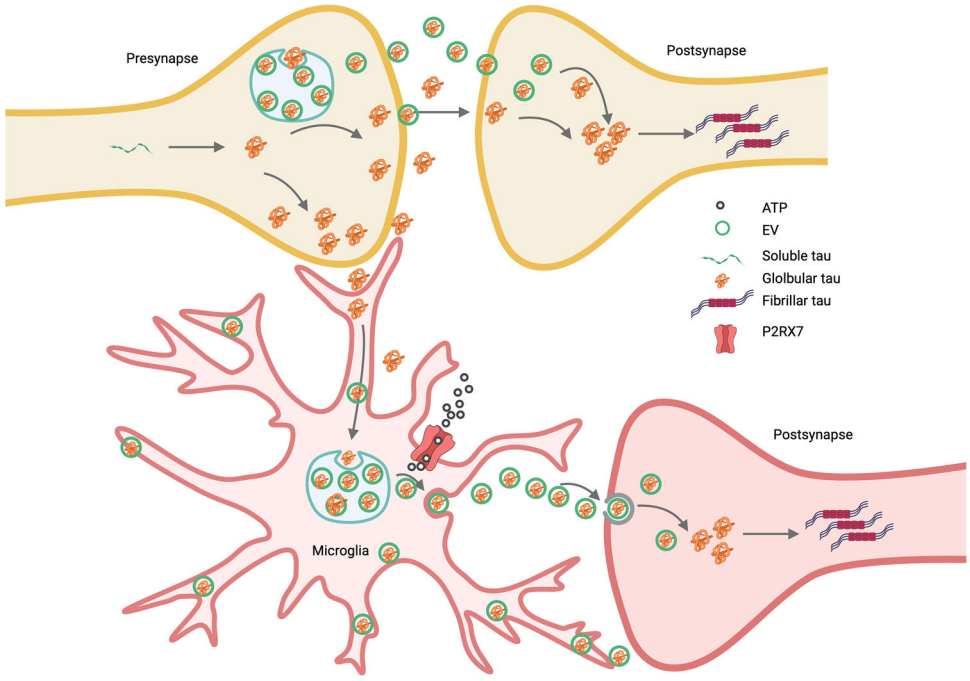

Figure 3. EV-mediated propagation of misfolded tau protein in the CNS. In AD, misfolded tau protein can be transmitted through a trans-synaptic pathway either in soluble or EV-contained forms. Accumulation of misfolded tau inactivates synapses, which are subject of synaptic pruning by microglia. Undigested tau seeds are secreted as either soluble or EV-contained form from microglia in P2RX7 sensitive manner.

Small tau-EVs are transported intra-axonally

In contrast, tau spreading mediated by microglial EVs likely occurs through an intracellular route. A recent cryoelectron tomography study unequivocally showed that tau is localized in the lumen, not at the surface, of EVs extracted from post-mortem AD patient brains (Fowler et al., 2023). Moreover, small tau-EVs were shown to be internalized by neurons. Those internalized EVs not sent to lysosomes for degradation were intra-axonally transported to presynaptic terminals and transmitted between interconnected neurons (Wang et al., 2017). But how exogenous tau-EVs engulfed by neurons can be released at synaptic sites? Small tau-EVs endogenously generated in neurons as intraluminal vesicles inside MVBs (pre-EVs) undergo activity-dependent release from AD brain nerve endings (synapses) upon MVB fusion with the plasma membrane (Miyoshi et al., 2021). Polanco and colleagues proposed that this secretory pathway can be hijacked by exogenous small tau-EVs to be released at the synapse. By super-resolution and electron microscopy, endosomes containing engulfed tau-EVs were shown to fuse with MVBs loaded with endogenous intralumenal vesicles, generating hybrid secretory endosomes competent for fusion, which could mediate the release of intact exogenous small tau-EVs at presynaptic terminals (Polanco et al., 2018). Interestingly, by the same secretory mechanism, small Aβ-EVs taken up by neurons can be transmitted across the synapse (Sardar Sinha et al., 2018). Thus, a model emerges in which both large and small Aβ/tau-EVs exploit extracellular and intracellular neuronal trafficking to move across the synapse and spread Aβ/tau pathology (Fig. 4).

Mechanisms of Axonal Maintenance by the Transfer of Extracellular Vesicles from Myelinating Glia

EVs and glial support for axonal integrity

Oligodendrocytes wrap CNS axons with the multilamellar myelin sheath forming a functional axon–myelin unit (Nave and Werner, 2014; Stassart et al., 2018). While myelin enables fast axonal impulse conduction through its insulating properties, it also shields the axon surface from the local parenchyma and thus forms the direct axonal microenvironment along its length. Both axons and myelin form a local and highly specialized subcompartment within the cell that needs to be physiologically and structurally maintained and is particularly vulnerable to degeneration processes. Due to this special architecture, myelinated axons require specific support from oligodendroglia to maintain their energy homeostasis and structural integrity (Duncan et al., 2021; Li and Sheng, 2023). It is becoming increasingly evident that in addition to metabolic support through delivery of energy metabolites via transporters (Funfschilling et al., 2012; Lee et al., 2012), oligodendrocyte-derived EVs serve as delivery route for complex molecular cargo such as enzymes providing axons with key factors for axonal maintenance (reviewed in detail in Kramer-Albers and Werner, 2023). The molecular

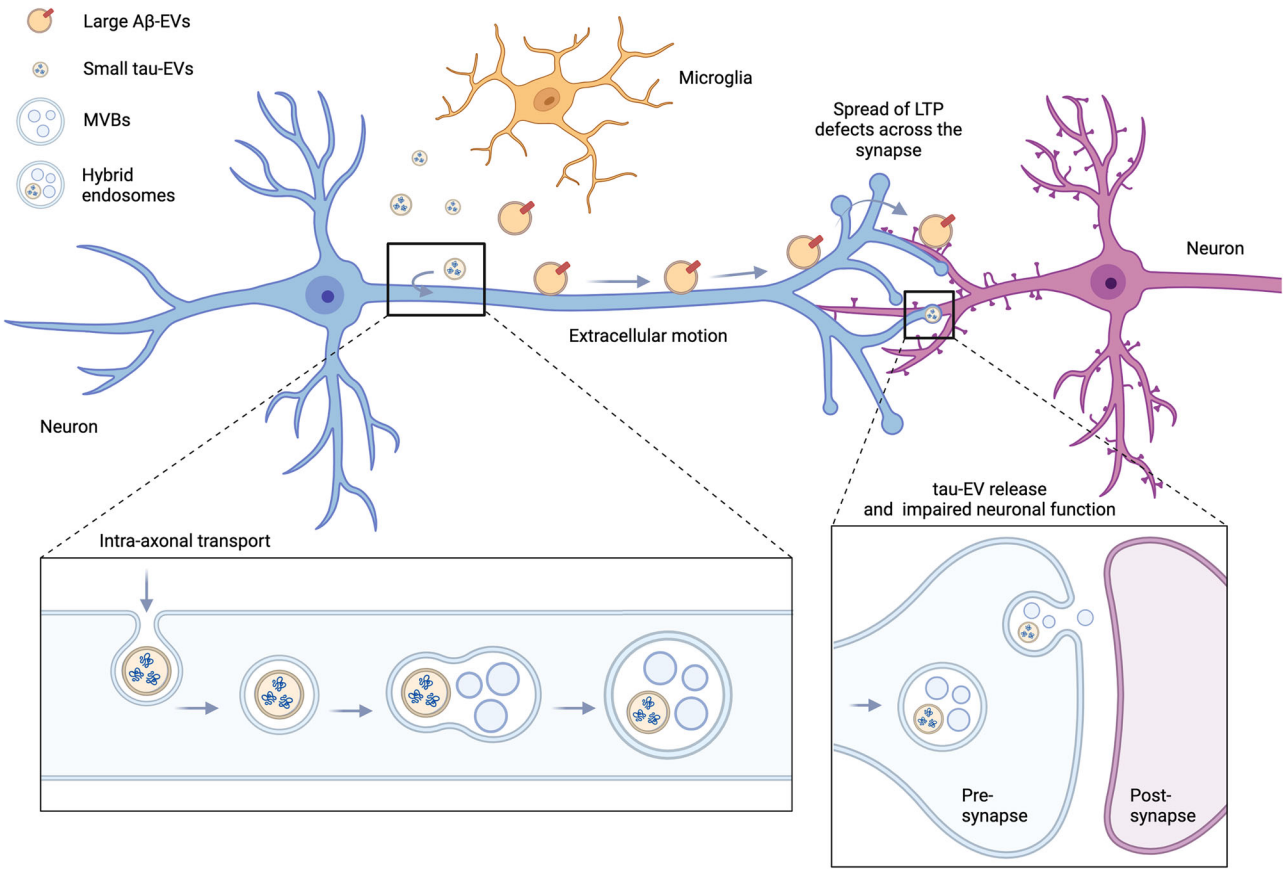

Figure 4. Extracellular and intracellular trafficking of microglial EVs carrying misfolded Aβ/tau proteins across the synapse. Large Aβ-EVs produced by microglia transfer Aβ cargo across the synapse by moving at the surface of axonal projection and affect LTP. Microglia-derived small tau-EVs are taken up by neurons into endosomes, which can fuse with MVBs generating hybrid secretory endosomes. These hybrid MVBs are transported anterogradely inside axons and mediate the release of internalized microglial small tau-EVs along with endogenous small EVs upon fusion with the presynaptic membrane. Transcytosis of tau-EVs promotes impairment of neuronal function.

mechanisms of EV release and mode of action have recently been deciphered, although there is certainly still much to be revealed, particularly regarding different EV cargos and their relevance for axonal health.

EV delivery to axons in response to axo-myelinic neurotransmission

Myelinating oligodendrocytes release EVs from the innermost noncompacted myelin layer in response to neuronal glutamatergic signaling (Fig. 5). This periaxonal cytoplasmic region of myelin contains MVBs (Frühbeis et al., 2013), which can be transported from the oligodendrocyte soma through the myelin compartment via cytoplasmic "myelinic" channels serving as conduits for organelles (Chapple et al., 2024). The biogenesis of multivesicular bodies in oligodendrocytes depends on ceramide generated at the MVB limiting membrane by neutral sphingomyelinase and the small GTPase Rab35 (Trajkovic et al., 2008; Hsu et al., 2010). Fusion of MVBs with the oligodendroglial plasma membrane leads to the secretion of small EVs termed exosomes. However, the exact mechanisms driving the fusion process are still unknown. Intriguingly, oligodendroglial exosome release is triggered by axo-myelinic neurotransmission involving the release of glutamate from electrically active neurons followed by activation of NMDA receptors on oligodendrocytes and influx of Ca^{2+} (Frühbeis et al., 2013). Once released, oligodendroglial exosomes are taken up by neurons via dynamin-dependent endocytosis, and the cargo is functionally available to the axonal compartment. Overall, this implies that axons import functional biomolecules from myelinating oligodendrocytes via exosomes, which is promoted by their electrical activity.

Oligodendrocyte EVs promote axonal transport and energy homeostasis

Evidence from in vitro and in vivo experiments indicates that exosome transfer from oligodendrocytes to neurons is important for axons to be maintained. Neurons receiving oligodendrocyte-derived exosomes are resilient to various stress conditions such as nutrient deprivation and oxidative stress and are able to maintain their energy homeostasis, typically challenged under these conditions (Frühbeis et al., 2013; Frohlich et al., 2014). Furthermore, oligodendroglial exosomes promote fast axonal transport (Fruhbeis et al., 2020), which plays a key role in axonal maintenance and is compromised in many neurological diseases including multiple sclerosis (Sorbara et al., 2014; Sleigh et al.,

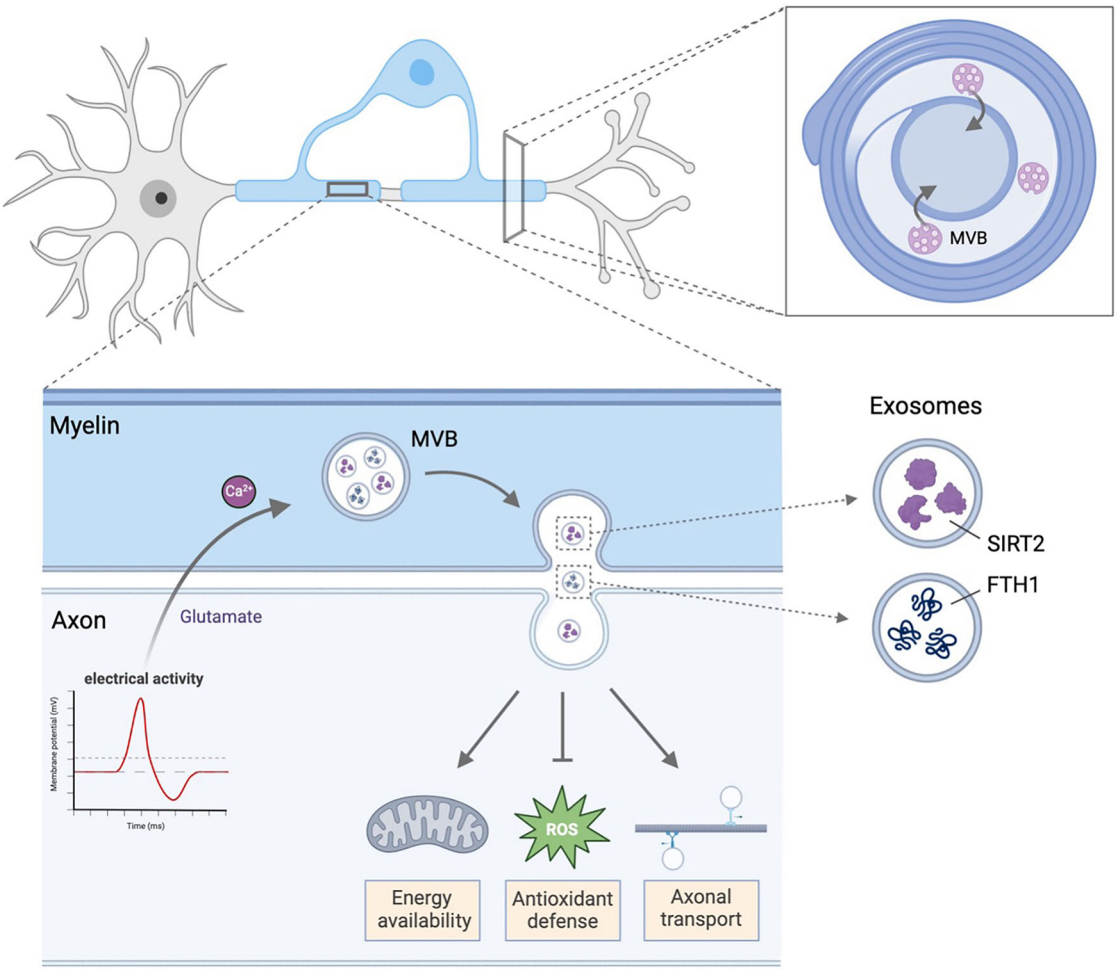

Figure 5. Functions of oligodendrocyte-derived EVs in axonal maintenance. Myelin produced by oligodendrocytes provides electrical isolation and prevents the uptake of nutrients and other extracellular interactions by axons. Oligodendrocyte-derived EVs support axons by providing biomolecules that are important for basic axonal functions and provide a pathway for external supply. Oligodendrocyte-derived EVs (exosomes) are released from periaxonal MVBs in response to neuronal activity and glutamatergic axon-to-myelin signaling. Exosomes are internalized by neurons and provide essential support for the maintenance of axonal integrity by increasing ATP availability, providing antioxidant defense, and promoting axonal transport. SIRT2 and FTH1 are relevant exosome cargos improving mitochondrial ATP production and protecting cells from ferroptotic damage, respectively. MVB, multivesicular body, ROS, reactive oxygen species, FTH1, Ferritin heavy chain 1.

2019). Mouse models with a progressive axonal degeneration due to absence of the exosomal cargo proteins PLP and CNP exhibit impaired exosome release from oligodendrocytes, and these mutant exosomes lack the ability to promote axonal transport (Fruhbeis et al., 2020). Vice versa, mice with diminished oligodendroglial exosome release due to absence of the Rab35-GTPase specifically in oligodendrocytes also develop neurodegeneration (Mukherjee et al., 2020). Together, these observations link oligodendroglial exosome dysfunction with axonal degeneration and suggest that proper exosome secretion is crucial for axonal health. Ongoing research, using genetic mouse models that are designed to identify the target cells of oligodendrocyte-derived EVs, will allow to reveal the prevalence of oligodendrocyte-to-neuron EV transfer in the brain (Krämer-Albers, unpublished).

Functional cargoes of oligodendrocyte-derived EVs

Among exosomal cargos, the proteins SIRT2 and Ferritin Heavy Chain 1 (FTH1) both were identified being functionally relevant for axonal homeostasis. SIRT2 is a NAD^+-dependent deacetylase that upon exosomal delivery deacetylates mitochondrial proteins including adenine nucleotide translocases 1 and 2 (ANT1/2), mediating an increase in axonal ATP levels and energy supply (Chamberlain et al., 2021; Kramer-Albers, 2021). FTH1 is an iron-binding protein protecting cells from ferroptotic damage through storage of iron in a soluble nontoxic state. Interfering with oligodendrocyte to neuron transfer of FTH1 by conditional deletion of its gene in oligodendrocytes causes oxidative damage in neurons and subsequently neuronal death (Mukherjee et al., 2020). Thus, FTH1 delivery via oligodendroglial exosomes provides antioxidative defense by protecting axons from iron overload and reactive oxygen species accumulation. Future research is expected to reveal further molecular components among the cargo with functions in glia–axonal support. For example, those proteins that are changed in both exosomes derived from PLP- and CNP-deficient oligodendrocytes could play a presently unrecognized role in long-term axonal homeostasis (Fruhbeis et al., 2020).

Implications for neurodegeneration and aging

In conclusion, oligodendroglial EVs secreted as exosomes play a vital role in maintaining axonal integrity and function. By increasing energy availability and protecting against oxidative stress, these exosomes ensure the long-term health of axons. Notably, oligodendroglial and myelin dysfunction is widely identified as a driver of axonal degeneration not only in myelin diseases but also in other neurodegenerative diseases and during aging (Mot et al., 2018; Depp et al., 2023). Thus, oligodendrocyte-to-neuron delivery of EVs may play a wider role in neurodegenerative processes, offering a promising avenue for future research and therapeutic development.

Conclusion

Since landmark studies implemented EVs as mediators of cell–cell communication and delivery vehicles for RNAs in the first decade of the century, tremendous progresses have been made in understanding EV biology and functions (Ratajczak et al., 2006; Valadi et al., 2007; Skog et al., 2008). The mammalian CNS is unique in its composition of highly heterogeneous cell populations, which are organized by complex cell–cell and cell–matrix interactions. Thus, EV-mediated intercellular communication in the CNS is particularly advantageous because it includes cell type-specific (or enriched) molecules, can protect signals, is mobile over long distances, and selectively targets recipient cells. This is distinct from canonical constitutive and regulated secretion pathways including synaptic vesicle release (for neurotransmitter signaling) in the CNS. As we illustrated above, cell type-specific EV signaling has been increasingly established in regulating CNS development, plasticity, homeostasis, and especially pathology. Recent progresses in examining various cargoes that are either on the surface or encapsulated in CNS-derived EVs also bring us closer, more than ever, to be able to monitor progression of specific neurodegenerative diseases. While this is an exciting time to study EV biology and functions in the CNS, there are also challenges to be addressed. Very little is known about how to regulate cell type-specific EV biogenesis and secretion, preventing effective approaches to either enhance or block its release in a cell type-specific manner in the CNS. In addition, isolation of EVs from in vivo brain and spinal cord tissues remains challenging and requires cautious follow-up. Thus, additional new in vivo tools and high-resolution imaging techniques will need to be developed to unravel EV signaling in the healthy and diseased brain.

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