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Astroglial vesicular network: Evolutionary trends, physiology and pathophysiology

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Abstract

Intracellular organelles, including secretory vesicles, emerged when eukaryotic cells evolved some 3 billion years ago. The primordial organelles that evolved in Archaea were similar to endolysosomes, which developed, arguably, for specific metabolic tasks, including uptake, metabolic processing, storage and disposal of molecules. In comparison to prokaryotes, cell volume of eukaryotes increased by several orders of magnitude and vesicle traffic emerged to allow for communication between distant intracellular locations. Lysosomes, first described in 1955, a prominent intermediate of endo- and exocytotic pathways, operate virtually in all eukaryotic cells including astroglia, the most heterogeneous type of homeostatic glia in the central nervous system. Astrocytes support neuronal network activity in particular through elaborated secretion, based on a complex

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intracellular vesicle network dynamics. Deranged homeostasis underlies disease and astroglial vesicle traffic contributes to the pathophysiology of neurodegenerative (Alzheimer's disease, Huntington's disease), neurodevelopmental diseases (intellectual deficiency, Rett's disease) and neuroinfectious (Zika virus) disorders. This review addresses astroglial cell-autonomous vesicular traffic network, classified into primary and secondary vesicular network defects in diseases, targets for developing new therapies for neurologic conditions.

Key words: Vesicle network; Endocytosis; Exocytosis; Lysosome; Astrocyte; Secretory vesicle; Traffic, Rett's disease, Intellectual deficiency, Neurodegeneration.

Abbreviations:

AD – Alzheimer's disease

AQP4 - aquaporin 4 ALS - amyotrophic lateral sclerosis

ANP – atrial natriuretic peptide

AxD - Alexander's disease

BDNF – brain derived neurotrophic factor

BBB - blood-brain barrier

CNS - central nervous system

cAMP - cyclic adenosine monophosphate

EEA1 - early endosome antigen 1

ESCRT - endosomal sorting complexes required for transport

FTY 720 – fingolimod

HIV-1 - human immunodeficiency virus 1

HD - Huntington's disease

ID - intellectual deficiency

IDE - Insulin degrading enzyme

IFN- γ - interferon- γ

INCL - infantile neuronal ceroid lipofuscinosis

iPSC – inducible pluripotent stem cells

LAMP-1 - lysosome associated membrane protein 1

LC - locus coeruleus

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LRP1- low density lipoprotein receptor-related protein 1

Mecp2 - methyl-CpG-binding protein 2

MHC II - major histocompatibility complex II

MVB - multivesicular body

NP - Niemann–Pick disease

PPT1 - palmitoyl protein thioesterase 1

SNARE – soluble NSF attachment protein receptor

TBEV - Tick borne encephalitis virus

V-GLUT1 - vesicular glutamate transporter

ZIKV – Zika virus

XLID - X-linked forms of ID

The gliocrine system: astrocytes as secretory elements of the CNS

Astroglia are the class of neural cells, heterogeneous in form and function, primarily responsible for the homeostasis of the central nervous system (CNS). Astroglial functions also include control of synaptogenesis and regulation of synaptic connectivity, integration and synchronization of neuronal networks and the maintenance of blood-brain barrier (BBB) integrity¹⁻¹⁸. In addition, astrocytes represent the fundamental element of defensive system of nervous tissue; insults to the brain or spinal cord trigger reactive astrogliosis, which limits the damage and facilitates post-lesion regeneration of neural networks^{3, 11, 13} although it may be also neurotoxic in some circumstances¹⁹. Moreover, astrocytes are part of the glymphatic system, regulating the convective removal of waste accumulated in the extracellular space²⁰.

Signalling between astrocytes and other cellular elements of the CNS is mainly mediated through the release of chemical messengers. Astroglial cells secrete surprisingly large number of molecules, which include neurotransmitters and their precursors, neurohormones, trophic, plastic and growth factors, scavengers of reactive oxygen species, immunoactive molecules and pro-inflammatory factors and many more^{16, 21, 22}. Mechanisms of astroglial secretion are complex and include vesicular exocytosis, diffusion through plasmalemmal channels and plasmalemmal transporters^{12, 16}. Thus astrocytes represent a gliocrine system²², responsible for regulation of the CNS microenvironment through secretion of neuroactive compounds. The role for secretory organelles is, however, substantially wider than simple export of various cargos; different types of cytosolic vesicles contribute to endocytosis and delivery of proteins to plasmalemma, whereas impairment of vesicular traffic results in multiple neuropathologies.

Evolution of secretory organelles

Life forms belong to the Domains (also known as Superkingdoms) of Bacteria, Archaea and Eukarya²³,²⁴. It is almost universally accepted that secretory organelles exist solely in eukaryotic cells, which notion, however, requires critical assessment. The origin and appearance of eukaryotes remains debatable^{25, 26} and their immediate predecessors (for example, archaeobacteria or eubacteria²⁷) are not yet unequivocally identified. Similarly disputed is the geological age of eukaryote emergence; eukaryotic signatures have been recovered in fossils of 3 to 3.5 billion years of age²⁸, this being substantially older than generally accepted time of eukaryote emergence ~ 2 billion years ago. This may indicate a very early divergence of the Domains of Life. Incidentally, the intermediate form lying in between Archaea and Eukarya, the "Chronocyte", has also been suggested²⁹.

Cytological structure of Bacteria and Archaea is relatively uncomplicated. How Eukarya, characterized by a significant degree of cellular structural complexity developed from Archaea³⁰, is unclear, although most hypotheses assume a prokaryote-to-eukaryote transition or else an integration of an archaeobacterium into a eubacterium. Conceptually, the eukaryote evolution is linked to the presence of mitochondrion-like structures and/or mitochondrial proteins. It seems, however, possible that aerobic and anaerobic eukaryotes, harbouring mitochondrial homologues of various sorts, have co-existed throughout eukaryote history³¹.

The hypothesis that Eukarya emerged from an ancestral Archaea has recently gained interest, in particular after subcellular organelles other than mitochondria were recognised in the early life forms. Indeed, the discovery of 'Lokiarchaeota', a novel phylum, with genes encoding an array of eukaryotic signature proteins that are required for membrane plasticity supports this view³⁰. Specifically, this ancestral Archaea expressed genes encoding proteins, which in Eukarya contribute to remodelling of cell shape and membrane deformation including phagocytosis, a form of endocytosis, where molecules or particles are transported into the cell involving vesicles pinching off the plasma membrane³². This process requires cytoskeletal elements such as actin; the Lokiarchaeum genome encodes five actin homologues with high similarity to eukaryotic actin and actin-related proteins³⁰. Key regulators of actin cytoskeleton dynamics are small GTPases, which are essential for vesicle traffic³³. A substantial presence of Ras-superfamily GTPases, which account for nearly 2% of the proteome, have been identified in Lokiarchaeota³⁰. This Archaeal phylum also contains the genes for endosomal sorting complexes required for transport (ESCRT) proteins, components of the eukaryotic multivesicular body (MVB) belonging to the endosome pathway required for the biogenesis of lysosomes, directing plasma membrane proteins for lysosomal degradation, acting in viral budding, in cytokinesis, autophagy, and other pathways³⁴. Identification of archaeal genes involved in membrane remodelling and vesicular trafficking indicates that the emergence of subcellular organelles, resembling endolysosomal structures, was already under way before the acquisition of the mitochondrial endosymbiont³⁰.

The lysosomal network

Lysosomes are dedicated organelles of endo- and exocytotic pathways, which operate in nearly all animal and plant cells. The term lysosome was introduced in 1955³⁵ from Ancient Greek "λύσις" - "to loosen" and "soma" - body i.e. "lytic or digestive body" since several degrading enzymes have

been found in this structure ^{36, 37}. To visualize this new organelle a staining method for acid phosphatase was used, and the location of these enzymes in the lysosome was confirmed by using electron microscopy ³⁸. An important insight about the degrading function of lysosomes, which contains many hydrolytic enzymes, came from the discoveries that molecules present in the extracellular space can enter the cell via endocytosis. Fragments of the labelled extracellular proteins were found localised in lysosomes ³⁹.

Until recently, it was thought that lysosomes are merely responsible for catabolism, since they contain hydrolytic enzymes. Besides degradation of biomolecules, however, the lysosome is involved in energy metabolism, in secretion, in delivery of membrane receptors to the plasma membrane, in plasma membrane repair and in cell signalling. All this is possible as lysosomes actively fuse (see Figure 1) with other cellular structures including the plasmalemma, late endocytotic vesicles, autophagosomes that haul intracellular material for degradation; in addition membrane transporters translocate metabolites, ions and soluble substrates into and out of lysosomes ³⁷. The central position of lysosome is not only linked to endocytotic entry of molecules into the cell but also to exocytotic exit of molecules (Fig. 1). Incidentally, the terms autophagy (for lysosomal degradation of material of the intracellular origin), endocytosis (for lysosomal digestion of material of the extracellular origin) and exocytosis were all introduced at a meeting in London in 1963 ³⁶, which followed lysosomes discovery. The complex lysosomal vesicular network is also present in astrocytes.

Astrocytes and vesicle traffic

Astrocytes actively participate in neurochemical signalling in the CNS, a property considered no longer exclusive to neurones. Astroglia express virtually all types of receptors ^{40, 41} to neurotransmitters and neuromodulators linked to the second messenger excitability involving cytosolic ionic signals ⁴²⁻⁴⁵ and cyclic adenosine monophosphate (cAMP) pathways ^{22, 46}. Astrocytes are also capable of secreting numerous signalling molecules.

Neurochemical signalling involves vesicle-related processes and the first observation of these functional subcellular structures in astrocytes was made over 100 years ago by microscopic studies conducted by Hans Held ⁴⁷ and Jean Nageotte ⁴⁸, who both proposed that glial cells act as secretory entities in the CNS. Existence of secretory organelles in astroglia has been confirmed by advanced biophysical, electrophysiological and quantitative high-resolution optical microscopy studies ^{16, 49, 50}. In contrast to vesicle-based fast neuronal signalling, vesicular regulated exocytosis in astrocytes occurs in a much slower time-domain, with temporal characteristics closely related to the slow CNS processes including neurodevelopmental plasticity, memory formation and homeostatic metabolic support; all these being strongly influenced by the relatively slow delivery of metabolic precursors for morphological and functional CNS plasticity ^{51, 52}.

The integrating role of glial communication in the CNS resembles, to some extent, the function of the endocrine system, a master regulator of bodily functions, which operates substantially slower than rapid, often sub-millisecond, synaptic neuronal signalling. Therefore, astroglia were proposed to constitute the gliocrine system within the CNS ^{16, 22}. Another similarity between the endocrine and the gliocrine systems lies in the manner of signalling molecules delivery to the appropriate targets. Hormones are transported by blood circulation, while gliosignalling molecules, secreted by astroglia into the interstitium, can be convectively transferred by the recently described glymphatic apparatus ²⁰. Thus, astroglial vesicular networks contribute to the tissue-wide array of processes, all fundamental to normal and pathological CNS conditions.

Astroglial endocytosis

Endocytosis consists of many processes, and contributes to a multitude of cellular functions including uptake of extracellular nutrients, regulation of cell-surface receptor presence, which shapes the plasmalemmal signalling landscape, and antigen presentation³². Astrocytes are facultative antigen presenting cells⁵³, especially during pathological conditions, when they become reactive.

Astrogliosis, an active defensive response of astrocytes is associated with various diseases/disorders; astrogliotic remodelling facilitates resolution of the pathology, although under some circumstances it may be maladaptive and deleterious^{13, 19}. Discrimination of stages of astrogliosis is difficult, since there is a gradient of changes in structure, expression of genes and function. However, exposure of astrocytes to interferon- γ (IFN- γ) induces the expression of major histocompatibility complex II (MHC II) molecules. These molecules traffic within astrocytes by endolysosomal vesicles along the cytoskeletal intermediate filaments⁵³ and reach the plasmalemmal surface following the fusion of these organelles with the plasma membrane. Lysosomal destination for fusion with the plasma membrane is to some degree determined by their previous interaction with autophagosomes³⁷ and is regulated by β -adrenergic receptor signalling, which inhibits the expression of MHC II molecules in astrocytes^{54, 55}. Whether this process provides toxic or beneficial effects in the pathological process in the damaged tissue remains to be determined.

Extracellular debris removal by astroglial endocytosis

The removal of waste materials and debris from the extracellular space is in part the function of endocytosis coupled with the convective flow of the glymphatic system, an important clearance mechanism, depending on aquaporin 4 (AQP4) water channel⁵⁶⁻⁵⁸. Although the position of AQP4 in the brain appears to be important in determining water transport in the brain⁵⁹, it is still unclear how AQP4 participates in the convective flow of extracellular solution through the brain and spinal cord parenchyma, which appears key in ensuring CNS homeostasis and contributes to pathologic conditions as occurring in cerebral ischemia, neurotrauma, cytotoxic oedema, epilepsy, Parkinson's and Alzheimer's diseases, in which the BBB is compromised⁶⁰⁻⁶². Debris that accumulates in the CNS due to transfer from the systemic circulation through the BBB or due to pathological events within the CNS itself, coupled to diffusion, fluxes along the relatively long tortuous extracellular space of the brain interstitium and may be taken into astroglial vesicle network by endocytosis.

Astroglial endocytic and lysosomal network can be fundamental for the removal of cellular detritus and toxic macromolecules both in physiological and pathological context. In a multitude of diseases/conditions associated with myelin destruction, which include multiple sclerosis, progressive multifocal leukoencephalopathy, metachromatic leukodystrophy and subacute infarct, astrocytes accumulate myelin debris by means of cell surface scavenger receptor low density lipoprotein receptor-related protein 1 (LRP1) receptor-mediated endocytosis⁶³. Myelin fragments were rapidly (within 120 min) translocated from endosomes to lysosomes, where they underwent degradation. Astrocytes can remove myelin in physiological context, for example during developmentally regulated shortening of optic nerve in *Xenopus laevis*⁶⁴. In *Drosophila* larvae astroglial cells act as primary phagocytotic elements critical for the removal of neurites during neuropil development⁶⁵ as

well as pruning of axons in developing, mushroom bodies⁶⁶, with the removed material entering endo-lysosomal degrading pathway. Astrocytes also employ endocytosis for the accumulation of signalling molecules, for example neurokinin B⁶⁷ or pro-brain derived neurotrophic factor (BDNF)⁶⁸.

In neurotrauma, for example, cytoplasmic proteins exit into the extracellular space. This may result in additional toxicity, with these proteins exerting further damaging effects on neighbouring cells. The capacity of internalization of S100B, a protein secreted by astrocytes⁶⁹, was studied recently⁷⁰. Fluorescently labelled S100B (S100B-Alexa Fluor[®] 488) was added to the cultured astrocytes and cells were monitored by time-lapse confocal microscopy. The S100B was found to enter cells, with subsequent trafficking with endocytotic vesicles. The entry of S100B-Alexa⁴⁸⁸ involved, at least in part, the “scavenger receptor” RAGE, since pre-treatment with anti-RAGE antibody partially prevented the uptake of S100B-Alexa Fluor[®] 488. Endocytotic uptake of S100B-Alexa Fluor[®] 488 was also suppressed by a dynamin inhibitor Dynole 34-2. As described previously⁷¹, endolysosomal traffic exhibits directional and non-directional vesicle mobility, and this was also observed for endocytotic vesicles taking up S100B-Alexa Fluor[®] 488⁷⁰. Directional mobility of S100B-Alexa Fluor[®] 488-positive vesicles increased over time as did the co-localization of this protein with a lysosomal marker, indicating the transport of the internalized protein into this compartment⁷⁰.

Exocytotic vesicles in astroglia

Although astroglial vesicle-mediated release of chemical messengers is well documented, this mechanism, however, is distinct from that operational in neurones, especially when the kinetics is considered^{16, 49, 72, 73}. Following the pioneering studies which revealed astroglial release of glutamate in a Ca²⁺-dependent manner⁷⁴, the role of vesicular secretion of gliosignalling molecules was analysed in vesicles containing fluorescently labelled atrial natriuretic peptide (ANP)⁵⁰ and by monitoring membrane capacitance, a parameter linearly related to membrane area⁷⁵ and sufficiently sensitive to detect unitary vesicle fusion/fission events in real-time in isolated^{76, 77} and also in astrocytes in tissue slices⁷⁸. While there has been comprehensive coverage on the size and nature of vesicles in astrocytes elsewhere^{10, 16, 18}, using electron microscopy and fluorescence markers^{68, 79-82}. Here, we focus on a recent study, where the super-resolution fluorescence microscopy was combined with the high-resolution membrane capacitance monitoring⁸³, compellingly demonstrated the existence of two populations of secretory vesicles (distinct in their size) in cultured and freshly isolated rat astrocytes. Small vesicles (~ 70 nm in diameter) mainly contain amino acids glutamate (based on labelling of the vesicular glutamate transporter 1; V-GLUT1) and D-serine and/or peptides (brain derived neurotrophic factor - BDNF and ANP), whereas ATP and some peptides are present mainly in larger (~ 200 nm in diameter) lysosome-associated compartments (marked by antibody against the lysosomal associated membrane protein LAMP-1 and by quinacrine (which binds to adenine nucleotides), as was already reported previously⁸⁴. This separation of vesicular populations was not dependent on animal age, since similar results were obtained in astrocytes deriving from post-natal as well as from adult animals. The larger vesicles that contain peptides and ATP may well be generated by the fusion of peptidergic vesicles that arise from the Golgi cisternae with some endosomes and/or lysosomes. Vesicles of all sizes in astrocytes interact with the plasma membrane, as was monitored by discrete increases in membrane capacitance. As was already noted for

endocrine cells⁸⁵, transient exocytotic fusions were more abundant than the full-fusion exocytotic events, especially after stimulation of cells, which facilitates conversion of transient fusion events to full-fusion predominantly in larger vesicles⁸³. This vesicle size-dependent mechanism of unitary exocytotic events is not due to a different density of SNARE molecules in larger and smaller vesicles⁸⁶, but it more likely reflects vesicle size-dependent stability of fusion pores, which appear more stable in smaller vesicles⁸⁷.

Regulation of vesicle network dynamics in astrocytes

First measurements of single vesicle peptidergic release from astrocytes⁵⁰, found that individual vesicles exhibit high mobility with elongated directional and contorted non-directional tracks. This was further corroborated by quantification of vesicle paths⁸⁸. Similar dual-modal vesicle mobility has been observed in other cell types, such as chromaffin and neuroendocrine cells⁸⁸⁻⁹¹. In contrast to other cell types, however, vesicle traffic in astrocytes exhibited a remarkable regulation by changes in $[Ca^{2+}]_i$ ^{92, 93}. Increase in $[Ca^{2+}]_i$ reduced mobility of peptides- and ATP-containing vesicles⁷¹, while increasing the mobility of vesicles carrying amino acids and labelled by VGLUT1⁹⁴. Recycling vesicles, which take-up extracellular markers (such as antibodies that bind to vesicle cargo or vesicle luminal markers) and subsequently release them by exocytosis, also demonstrate similar vesicle dynamics, both in cultured astrocytes⁹⁵ and in the brain slices⁹⁶. Increase in astroglial $[Ca^{2+}]_i$ arrests movements of recycling vesicles⁹⁵. These vesicle type dependent, regulatory mechanisms may well change in pathologic conditions^{2, 92}.

Astroglia as a central element of neuropathology

Astroglia contribution to neuropathology is multifaceted and most likely disease-specific. The very nature of astrocytes as homeostatic cells makes them central elements in neural tissue response to various insults. In the context of neurological diseases astroglia may undergo pathological remodelling (for example, in Alexander disease - AxD), degeneration with loss of function (in psychiatric diseases or at the early stages of neurodegenerative diseases such as amyotrophic lateral sclerosis - ALS, or Alzheimer's disease - AD) or reactive astrogliotic remodelling instigated by neurotrauma or accumulation of extracellular pathological protein deposits such as Levy bodies of senile plaques^{11, 13, 19, 21, 40, 97-102}. These principal pathological metamorphoses can develop separately or in combination, and moreover distinct astrogliopathological changes may emerge at different stages of neurological disorders. Degeneration and atrophy of astrocytes, which is manifested by a decrease in the number of cells and dwindling of their morphological profiles is often associated with compromised glutamate clearance that seems to be a converging point in many diseases associated with glutamate neurotoxicity. The impaired astroglial glutamate uptake plays a critical role in neurological disorders with broad clinical manifestations, which include ALS, Wernicke's encephalopathy, Huntington's disease (HD) and some psychiatric disorders^{3, 103}. Astrogliotic response is a similarly widespread feature of neuropathology⁹⁸. Astrogliosis represents a conserved defensive reprogramming of astroglial cells, which involves complex biochemical and functional remodelling and produces multiple reactive cellular phenotypes. Reactive astrocytes boost neuroprotection and are critical for post-lesion regeneration of the nervous tissue; inhibition of

astroglial reactivity usually exacerbates neuropathology. The common denominator of astroglipathological changes are associated with morphological remodelling aimed towards atrophy or hypertrophy^{104, 105}. These relatively rapid morphological changes may involve modified vesicular trafficking^{46, 92}; similarly, both astrodegeneration and astroglial reactivity result in changes in astroglial vesicular intracellular networks.

Vesicular dysfunction in neurologic diseases

Vesicle traffic associates with numerous functions of astrocytes including regulation of the plasma membrane density of receptors, pumps, transporters, is involved in changes in morphological plasticity and in cell degradation pathways. Impairment of the vesicle network dynamics hence may contribute to astrogliopathies⁹².

Primary and secondary vesicle network defects

In relation to vesicle network disturbances, in principle, there are two main groups of disorders, depending on the defect within the astroglial vesicular network. First, vesicle traffic may be **primarily** affected due to altered regulation of vesicle mobility including altered association of vesicles with the cytoskeletal elements, mutated cytoskeletal elements, and/or disturbances in the vesicle membrane merger. These alterations are becoming uncovered only recently when experimental approaches enabling direct monitoring of vesicle traffic at single vesicle level in real-time in astrocytes have been introduced^{76, 88, 106} and due to advanced genetic and posttranslational tubulin studies¹⁰⁷. Examples of neurological diseases with primary vesicle network defects are listed in Table 1. Impaired vesicle mobility may result from alterations in cytoskeleton fabrics. Such alterations accompany reactive gliosis with overexpressed intermediate filaments¹⁰⁸. Similarly, cytoskeleton is impaired in AxD, associated with sporadic mutations of glial fibrillary acidic protein¹⁰⁹. Unstable microtubules contribute to the Rett's disease¹¹⁰. General defects in vesicle traffic possibly are observed in neurodegeneration including AD¹¹¹, HD¹¹², and in ALS^{113, 114}. Mutated regulatory proteins affect vesicle dynamics¹¹⁵ in intellectual deficiency (ID)¹¹⁶. Vesicle dynamics may be also altered by virus infection such as the Tick borne encephalitis virus (TBEV) infection¹¹⁷ or autoimmune Devic's disease (neuromyelitis optica)^{118, 119} where vesicle dynamics of AQP4 water channel is affected^{120, 121}. Alterations in vesicle dynamics may develop as side effects of medication, for example, in multiple sclerosis treated with FTY 720¹²² or when using ketamine, an anaesthetic and antidepressant¹⁰⁶, which also affects vesicle merger with the plasma membrane¹²³.

The other vesicle network defects, termed **secondary**, are associated with abnormalities of carried cargo. Enzymatic deficits result in impaired processing of metabolic intermediates, which are transported in vesicles leading to accumulation of the non-degraded cargo in the vesicular compartment that may disrupt (jam) the vesicle network as a whole (Figure 1). In support that the vesicle network defects are of two kinds, primary and secondary, are experiments, where a combination of cytoskeleton alteration and an enzymatic deficit was introduced to study infantile

neuronal ceroid lipofuscinosis (INCL), an inherited neurodegenerative disorder, caused by mutations in the lysosomal hydrolase, palmitoyl protein thioesterase 1 (PPT1), revealed that if both the intermediate filament cytoskeleton (deleting GFAP and vimentin) and the PPT1 were impaired, the INCL progressed more rapidly¹²⁴.

Malfunction of hydrolitic enzymes, such as PPT1, impairs lysosomal degrading pathway; this impairment lies at the core of extended (more than 60 members) family of lysosomal storage diseases^{125, 126}. Roughly two thirds to three quarters of lysosomal storage diseases are neuropathic¹²⁷. Neurological disorders resulting from lysosomal pathology include neuronal ceroid lipofuscinoses (or Batten disease), Krabbe disease, Gaucher disease, Sandhoff disease, Niemann-Pick (NP) type C, Tay-Sachs disease, etc.¹²⁸. In Tay-Sachs disease there is a deficiency of hexosaminidase A, a vital lysosomal hydrolytic enzyme, degrading glycolipids¹²⁹. As a result, these lipids accumulate, and this intracellular accumulation affects normal cell function and interferes with normal tissue processes, eventually leading to premature cell death. Sandhoff disease is characterized by deficiency of hexosaminidases A and B, resulting in excessive lysosomal accumulation of GM2 gangliosides and oligosaccharides containing glucosamine residues¹³⁰. The Niemann-Pick (NP) diseases are a subfamily of lipid storage disorders in which harmful quantities of lipids or fatty acids accumulate in the liver, bone marrow, lungs, spleen, and CNS. In the type A of NP diseases there is a deficiency of sphingomyelinase¹³¹, hence sphingomyelin accumulates in lysosomes, leading to the enlargement of cells. The Gaucher disease reflects upon deficiency of acid-beta-glucosidase, leading to accumulation of glucosylceramide¹³², whereas Krabbe disease is associated with the deficiency of β -galactocerebrosidase¹²⁷. In the context of lysosomal storage diseases, astrocytes demonstrate either reactive phenotypes or apoptotic death, both of which possibly associate with abnormalities of the lysosomal network¹²⁷. Astroglial reactive remodelling sometimes occurs at the early pathological stages preceding (and possibly precipitating) neuronal death; such an early activation is observed, for example, in juvenile Batten disease¹³³. Considerable lysosomal pathology was observed in astrocytes in the context of multiple sulfatase deficiency that cause severe neurodegeneration. Deficient astroglial lysosomal network affects autophagy and is linked to decreased neuronal survival¹³⁴.

In contrast to secondary vesicle network impairments, vesicle dynamics in primary network defects, may be directly affected by changes in cytoskeleton along which vesicles are transported. As mentioned previously, vesicles exhibit directional and non-directional mobility. The former depends on intact cytoskeleton¹⁰⁸ and impairments in the linking of vesicles to cytoskeleton as well as changes in cytoskeleton may lead to pathology. This means that vesicle network dynamics may be a target for treatments.

Neurotropic viruses use endocytosis to enter astrocytes

The rise of Zika virus (ZIKV) epidemic in the Central and South Americas enticed refreshed interest into how the virus, linked with microcephaly (severe under-development of the cerebral cortex in newborn babies from infected mothers¹³⁵), instigates this neurodevelopmental disorder. As shown previously the tick-borne encephalitis virus (TBEV), a human pathogen that causes neuroinfections in

Europe and Asia¹³⁶⁻¹³⁸, infects astroglia, after entering astrocytes by endocytosis¹¹⁷. The TBEV, as well as ZIKV are members of the *Flaviviridae* family^{135, 139}. A similar mechanism of entry into astrocytes, (although it is yet to be fully characterised) underlies ZIKV neurotropism. Again, similar mechanism underlies astroglial infection with human immunodeficiency virus 1 (HIV-1), which enters astroglia by endocytosis, however the HIV-1 virus seems to be effectively destroyed in the astroglial lysosomal pathway¹⁴⁰.

Mechanisms of TBEV entry into rodent astrocytes are complex¹¹⁷. First, to invade the CNS, a neurotropic virus must negotiate the BBB, which protects the CNS tissue from the direct ingress of viruses circulating in the blood^{138, 141}. Astrocytes are a key component of the BBB, with astroglial endfeet providing almost complete coverage of the intracranial vasculature. Astrocytes are positioned between synapses and blood vessels, contributing to neurovascular coupling^{142, 143}. Infection with TBEV does not affect astroglial viability; to the contrary, astrocytes likely serve as a reservoir for TBEV supporting re-infection¹¹⁷. Second, preferential viral infection of astroglia likely reflects the fact that astrocytes provide a favourable environment for virus replication, possibly because since they are the site with a special adaptation of glycolysis. Aerobic glycolysis, characteristic for astroglia, also exists in rapidly dividing cells and in cells undergoing plastic morphological changes, despite the presence of adequate levels of oxygen, a phenomenon known as "the Warburg effect"¹⁴⁴. This form of metabolism is far from being efficient for ATP production; however, it provides biosynthetic intermediates thus being advantageous for developing and growing tissues¹⁴⁵. Aerobic glycolysis, primarily taking place in astrocytes in the CNS, is operative during neurodevelopment and even in adulthood in some areas of the CNS¹⁴⁶. Therefore, this property of astrocytes may be associated with the preferential infection of astrocytes by neurotropic viruses, such as ZIKV.

Intra-astrocytic traffic of TBEV within the endosomal system was monitored in real-time¹¹⁷. As depicted on Figure 2, TBEV particles associate with early endosomes and later, during the post-infection time, also with lysosomes as revealed by co-localization of TBEV particles with endosomal (early endosome antigen 1, EEA1) and lysosomal LAMP1 markers. Similarly to secretory vesicles⁸⁸, endocytosed TBEV-containing vesicles exhibit two forms of mobility in living astrocytes, directional and non-directional. The former likely reflects vesicular transport with protein motors along the cytoskeletal elements, such as microtubules, actin and intermediate filaments, including astroglia-specific glial acidic fibrillary protein (GFAP)¹⁰⁸. In contrast, the mobility of non-directional vesicles is determined by free diffusion⁸⁸. Prolongation of the post-infection time not only increased the number of TBEV particles within an astrocyte, but it also resulted in increased TBEV particle mobility¹¹⁷.

Endocytosis was recently confirmed to be the mechanism of ZIKV infection of astrocytes and microglia¹⁴⁷. The membrane protein Axl, a member of the Tyro3 Axl Mer (TAM) family of proteins, exhibiting tyrosine kinase receptor activity, regulating innate immunity and the removal of apoptotic cells¹⁴⁸, plays a role in targeting glia¹⁴⁷. Since Axl is abundantly expressed in glial but not in neural progenitor cells, this indicates a preferential cellular tropism for ZIKV infection¹⁴⁷. Future

experiments should aim to confirm that neurovirulence of ZIKV and other flaviviruses is associated mainly with astroglia, including radial glia, which are fundamental for the development of the human neocortex, which involves the activity of locus coeruleus (LC)¹⁴⁹. The release of noradrenaline from LC neurones activates aerobic glycolysis in astrocytes, monitored as an increase in cytosolic glucose concentration¹⁵⁰, leading to the production of L-lactate¹⁵¹. The increased levels of L-lactate feed back to the LC neurons¹⁵², an interesting glia-neuronal communication where L-lactate serves as a signal.

Neurodegeneration and vesicle traffic deficits

Neurodegeneration starts decades before clinical presentation, and may involve defective vesicle traffic. The initial changes might be small, but over a long time-period, significant transformations of cells and, subsequently, of tissue function develop. Small initial changes have been detected in astrocytes from 3xTg-AD mice, an animal model for AD, expressing three mutant genes for amyloid precursor protein (APP_{Swe}), presenilin 1 (PS1_{M146V}) and microtubule-associated protein Tau (Tau_{P301L})¹⁵³. These astrocytes, which (according to the disease model genetic design) express only the PS1_{M146V} mutation, exhibit impaired vesicle traffic¹¹¹. Mobility of peptidergic and endolysosomal vesicles in astrocytes, isolated during early pre-symptomatic state, revealed that spontaneous as well as stimulated, Ca²⁺-dependent, vesicle mobility was diminished. The impaired vesicle traffic was observed also in wild type rat astrocytes transfected with familial AD-associated mutated PS1_{M146V} gene. Eventually, altered properties of vesicle mobility may result in reduced peptide secretion, contributing to the development of neurodegeneration¹¹¹. Moreover, a recent study where mutated huntingtin caused impaired peptidergic secretion in HD¹¹², is supporting the view that vesicle traffic defects may lead to neurodegeneration⁹².

In addition to a reduced capacity of vesicular release of peptidergic and various chemical messengers from astrocytes, other vesicle-based mechanisms might contribute to the evolution of neurodegeneration. A prominent histopathological sign of AD is the extracellular accumulation of β -amyloid, which may be associated with a reduced capacity for removal or degrading mechanisms¹⁵⁴. An important factor that may decrease β -amyloid deposits is the release of proteolytic enzymes from exocytotic lysosomes, which degrade the β -amyloid peptide in the extracellular space. Insulin degrading enzyme (IDE) is such an enzyme, secreted from neurones in the healthy brain¹⁵⁵. In astrocytes the secretion of IDE can be increased by an autophagy-dependent way by statins, a process likely leading to statin-induced degradation of extracellular β -amyloid peptide A β 40, which depends on IDE secretion from primary astrocytes¹⁵⁶. Therefore, it appears, that in AD the function of IDE secretion by astrocytes is reduced in comparison to astrocytes in healthy subjects, consequently leading to increased deposits of β -amyloid. The mechanism of reduced secretion of IDE in AD is possibly mediated by an impaired interaction between autophagosomes and lysosomes¹⁵⁴, an interaction needed to facilitate lysosomes to become competent for exocytosis³⁷. The reduced capacity for release of IDE by astrocytes may mirror a general vesicle traffic defect associated with a lysosomal involvement in injury repair in astrocytes¹⁵⁷.

In the vicinity of plaques and β -amyloid deposits astrocytes attain a reactive phenotype with over-expressed intermediate filaments¹⁵⁸. Astrocytes exposed to IFN- γ , a pro-inflammatory cytokine, start to express in their surface membrane MHC-II molecules, turning astrocytes into antigen-presenting cells. These molecules are delivered to the plasma membrane by endolysosomes^{53, 159}. Vesicle delivery of MHC-II molecules to the plasma membrane involves a cytoskeletal network that exhibits upregulated intermediate filaments, a characteristic of astrogliosis¹³. However, microtubules^{160, 161}, actin¹⁶² and their motor proteins^{160, 163} have all been reported to play a role in MHC-II-vesicle traffic. Up-regulation of cytoskeletal structures, in particular intermediate filaments, speeds up vesicle mobility and makes antigen presentation more efficient⁵³. Noradrenaline, released from LC neurones is an endogenous suppressor of antigen presentation by astrocytes⁵⁴; whether this inhibition involves reduced vesicle traffic, remains to be studied.

Neurodevelopment-related diseases and vesicle traffic

Vesicle traffic is strongly involved in neurodevelopment¹⁶⁴, for example, by regulating cell migration during the neocortex formation¹⁶⁵. Thus, when a neurotoxic attack or a viral infection impairs vesicle dynamics, neurodevelopmental defects (such as the microcephaly associated with ZIKV infection) may follow¹³⁵.

In neurodevelopmental diseases, such as in intellectual deficiency (ID), a form of mental retardation characterised with low (< 85) IQ, vesicle traffic disturbances may be the key cellular defect. The only manifest sign in nonspecific ID is a reduced cognitive potential, which when presented together with other clinical signs and symptoms may compose a syndrome (e.g., Down syndrome), or may be associated with mitochondrial, developmental or metabolic deficits¹⁶⁶. Symptoms of X-linked forms of ID (XLID), which appear early in life, are affecting a fair number of subjects; XLID is present in around 2% of the population¹⁶⁷. One of the first mutated genes discovered in patients with XLID is *GDI1*¹⁶⁸, which encodes the α GDI, a regulatory protein of monomeric GTP-ases, such as the family of Rab proteins. The α GDI protein retrieves the inactive, GDP-bound form of Rab proteins, from the membrane. Rab GTPases are important vesicle traffic regulators¹⁶⁹. Therefore, when the causal role for *GDI1* mutated gene in ID was identified, this suggested that vesicular traffic in neuronal synapses is affected^{170, 171}. Astrocytes express α GDI, and vesicle traffic in astrocytes carrying a mutation in *GDI* revealed an impaired vesicle phenotype; vesicle directionality index was reduced, revealing a less efficient coupling of vesicles to the cytoskeleton¹¹⁶. These results are consistent with previously published data, where the role of mutated Rab 4 and Rab 5 monomeric GTP-ases, both regulated by α GDI protein, were studied (Potokar et al., 2012). How the impaired endolysosomal traffic affects the function of astrocytes in ID remains to be clarified.

Abnormal astrocytic vesicle traffic underlies another autistic spectrum disorder, the Rett's syndrome, a rare X-linked neurodevelopmental disease¹¹⁰. Mutations in the methyl-CpG-binding protein 2 (*Mecp2*), encoding a multifunctional protein that binds to methylated DNA, acting as a transcriptional regulator are believed to be responsible. When *Mecp2* is re-expressed specifically in astrocytes in the *Mecp2*-null mice, locomotion and anxiety levels

were significantly improved, respiratory abnormalities were restored to a normal pattern and lifespan was greatly prolonged compared with knock-out mice ¹⁷². Microtubule-dependent vesicle transport is altered in Mecp2-deficient astrocytes isolated from newborn Mecp2-deficient mice and from human MECP2 p.Arg294* iPSC-derived astrocytes ¹¹⁰. Administration of microtubule-stabilizing drug (Epothilone D), that passes BBB restored microtubule dynamics in Mecp2-deficient astrocytes and in human MECP2 p.Arg294* iPSC-derived astrocytes *in vitro*. Therefore, drugs that target not only the fusion/fission of vesicles with the plasma membrane as does ketamine, but also molecules that target the integrity of the cytoskeleton are valid targets for new innovative treatments of impaired vesicle networks to be used in future therapies.

Vesicle network dynamics, a target of drugs for treating neurological disturbances

Several drugs, currently used to treat neurological disease, have an effect on astrocytic vesicle dynamics. Vesicle mobility in astrocytes is decreased by fingolimod or FTY720 ¹²². This drug (Gilenya, Novartis), which is hydrophobic in nature, accumulates in the white matter in the CNS and is used for the treatment of multiple sclerosis ¹⁷³. The marketing authorization holder claims that the mechanism of action of this drug is an inhibition of the egress of lymphocytes into the CNS in its phosphorylated form, acting via the sphingosin 1 receptor (SP1R) ¹⁷⁴. However, this molecule also acts in the unphosphorylated form directly on astrocytes where it affects astrocytic vesicle dynamics ¹²² and likely also antigen presentation by astrocytes, that associates with neuroinflammation ⁵³. FTY 720 treatment revealed that traffic of all vesicle types studied were affected ¹²², hence this drug may modulate the release of other proinflammatory factors from astrocytes, including eicosanoids, which are released in the CNS by an ATP-dependent mechanism ¹⁷⁵. Regulated release of ATP from astrocytes ⁸⁴ participates in the neuroinflammatory CNS states and alterations of traffic of ATP carrying organelles may additionally affect neuroinflammatory response in diseased conditions.

Another established drug, ketamine, an anaesthetic and an antidepressant, affects the vesicle traffic and BDNF release from astroglia ¹⁰⁶. High-resolution membrane capacitance measurements revealed that in the presence of clinically relatively low concentrations, ketamine induces fusion pores to enter into a stable, narrow flickering state ¹²³. In the presence of ketamine, the fusion pore attains a diameter that is too narrow to permit the discharge of the peptides contained in the vesicle lumen ^{106, 176}. These results indicate that the fusion pore, an intermediate stage of the secretory vesicle traffic, is a target for therapy.

Conclusions

Vesicular network consists of many pathways, where endocytotic and exocytotic vesicles merge and give rise to new vesicles, essentially intersecting with the lysosome, present in nearly all eukaryotic cells including astrocytes. Thus lysosomes, being at the crossroad of many vesicle-based processes, not only contribute to their classically assigned function – degradation of molecules - but instead, control the whole vesicle network, contributing to important subcellular physiological events, including the modulation of the metabolic and signalling capacity of astrocytes, as well as determining the morphological plasticity and adaptability to pathologic changes. By understanding more fully the mechanisms of vesicle network traffic and the interaction of vesicles with the

cytoskeleton, at various stages, from how endocytotic mechanisms prime some lysosomes to becoming exocytotic vesicles, to how vesicles containing peptidergic cargo, emerging from Golgi, interact with antigen presentation systems and phagocytosis. Clearly, as addressed in this review, all these processes may well form the basis for neurological dysfunction and represent targets for development of new therapies.

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Conflicts of Interest

The authors declare no conflict of interest.

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Table 1 Neurological diseases associated with primary and secondary vesicle network defects

Diseases and neuropathological states	Astroglial Vesicle Network Defect		Mechanism/Comment	Reference
	Primary	Secondary		
Tay Sachs disease, a member of the lysosomal storage diseases		X	Hexosaminidase A deficiency	¹²⁹
Sandhoff disease, a member of the lysosomal storage diseases		X	Hexosaminidase A and B deficiency	¹²⁷
Nieman-Pick's Type A disease, a lipid storage disorder		X	Sphingomyelinase deficiency	¹³¹
Alexander's disease	X		Cytoskeletal defect	¹⁰⁹
Devic's disease (Neuromyelitis optica)	X?		Autoantibodies against AQP4, affecting AQP4 vesicle dynamics	¹¹⁸⁻¹²⁰
Alzheimer's Disease	X		General vesicle traffic defect	¹¹¹
Intellectual Deficiency	X		General vesicle traffic defect	¹¹⁶
Rett's disease	X		General vesicle traffic defect/cytoskeletal defect	¹¹⁰
Glycogen Storage Disease		X	Glucose-6- phosphatase deficiency	¹⁷⁷
Neuroinfection by viruses (Tick-borne encephalitis virus - TBEV)	X?		Affecting endocytosis and endolysosomal traffic dynamics	¹¹⁷
Amyotrophic lateral sclerosis (ALS)	X?		Signalling and vesicle traffic defect	^{113, 114}
Huntington's Disease	X		Mutated huntingtin affects vesicle traffic	¹¹²
Ketamine treated psychiatric disease	X		Affecting vesicle merger with the plasma membrane/signalling	^{106, 123}
Fingolimod (FTY 720) treated multiple sclerosis	X		Affecting vesicle dynamics	¹²²

Reactive gliosis	X		Overexpression of intermediate filaments affects vesicle dynamics	¹⁰⁸
Microcephaly, lissencephaly	X		Tubulin cytoskeleton alterations in radial glia leading to abnormal cell division and migration	¹⁰⁷
Gaucher disease		X	deficient acid-beta-glucosidase, leading to accumulation of glucosylceramide	¹³²
Krabbe disease, a form of leukodystrophy		X	deficiency of β -galactocerebrosidase leads to build-up of unmetabolized lipids affecting the growth of the myelin sheath	¹²⁷
Infantile neuronal ceroid lipofuscinosis (INCL), neurodegenerative disease		X	Deficiency of palmitoyl protein thioesterase 1, a lysosomal enzyme	¹²⁴
Batten disease, neuronal ceroid lipofuscinoses, a form of lysosomal storage diseases		X	Lipofuscin materials build up in neuronal cells and many organs	¹³³

Figure legends

Figure 1. The vesicular-lysosome network.

Intracellular secretory organelles (synaptic-like vesicles, dense-core vesicles and primary lysosomes) originate from the endoplasmic reticulum and Golgi complex. Primary lysosomes fuse with endosomes, phagosomes and autophagosomes and convert to secondary lysosomes which undergo exocytosis thus expelling products of degradation. The multivesicular bodies contain exosomes that may carry various signalling factors.

Figure 2. Time-dependence of the number of endosomes and lysosomes associated with DiD-TBEV particles in astrocytes.

(Ai,ii). An astrocyte with DiD-labelled TBEV vesicles (TBEV) incubated at 37 °C for 4 h and 18 h and with labelled early endosomes (EEA1) Overlays represents overlapped TBEV and EEA1 fluorescent signals, indicating the association between TBEV and endosomes. Bars: 5 μ m. (Aiii) Prolonged

incubation increased the average number of TBEV labelled vesicles per cell and also the average number of vesicles co-labelled with TBEV and EEA1. Black bars - TBEV labelled vesicles, white bars - TBEV and EEA1 co-labelled vesicles, $*P<0.05$. (Bi,ii) An astrocyte with DiD-labelled TBEV vesicles (TBEV) incubated at 37 °C for 4 h and 18 h and with LAMP1-labelled lysosomes (LAMP1-lysosomal associated membrane protein 1). Overlays represent overlapped TBEV and LAMP1 fluorescent signals, indicating the association between TBEV and lysosomes. Bars: 5 μ m. (Biii) Prolonged incubation increased the average number of TBEV labelled vesicles per cell and the average number of vesicles co-labelled with TBEV and LAMP1. Black bars - TBEV labelled vesicles, white bars - TBEV and LAMP1 co-labelled vesicles, $***P<0.001$. (C) Astrocyte co-labelled with anti-LAMP1 and anti-EEA1. In a single, 1 μ m thick optical slice, lysosomes and early endosomes appear to be largely different in size due to different position in z-axis and variable antibody attachments. Arrowheads point to large late lysosomes (LAMP1) and early endosomes (EEA1). Bar (bar inset): 5 μ m (2.5 μ m). n (n) = number of cells (number of vesicles). Reprinted with permission ¹¹⁷.

Figure 3 Secretory vesicles studied by STED and SIM microscopies in acutely isolated rat astrocytes.

(A) Confocal and STED microscopy images of immunostained D-serine-, V-GLUT1-, ANP- and BDNF-positive vesicles in acutely isolated astrocytes. Histograms display STED-acquired vesicle diameter distributions for 1788 (D-serine), 6787 (V-GLUT1), 1747 (ANP) and 798 (BDNF) vesicles (2 cells per staining). The black curves show Gaussian fits of the diameter distributions; the numbers next to the distribution peaks indicate the average vesicle diameter (expectation value $\pm$ SEM). Recalculated values taking into account the microscope's optical resolution (45 nm) are 80.8 nm for D-serine, 88.4 nm for V-GLUT1, 85.9 nm for ANP and 86.8 nm for BDNF. Scale bar, 500 nm.

(B) Wide-field microscopy and SIM were used to determine the vesicle diameter of immunostained LAMP1 endolysosomes and ATP-loaded vesicles (quinacrine dihydrochloride). Histograms show SIM-acquired vesicle diameter distributions for 557 (LAMP1, 2 cells) and 445 (quinacrine, 2 cells) vesicles in acutely isolated astrocytes (upper two panels) and 338 (LAMP1, 3 cells) and 333 (quinacrine, 6 cells) vesicles in astrocytes isolated from 7- to 8-week-old rats (lower two panels). The black curves show Gaussian fits of the diameter distributions; the average vesicle diameter (expectation value $\pm$ SEM) is labelled next to the distribution peaks. Scale bar, 500 nm. Modified with permission ⁸³.