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Biogenesis and secretion of mitovesicles, small extracellular vesicles of mitochondrial origin at the crossroads between brain health and disease

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Abstract

In the brain, mitochondrial components are released into the extracellular space via several mechanisms, including a recently identified type of extracellular vesicles called mitovesicles. While vesiculation of neuronal mitochondria yields various intracellular types of vesicles, with either a single or a double membrane, mitovesicles secreted into the extracellular space are a unique subtype of these mitochondria-derived vesicles, with a double membrane and a specific set of mitochondrial DNA, RNA, proteins, and lipids. Based on the most relevant literature describing mitochondrial vesiculation and mitochondrial exocytosis, we propose a model for their secretion when the amphisome, a hybrid endosome–autophagosome organelle, fuses with the plasma membrane, releasing mitovesicles and exosomes into the extracellular space. In aging and neurodegenerative disorders, mitochondrial dysfunction, in association with endolysosomal abnormalities, alter mitovesicle number and content, with downstream effect on brain health.

Introduction

Extracellular vesicles (EVs) are membrane-bound vesicles that are released into the extracellular space [1,2], mediating physiological mechanisms that are critical for

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Declaration of Competing Interest

None.

maintaining cell homeostasis, including waste disposal, cell-to-cell communication, and exchange of trophic factors [3,4]. However, EVs also play a pathogenic role in disease propagation by spreading toxic molecules, including protein aggregates, from one cell to the other, both within the same organ [1] and in an inter-organ fashion [5]. Classically, EVs were divided into two main subgroups, the larger (100–1000 nm) plasma membrane–derived ectosomes and the smaller (50–200 nm) late endosome/multivesicular body (LE/MVB)–derived exosomes [2], although more recent works have demonstrated that their heterogeneity is higher than what originally anticipated, including subpopulations of EVs of mitochondrial origin [6]. Several research groups demonstrated the presence of mitochondrial components in the extracellular space [7,8], with differences in their nature, characteristics, functions, biogenesis, and secretion mechanisms. Some groups suggested that whole mitochondria are transferred between cells [9–11]. Others proposed either the encapsulation of mitochondria within large ectosomes [12,13] or the incorporation of undegraded mitochondrial molecules into exosomes [7,14,15]. We recently modified the method of isolation of EVs from brain tissue and identified a separate type of small (median diameter under cryogenic electron microscopy: ~110 nm; 25th to 75th percentiles: 90–140 nm [6,16]) mitochondria-derived EVs that do not contain endosomal, exosomal (e.g. tetraspanins), or plasma membrane components and therefore cannot be classified as exosomes or ectosomes [6]. Using this method, fractions enriched in ectosomes and exosomes do not contain any mitochondrial components [6]. On the other hand, these EVs are also different from nude mitochondria, both in their morphology (mitovesicles are one order of magnitude smaller, bear a narrower intermembrane space, lack *cristae*, and are spherical; Figure 1), and cargo (e.g. mitovesicles do not contain TOMMs and ribosomes, unlike mitochondria [6,16]; see section ‘Characterization of mitovesicles’ for more details). To stress their uniqueness, we named them ‘mitovesicles’, a portmanteau of the words ‘*mito*chondrion’ and ‘extracellular *vesicles*’.

The impact of mitochondria-derived EVs in physiology and disease is unclear. Once taken up by recipient cells, both proinflammatory [10,17,18] and anti-inflammatory [19,20] activities have been documented. Similarly, some studies supported the idea that EVs containing mitochondrial material promote cell metabolism [9], while others have shown an opposite effect [5,21]. We have recently shown that brain mitovesicles impair long-term potentiation in otherwise normal hippocampi when used at a concentration three times higher than what found in the physiological brain [16], suggesting that mitovesicles have a role in neurotransmitter metabolism and synaptic regulation. Molecular mechanisms driving mitochondria-derived EV production and secretion are controversial, as well. For instance, some groups reported that the overactivation of the PINK1/Parkin axis suppresses the release of mitochondria-derived EVs [11,20], whereas other studies showed the contrary [5,15].

A possible model to reconcile all these discrepancies postulates the existence of multiple subtypes of mitochondria-derived EVs. As there is no gold standard to date to purify mitochondria-derived EVs, this model implies that differences in the isolation protocol may lead to the enrichment of one or multiple subpopulations of mitochondria-derived EVs, potentially with different downstream effects. For example, in some studies, mitochondrial components were analyzed in samples containing both large and small EVs [20,22]; in other studies, an intermediate centrifugation speed (e.g. 60 000*g*) was used to pellet EVs

without prior removal of larger materials [11], whereas in other studies only small EVs (e.g. 100 000*g* pellets) were analyzed after discarding the 10 000*g* large EV pellet [6]. Thus, it is conceivable that divergences in purification steps likely cause the enrichment of EV pools with different characteristics. In addition, mitochondria-derived EV studies have been challenged by the absence of a clear method to separate these EVs from other types of EVs of similar size. In most studies, heterogeneous EV pools are generated using size-based techniques, including differential centrifugation and size-exclusion chromatography. However, mitochondria-derived EVs comprise only a small fraction of the total EV populations (up to 1–2%, according to the source or the subtype analyzed [6,10,23]) and overlap in size and therefore co-isolate with other known EVs using these techniques [6,10,13]. Herein, we will focus exclusively on mitovesicles, the subpopulation of small EVs of mitochondrial origin with neither ectosome nor exosome proteins, given their relative novelty and potential functions *in vivo* in the brain [6,16]. Based upon published relevant data, we propose a model for mitovesicle biogenesis and secretion and discuss the potential role for these EVs in the brain during aging and in neurodegenerative diseases, focusing on Alzheimer's disease (AD) and Down syndrome (DS).

Main text of the review

Characterization of mitovesicles

In 2019, Jeppesen et al. demonstrated that ectosomes can be separated from exosomes accurately after their release *in vitro* by exploiting the density differences among these EVs, rather than their size [24]. Using a similar strategy, we developed a novel density-based technique to fractionate small EVs upon their isolation from brain tissue [6,16,23,25–27], and we focused our attention on mitochondria-derived EVs. We showed that in the brain, mitochondrial components are not found in exosome- or ectosome-enriched fractions and that these newly identified EVs of mitochondrial origin (mitovesicles) do not carry endosomal, exosomal, cytosolic, or plasma membrane proteins [6]. In addition, these EVs have exclusive morphometrical features when compared with ectosomes and exosomes, including a double concentric membrane and an electron-dense core (Figure 1). An independent study later found EVs with the same morphometrical features, likely mitovesicles, in small EVs isolated from the cell culture medium of mouse embryonic fibroblasts [20], suggesting the existence of mitovesicles outside of the brain. Moreover, mitovesicles contain mitochondrial DNA (mtDNA) and mitochondrial RNA (mtRNA), and their membranes carry a different set of lipids when compared with other brain EVs, including cardiolipin [6], further reinforcing mitovesicle uniqueness. Although mitochondrial proteins and components are exclusively associated with mitovesicles in small EVs isolated from the physiological brain [6], this may not be a universal trait. In other solid tissues, especially under disease conditions such as cancer, several types of mitochondria-derived EVs may be present, including EVs that can be immunocaptured with antibodies for the mitochondrial proteins mt-CO2 and FACL4 and that contain both ectosome (Annexin A1, A2, V) and exosome (tetraspanins) proteins [22,28], suggesting multiple, complex, and possibly tissue-specific routes for mitochondrial exocytosis.

Mitovesicle biogenesis and secretion

The presence of cardiolipin in mitovesicle membranes suggests a distinct mechanism for the biogenesis of these EVs when compared with exosomes and ectosomes. Cardiolipin is found almost exclusively in the inner mitochondrial membrane and in traces in the outer mitochondrial membrane [29], indicating that mitovesicles likely bud directly from these organelles. This speculation implies that mitochondria are able to vesiculate and generate intracellular vesicles with the same morphological features and size (50–300 nm) of mitovesicles, eventually released into the extracellular space (Figure 2). These intracellular mitochondria-derived vesicles (MDVs) were previously described, both *in vitro* and *in vivo*, including in the brain [30–36]. It was shown that MDVs traffic to the endolysosomal system, and it was suggested that this is an alternative route to mitophagy to deliver mitochondrial cargo to the lysosome for degradation [30–36]. However, most lysosomal markers, including LAMP1, RAB7, and Lysotracker (all used previously to mark lysosomes in MDV studies [32,35,37,38]), are not lysosome-specific and are also associated with LE/MVBs and amphisomes, organelles originating upon fusion of autophagosomes with LE/MVBs [39]. This discrepancy is further exacerbated in neurons, where only ~20% of $LAMP1^+$ organelles are fully acidified and can be considered as mature lysosomes [39]. Thus, it is conceivable that MDVs reach the LE/MVB/amphisome and are either transported to the lysosome for degradation or released into the extracellular space as mitovesicles when these organelles fuse with the plasma membrane (Figure 2).

Intracellular MDVs are highly heterogeneous and include at least three subgroups conventionally named after their differential cargo. Among them, $MAPL^+$ and $TOMM20^+/PDH^-$ (hereafter $TOMM20^+$) MDVs constitute approximately 90% of the total intracellular MDV population under physiological conditions, while a third type ($TOMM20^-/PDH^+$; hereafter PDH^+) accounts for the remaining 10% [32]. Whether $TOMM20^+$ MDVs are single or double membraned is unclear. Early reports suggested that $TOMM20^+$ MDVs are single-membraned vesicles that bud from the outer mitochondrial membrane (lacking matricial components such as mtDNA or proteins such as PDH and the ectopically expressed matricial protein OCT-DsRed2 [35]), in a process that is reminiscent of the generation of vesicles from bacteria [30]. More recently, this conclusion was questioned by proteomic analyses of purified $TOMM20^+$ MDVs, demonstrating the presence of some proteins from the inner mitochondrial membrane and from the matrix, although it is not clear whether these MDVs are organized with a structured double bilayer or whether these proteins are degradative cargo. Electron microscopy photomicrographs of $TOMM20^+$ cytosolic small vesicles suggested that these MDVs are single membraned [32,35]. The authors also demonstrated that $TOMM20^+$ MDVs are enriched in outer membrane components, including proteins involved in mitochondrial motility (such as MIRO1) and proteins of the BCL2 family (such as BAD), and carry intact TOM macrocomplexes formed by TOMM20, TOMM40, TOMM70, TOMM22, and TOMM7 multisubunits [32]. In addition, $TOMM20^+$ MDVs are largely characterized by lack of a transmembrane H^+ potential, inability to produce ATP, and absence of mtDNA [30,32,35,40].

Unlike $TOMM20^+$ MDVs, the brain extracellular mitovesicle proteome does not include TOMMs (such as TOMM20), mitochondrial motility proteins, and BCL2 family members

with the exclusion of BAX [6], albeit it encompasses matricial proteins, such as PDH subunits and all the Krebs' cycle components [6]. Lipidomic data confirm the different nature of the membranes that surround TOMM20$^+$ MDVs and mitovesicles, with cardiolipin being enriched in mitovesicles [6] but not in TOMM20$^+$ MDVs [32], again suggesting the presence of the inner mitochondrial membrane (where cardiolipin is enriched) within mitovesicles but not in TOMM20$^+$ MDVs. Moreover, mitovesicles contain mtDNA [6], similar to intracellular PDH$^+$ MDVs [40], and can produce a transmembrane H+ potential, as well as ATP in a cell-free system [6,23], similar to TOMM20-negative MDVs generated by *Saccharomyces cerevisiae in vitro* [41]. As TOMM20 cannot be detected in small brain EVs [6] and TOMM20$^+$ MDVs do not share molecular and morphological characteristics with extracellular mitovesicles, we postulate that TOMM20$^+$ MDVs are not secreted into the extracellular space in the brain. The absence of TOMM20 in small EVs may not be brain-specific, as it was described in several studies analyzing small EVs isolated from cell culture media [42,43], albeit it is unclear how common this molecular trait is among different cell sources. On the other hand, PDH$^+$ MDVs share with mitovesicles several molecular and morphological properties [30,35,37,38,44] and likely represent nascent intracellular mitovesicles. Moreover, signals and pathways stimulating PDH$^+$ MDVs production and mitovesicle biogenesis are identical, supporting the conjecture that PDH$^+$ MDVs and mitovesicles are the same entity prior to (PDH$^+$ MDVs) and after (mitovesicles) the release from the cell. Among others, (1) mitochondrial reactive oxygen species (ROS) induce the generation of PDH$^+$ MDVs [35,38] and positively correlate with mitovesicle and small mitochondria-derived EV production, both *in vitro* and *in vivo* [5,6,26,31,43,45]; (2) the number of both PDH$^+$ MDVs and mitovesicles is dependent on mitochondrial fission proteins, such as DRP1 and MFF, which mediate the pinch-off event through which the tip of a mitochondrial protrusion is detached from the body of the mitochondrion to generate a vesicle [26,32]; (3) the biology of both PDH$^+$ MDVs (but not TOMM20$^+$ MDVs [32,35]) and small mitochondria-derived EVs, including mitovesicles, is similarly affected by autophagy and mitophagy initiation alterations, including a positive correlation with the PINK1/Parkin axis, both *in vitro* [5,15,30,38,46–48] and *in vivo* in a chronic cocaine exposure model [26]; (4) blocking the activity of the lysosome, either pharmacologically or genetically, causes the intracellular accumulation of PDH$^+$ MDVs [30,32,34,35,37,38] and a higher extracellular release of small mitochondria-derived EVs [5,42,43].

As PDH$^+$ MDVs are preferentially trafficked to the late endocytic pathway, it is conceivable that mitovesicles are secreted when fully mature LE/MVB/amphisomes fuse with the plasma membrane (Figure 2). This speculation is partially supported by studies showing that knocking down RAB27A, a protein responsible for the docking of LE/MVB/amphisomes to the plasma membrane [49], inhibits the release of small mitochondria-derived EVs [15,42]. The mechanism by which intact PDH$^+$ MDVs reach the lumen of the LE/MVB/amphisome without losing their properties remains elusive. A simple model where nude PDH$^+$ MDVs fuse with the endosomal membrane to release the core vesicle into the lumen of LE/MVB/amphisomes is unlikely, as it implies the loss of the outer membrane of PDH$^+$ MDVs during the process. Likewise, incorporation through invagination from the surface of LE/MVB/amphisomes is equally unlikely as it would generate triple-membraned EVs. Therefore, we propose the involvement of the autophagy machinery as the shuttle

system to deliver PDH^+ MDVs to the endocytic pathway (Figure 2). Although no direct evidence is available to confirm this model, several indirect observations support it. For instance, $LC3^+CD81^+$ LE/MVB/amphisomes incorporate more mitochondrial proteins when lysosome acidification is blocked [42], a stimulus that was shown to promote mitochondria-derived EV secretion [5,42,43]. Moreover, autophagy-deficient mouse embryonic fibroblasts secrete less mitochondria-derived EVs when compared with autophagy-competent cells [50] while they produce the same number of PDH^+ MDVs [35], implying that the phagophore may play a role in secretion, not biogenesis, of mitovesicles. Accordingly, the trafficking of PDH^+ MDVs (but not $TOMM20^+$ MDVs) to the endocytic system depends on the core machinery that mediates the fusion of autophagosomes with the endolysosome [37,51], suggesting that nascent mitovesicles are delivered to the endocytic pathway via autophagosomes.

Thus, we hypothesize that mitovesicles are generated as the PDH^+ subtype of MDVs, incorporated into LE/MVB/amphisomes, and eventually released into the extracellular space, similar to the fate of intraluminal vesicles (ILVs) within LE/MVB/amphisomes, which are released as exosomes. Although we cannot rule out unknown alternative mechanisms, we also propose a role for the autophagosome as a shuttle system to deliver intact PDH^+ MDVs into the lumen of LE/MVB/amphisomes before secretion (Figure 2).

Mitovesicle roles in brain homeostasis and neurodegenerative disorders

The endocytic pathway plays a central role in the elimination of toxic molecules, including protein aggregates and cleavage products, that are key molecular players in the pathogenesis of neurodegenerative diseases [4,52–54], including in AD where endosomal abnormalities were described, preceding the onset of dementia [4,52–54]. Additionally, a faulty autolysosome acidification is typically found in AD brains and in mouse models of β-amyloidosis, as well as in DS [55], causing ILV accumulation and LE/MVB/amphisome enlargement (Figure 3) [4,56] and leading to a concomitant higher release of ILVs as exosomes (Figure 2, right) [4,57].

Mitochondrial dysfunction and oxidative stress are also hallmarks of AD [58]. Consistent with what described above, mitochondrial ROS and endosomal changes co-operatively support the generation and the release of a higher number of mitovesicles in AD and DS brains (Figure 2, right) [6]. How these changes affect the progression of the disease is far less clear. *In vitro* data show that the downregulation of RAB27A (and consequent depression of EV secretion) causes mitochondrial impairment [15], suggesting a cross-talk between EV secretion, endosomal function, and mitochondrial quality control mechanisms alternative to mitophagy. In our model, the higher mitovesicle secretion in AD may serve as a homeostatic attempt of the source cell to support metabolism by eliminating unwanted mitochondrial components that are not digested by defective lysosomes. This potential prosurvival mechanism leads over time to the accumulation of mitovesicles in the extracellular space, leading to a roughly threefold increase in their number in the brain of older people with DS when compared with age-matched controls [6]. Unlike other types of EVs, intact mitovesicles retain a metabolic activity in the extracellular space, at least in an artificially reconstructed extracellular environment [6], and the extracellular accumulation

of mitovesicles in AD and DS may enhance this cell-free catabolic activity. Accordingly, we speculate that mitovesicles in AD acquire toxic enzymatic activities once released into the brain extracellular fluids, partially due to their larger number, and partially due to AD-specific differences in their content, as was shown for DS [6]. In this perspective, the monoamine oxidases type A and B (MAO-A and MAO-B) are two potential candidates that can participate in pathogenic challenges in AD [59]. MAOs catalyze the degradation of monoamines generating ROS as a byproduct of the reaction [60] and are found on the mitovesicle outer membrane, where they retain their enzymatic activity [6]. Thus, higher diffusion of mitovesicles throughout the brain in AD may cause increased extracellular MAO activity, leading to alterations in monoamine levels and enhanced ROS production, ultimately causing neurotransmitter imbalance and spread of oxidative damage [16].

Sex differentially affects mitovesicles during aging

Mitochondrial dysfunction is a hallmark of aging [61], including impairment of the electron transport chain [62], changes in mitochondrial dynamics [63], and defective mitophagy [64], leading to age-dependent abnormalities in mitochondrial quality control. Thus, consistent with the observation that mitochondrial dysfunction promotes the secretion of mitovesicles [6], aging interferes with mitovesicle biology. *In vitro*, the secretion of small EVs from senescent cells is increased when compared with controls [65–67], and *in vivo* the overall secretion of brain mitovesicles increases during aging in a sex-independent fashion [45]. However, sex-specific changes were found when the cell types responsible for these changes were examined. Whereas the release of astrocytic mitovesicles ($SFXN5^+$) increases in the brain of both males and females, the number of neuronal mitovesicles ($OCIAD2^+$) increases with age exclusively in the brain of female mice [45].

As discussed above, we speculate that mitovesicle secretion has a role in mitochondrial quality control to protect against the accumulation of potentially harmful damaged mitochondria and to maintain a healthy pool of these organelles in the cell, particularly when lysosome activity is impaired. The observed increase in mitovesicle secretion in the aging brain may thus indicate age-related changes in mitochondrial function in both male and female brains, with a more pronounced effect in females. In addition, the observation that aging neurons in female mouse brains release a greater quantity of mitovesicles into the brain extracellular space, compared with male counterparts, highlights the ability of female brains to uphold the quality control system of mitochondria in neurons, ultimately contributing to brain homeostasis.

Conclusions

In addition to the existence of large ectosomes containing parts or whole mitochondria, the integration of data from several independent groups indicates that mitochondrial material in small EVs in the brain is mostly, if not exclusively, encapsulated by a specialized subtype of EVs originating directly from mitochondria: mitovesicles. In our model, mitovesicles are generated intracellularly as double-membraned PDH^+ MDVs and eventually secreted into the extracellular space together with exosomes when the LE/MVB/amphisomes fuse with the plasma membrane (Figure 2). In aging and neurodegenerative disorders, mitochondrial

stress co-operates with concomitant changes in the endosomal pathway, causing profound alterations in mitovesicle biology and potentially contributing to phenotypic manifestations.

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Data Availability

No data were used for the research described in the article.

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Papers of particular interest, published within the period of review, have been highlighted as:

• of special interest

•• of outstanding interest

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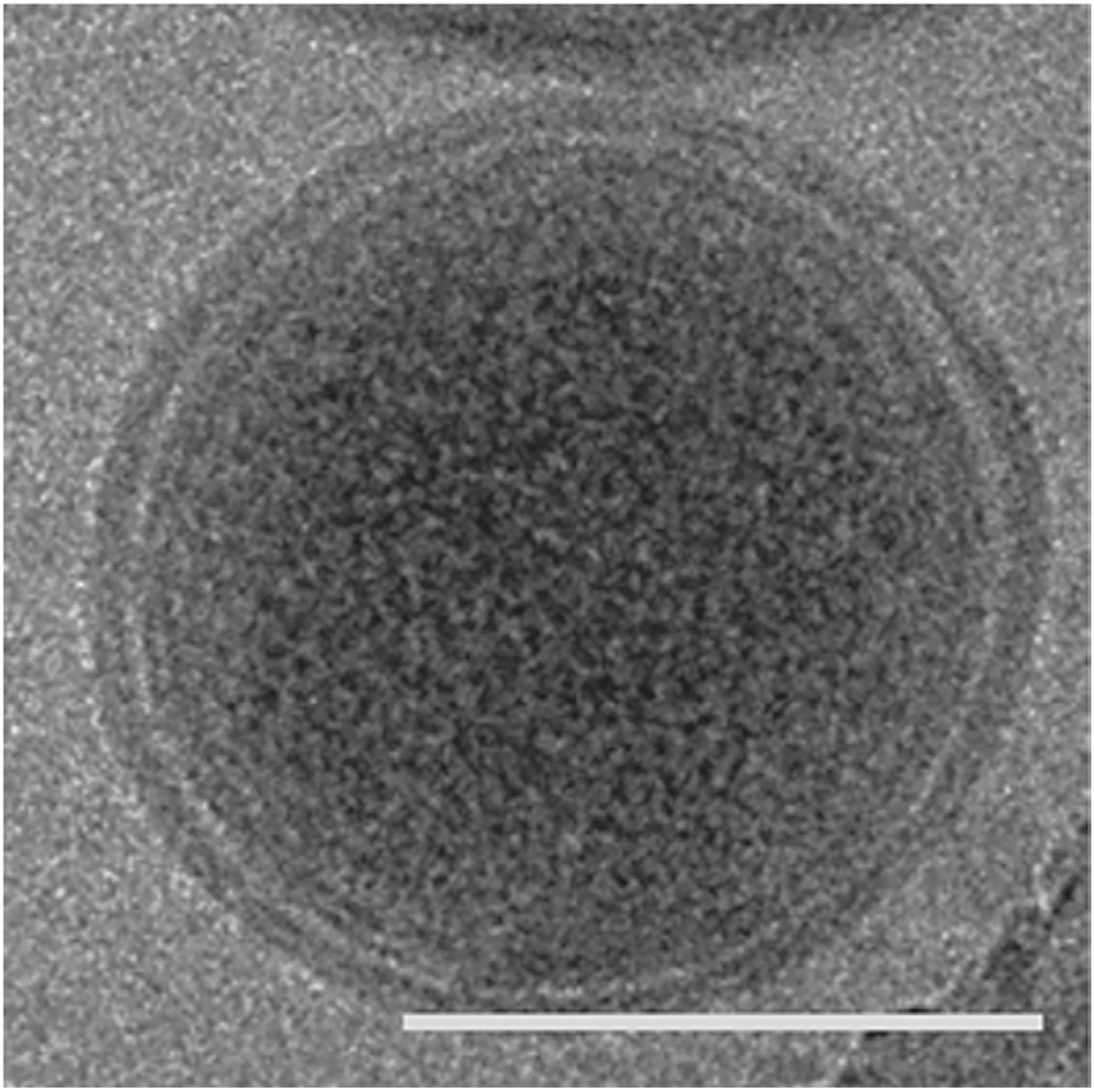

Figure 1.

Mitovesicles morphological properties. Representative cryogenic electron microscopy photomicrograph of a mitovesicle isolated from the brain of a C57BL/6 male mouse at 12 months of age. Note the double membrane and the electron-dense core. Scale bar = 100 nm.

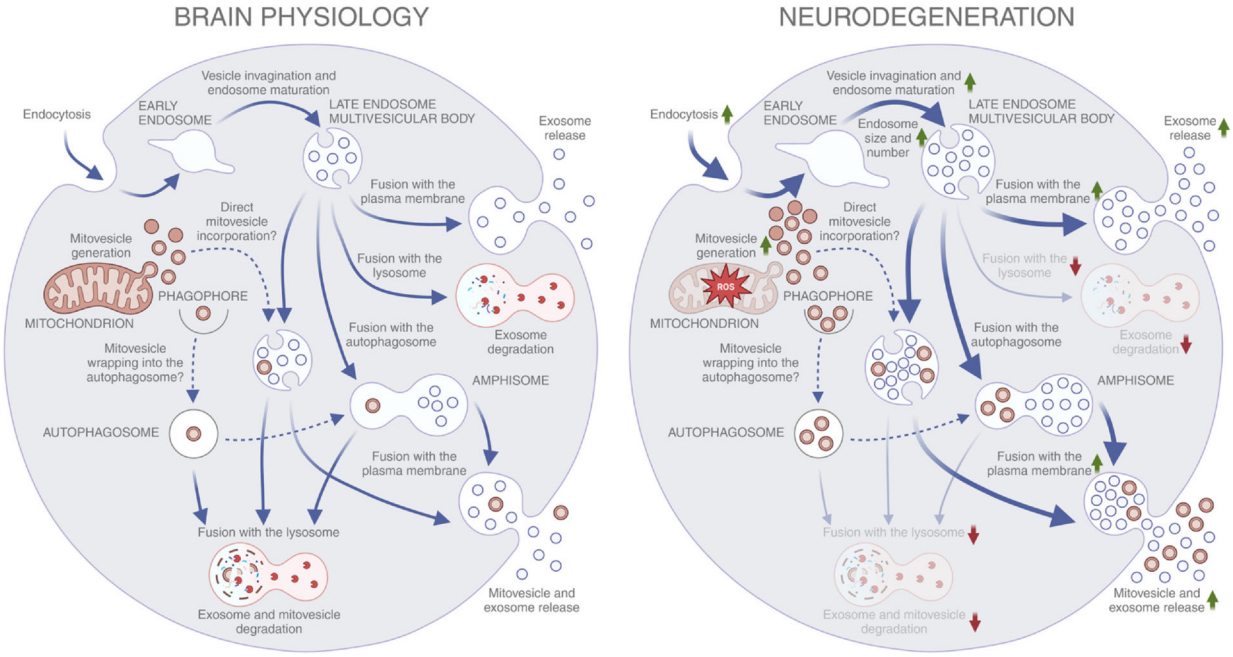

Figure 2.

A unified model for the secretion of exosomes and mitovesicles in the brain via the endocytic pathway. Under physiological conditions (left), upon endocytosis, early endosomes undergo a process of maturation through which they invaginate cytosolic material into the lumen and generate ILVs. Fully mature endosomes, also known as LE/MVBs (see Figure 3), eventually fuse with the plasma membrane (to release ILVs into the extracellular space), the lysosomes (for degradation of the same vesicles and other endocytic material), or the autophagosomes (to generate hybrid organelles called amphisomes). Double-membraned vesicles known as PDH^+ MDVs bud from the surface of mitochondria and are delivered to the endocytic pathway with a yet unidentified mechanism. We propose a role for the autophagosomes as shuttle systems to deliver intact PDH^+ MDVs into the lumen of the LE/MVB/amphisomes. During neurodegeneration (right), a higher endocytic flux drives the accumulation of enlarged early endosomes, while lysosomal dysfunctions hinder the degradation of ILVs, causing MVB enlargement and ILV accumulation, as well as higher exosome secretion. In parallel, higher production of mitochondrial ROS promotes the biogenesis of PDH^+ MDVs and their trafficking into the endocytic system. Given the defective functionality of lysosomes, PDH^+ MDVs will preferentially be released as mitovesicles into the brain extracellular space together with exosomes instead of being digested. Created with BioRender.com.

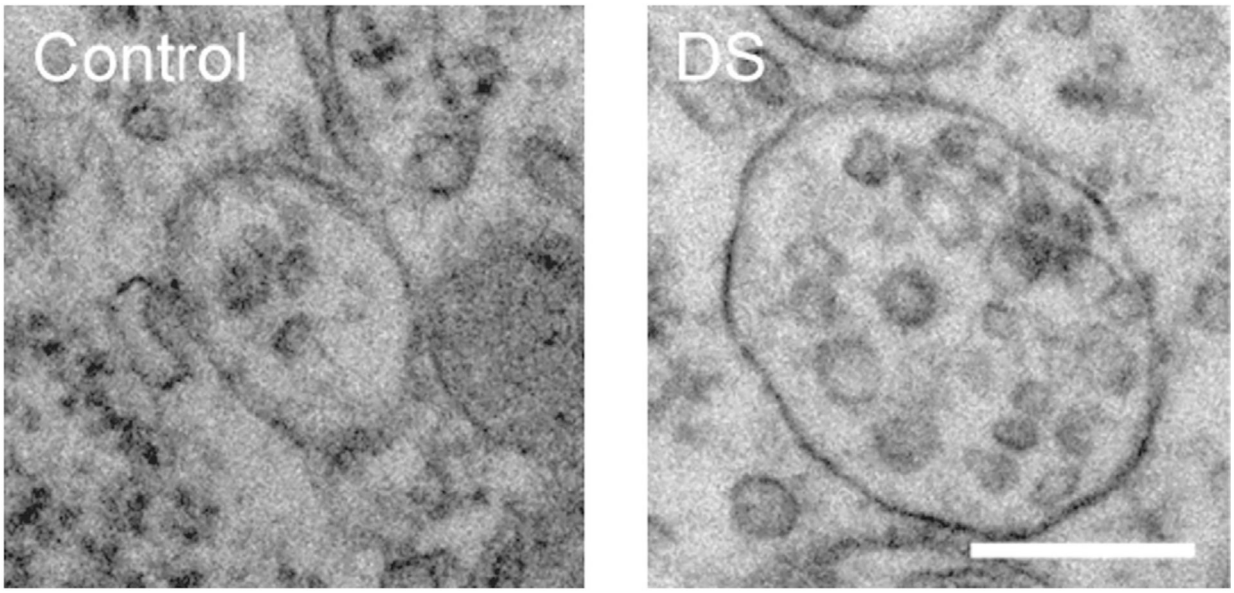

Figure 3.

LE/MVB enlargement and ILV accumulation in the brain of a mouse model of DS. Representative transmission electron microscopy photomicrographs of LE/MVBs in cortical neurons of a mouse model of DS (right) compared with a littermate diploid control (left) at 12 months of age. Scale bar: 500 nm. Quantifications and analyses are available in D'Acunzo et al., 2019 [56].