CNS-derived extracellular vesicles from superoxide dismutase 1 (SOD1)G93A ALS mice originate from astrocytes and neurons and carry misfolded SOD1

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Extracellular vesicles (EVs) are secreted by myriad cells in culture and also by unicellular organisms, and their identification in mammalian fluids suggests that EV release also occurs at the organism level. However, although it is clearly important to better understand EVs' roles in organismal biology, EVs in solid tissues have received little attention. Here, we modified a protocol for EV isolation from primary neural cell culture to collect EVs from frozen whole murine and human neural tissues by serial centrifugation and purification on a sucrose gradient. Quantitative proteomics comparing brain-derived EVs from nontransgenic (NTg) and a transgenic amyotrophic lateral sclerosis (ALS) mouse model, superoxide dismutase 1 (SOD1)G93A, revealed that these EVs contain canonical exosomal markers and are enriched in synaptic and RNA-binding proteins. The compiled brain EV proteome contained numerous proteins implicated in ALS, and EVs from SOD1G93A mice were significantly depleted in myelin-oligodendrocyte glycoprotein compared with those from NTg animals. We observed that brain- and spinal cord–derived EVs, from NTg and SOD1G93A mice, are positive for the astrocyte marker GLAST and the synaptic marker SNAP25, whereas CD11b, a microglial marker, was largely absent. EVs from brains and spinal cords of the SOD1G93A ALS mouse model, as well as from human SOD1 familial ALS patient spinal cord, contained abundant misfolded and nonnative disulfide-cross-linked aggregated SOD1. Our results indicate that CNS-derived EVs from an ALS animal model contain pathogenic disease-causing proteins and suggest that brain astrocytes and neurons, but not microglia, are the main EV source.

Amyotrophic lateral sclerosis (ALS)2 is a fatal neurodegenerative disease resulting in the progressive loss of motor neurons in the brain, brainstem, and spinal cord. The disease is characterized by progressive propagation of pathology spreading from the CNS foci in which symptoms first appear (1). Neuroanatomical propagation may occur by two distinct pathways: 1) contiguous propagation, which occurs side-to-side regionally through the extracellular matrix independent of synaptic connection and 2) network propagation, which occurs end-to-end, dependent on synaptic connections and axonal transmission in connected neural networks (1). The molecular mechanisms responsible for the above observations are the subject of intense study, among which prion-like propagated protein misfolding has emerged as a prominent candidate. Discovery of dominant mutations resulting in the ALS syndrome has implicated a number of proteins responsible for cellular toxicity and perhaps also spreading of pathology in ALS. Whereas the vast majority of clinical ALS cases are sporadic, mutations in human copper-zinc superoxide dismutase (SOD1) cause about 20% of inherited cases of ALS, with mutations in other genes such as TARDBP (encoding the protein TDP-43), FUS, and C9ORF72 (reviewed in Ref. 2), each accounting for a sizable percentage of cases. At the moment, there is experimental evidence in vitro supporting propagation of pathological conformations in both SOD1 (3–6) and TDP-43 (7, 8). These proteins have been shown to induce a pathologic conformation on their natively folded counterparts in a template-directed manner. Our previous work with transfected immortalized cell lines suggests that misfolded SOD1 protein can be transferred from cell to cell via both exosome-dependent and -independent routes (6). However, cell culture studies in vitro may not recapitulate the pertinent mechanisms in a living organism.

All of the cells of the nervous system, including neurons (9), oligodendrocytes (10), astrocytes (11), and microglia (12), have been shown to release membrane-bound vesicles into the extracellular milieu. Such vesicles have the same membrane topology as the cell and range in diameter from 30 to 2000 nm.

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This article contains Figs. S1 and S2 and Tables S1–S9.

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These extracellular vesicles (EVs), neuronal and otherwise, are currently the subjects of intense functional study worldwide. EVs mediate the secretion of an assortment of macromolecular cargo, including mRNAs, miRNAs, lipids, and proteins (13–16); interact with neighboring cells; and transmit their cargo from cell to cell (17–19). Likewise, an increasing body of work has demonstrated a functional role in health and disease of EVs (20–23), which are released by virtually all mammalian cells, as well as nonmammalian and bacterial cells (16).

Exosomes (EXs) and microvesicles (MVs) are the two main classes of EVs, characterized in part, and for the purpose of this study, by the centrifugation parameters at which they pellet, 100,000 × g and 15,000 × g, respectively. The most current studies, using nanoparticle tracking analysis, show these two vesicle pellets to be dominated by vesicles of the same size, ~100 nm in diameter, whereas the MV population has a larger size range, up to 2 µm, and contains more large (0.5–2-µm) vesicles (20–24). The mechanisms of vesicle biogenesis are not yet settled, with evidence for both direct budding from the plasma membrane (25) and release of vesicles from multivesicular endosomes (26). For recent comprehensive reviews of the various types of vesicles secreted by cells, see Refs. 27, 28, and 29.

In the past 5 years, investigations of EVs have been expanded from cell lines and mixed primary cell cultures to the collection of vesicles from the extracellular spaces within tissues (30, 31). These studies have focused on the EV population in Alzheimer’s disease (AD), deriving either from human brains or AD mouse model brains. Few publications have examined the molecular mechanisms responsible for ALS and its spreading pathology from the tissue-derived EV perspective (32, 33).

To better understand the role of EVs in an ALS-affected central nervous system, we employed a method of whole-tissue vesicle isolation (30). Herein, we establish a phenotypic profile of vesicles from whole mouse brains and spinal cords and investigate how model motor neuron disease modifies this phenotype.

Results
Extracellular vesicles were collected from mouse brain and spinal cord tissues

In 2012, Perez-Gonzalez et al. (30) showed that it was possible to collect vesicles from the extracellular space of whole mouse brains and, by extension, from human brain tissue. We tested this technique on brains from the ALS mouse model overexpressing human SOD1 with the G93A mutation (SOD1G93A) and non-transgenic (NTg) littersmates. NTg vesicles that had been pelleted at 100,000 × g (EX) floated through a sucrose gradient as canonical exosomes, primarily floating to ~30% sucrose fractions (Fig. 1A). This fraction contained classic exosomal markers (34), including prion protein (PrP) and SOD1 (Fig. 1A). To test for contamination from intracellular vesicles released by the isolation procedure, we examined markers of the Golgi apparatus (GM130) and Bcl-2, a marker of the mitochondria as well as apoptotic cells and vesicles (35). We also compared known protein amounts of purified vesicle lysates with equal and higher protein amounts of whole-brain homogenates. Whereas exosome markers were detected in the vesicle sample (GRP78, PrP, and SOD1), the markers of contamination were only evident in the brain homogenates (Fig. 1B).

EM confirmed a vesicular morphology of the 30% sucrose-purified vesicles (Fig. 1C). Vesicles were then floated through an iodixanol gradient for greater fractionation. For both NTg and SOD1G93A samples, the exosomal marker PrP was found in the center of the gradient, focused in and around fractions 5 and 6 (Fig. 1D). SOD1 showed a wider distribution, with strong signals in fractions 2 and 3 as well as 5–7, for both genotypes (Fig. 1D). In the SOD1G93A samples, two SOD1-reactive bands were evident (Fig. 1D, bottom), suggesting that both the human transgene and endogenous mouse SOD1 are present in the vesicles. Notably, SOD1 and PrP were also detected at the bottom of the gradient, in fraction 11 (Fig. 1D), which may represent dense debris from the digestion and extraction.

In ALS and mice transgenic for human mutant SOD1 (e.g., SOD1G93A), both upper and lower motor neurons were affected, with maximal pathology and motor neuron death occurring in the spinal cord (1, 36). We applied the methods for extracting brain-derived EXs (BDEXs) to mouse spinal cords, with the important modification that five spinal cords were pooled for every vesicle isolation. Moreover, we expanded our investigation to the sucrose gradient–purified MV as well, from both brains and spinal cords, to generate a comprehensive understanding of the CNS EV population. As can be seen in Fig. 1E, both brain and spinal cord–derived 100,000 × g (EX) and 15,000 × g (MV) pellets contained spherical structures with evident bilayers and morphology consistent with vesicles.

Immunoblotting of spinal cord MVs (Fig. S1) revealed a pattern of protein expression similar to that found in the BDEX (Fig. 1B), where vesicle markers SOD1, PrP, actin, GRP78/Bip, and CD9 were clearly represented, whereas contamination markers such as GM130, Bcl-2, and the neuronal nuclear protein NeuN were absent (Fig. S1).

Proteomic profiling describes a distinct vesicle surface proteome and demonstrates a loss of myelin oligodendrocyte glycoprotein in BDEX from SOD1G93A transgenic mice

To further characterize the BDEXs and potentially identify novel EV-associated proteins involved in SOD1-ALS, we performed advanced quantitative proteomics. Using a tri-labeled technique, we compared 1) the relative expression of proteins identified in the 100,000 × g pelleted and sucrose gradient–purified surface-depleted BDEXs from SOD1G93A and NTg mice (Fig. 2A); 2) proteins expressed on the surface of BDEXs from SOD1G93A and NTg mice (Fig. 2B); 3) the relative expression of proteins on the surface versus the BDEX for NTg mice (Fig. 2C); and 4) the relative expression of proteins on the surface of SOD1G93A vesicles versus NTg BDEXs (Fig. 2D). The relative quantitation is calculated as a ratio of expression for each protein and provided as the log2 -fold difference (1e-1). Significant enrichments of the ratios were calculated using a t test with false discovery rate corrections.

Approximately 1200 proteins were identified in the BDEX proteome (Table S1). Due to the remaining presence of transmembrane proteins, and likely incomplete surface trypsin digestion, these surface-depleted BDEX preparations were assumed to retain a signal from the majority of surface proteins,
an assumption supported by our results (Fig. 2E). Of the 1200 BDEX proteins, 268 were assigned a ratio in at least six of eight experiments (Table S2). Human SOD1<sup>G93A</sup> did not receive a quantifiable ratio owing to its complete absence from the NTg sample. Only one protein was found to be significantly enriched after the false discovery rate corrections; myelin-oligodendrocyte glycoprotein (MOG) was significantly enriched in the NTg vesicles (Fig. 2A), at a NTg/SOD1<sup>G93A</sup> ratio of ~1.29, prior to the log<sub>2</sub> transform. This slight (<30%) although significant enrichment is consistent with studies demonstrating that oligodendrocytes are lost in neurodegeneration (37). Some other proteins approached significance, including the enrichment of protein-disulfide isomerase in SOD1<sup>G93A</sup> BDEX, which has been linked to ALS pathologies (38, 39), and SNAP25 in NTg BDEX. SNAP25 mediates axonal repair, calcium signaling, and vesicle fusion and recycling (40, 41) and therefore has an impact on at least three processes critical to ALS pathogenesis. However, the absence of a large significantly enriched protein population in the transgenic vesicles suggests that the SOD1<sup>G93A</sup> genotype has little effect on the BDEX proteome, at least at 3 months of age, the age of disease onset in this animal model.

In neither SOD1<sup>G93A</sup> nor NTg BDEX did we find the ALS-associated protein TDP-43 or the dipeptide repeated sequences of C9orf72 (Table S1). TDP-43 inclusions have been shown in 97% of sporadic (but not SOD1 familial) ALS (42, 43), and the hexanucleotide repeat expansion-prone C9orf72 (44) is the most common genetic determinant of TDP-43 pathology. However, the ALS-associated RNA-binding protein (RBP) MATR3 and candidate genes hnRNPU and SRSF2 (45, 46) were identified in both SOD1<sup>G93A</sup> and nontransgenic vesicles (Table S1); RBM6, a predicted but not yet validated ALS-associated RBP, was also identified.
The vesicle surface proteome showed a similar pattern of enrichment to the BDEX proteome. Approximately 800 proteins were identified in the surface protein preparations (Table S3). We observed significant overlap between the BDEX proteome and the surface proteome; ~50% of the BDEX proteins were also identified on the surface (Fig. 2E). Of the 202 unique surface protein identifications (Fig. 2E), these were likely attributable to the surface-depleted nature of the BDEX sample.

Of these proteins, only 184 had quantifiable ratios in at least six of eight experiments (Fig. 2B and Table S4). None of these proteins displayed significant enrichments in either sample (Table S4). Importantly, SNAP25 and synaptophysin were found to be enriched in the vesicle proteome in both comparisons (Fig. 2C and D). This orientation is particularly meaningful; SNAP25 and synaptophysin coat the inner surface of vesicles and are critical for vesicle formation and function.

In both surface versus vesicle comparisons, ~60 proteins were relatively enriched on the vesicle surface. These included known membrane proteins such as cadherin-13 and transmembrane proteins like alastin-1. Perhaps surprisingly, many of the surface-enriched proteins were ribosomal subunits (Tables S5 and S6). In fact, after gene ontology and KEGG annotation of the data, a one-dimensional annotation enrichment test showed significant enrichment of the KEGG term “ribosome” with the surface of SOD1<sup>G93A</sup> vesicle surface proteins versus NTg whole vesicles. This term did not reach significant enrichment on the surface of NTg vesicles.

Whereas 60 proteins were enriched on the vesicle surface, only eight (Fig. 2C) and six (Fig. 2D) proteins were more concentrated in the NTg vesicle proteome. This may seem surprisingly low but is almost certainly because many proteins that are exclusively luminal (absent from the surface) cannot be analyzed by ratio analysis and are thus automatically excluded from the quantitative analysis. Interestingly, SNAP25 and synaptophysin were found to be enriched in the vesicle proteome in both comparisons (Fig. 2C and D). This orientation is particularly meaningful; SNAP25 and synaptophysin coat the inner surface of vesicles.
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Leaflet cytoplasmic side of the plasma membrane (47). Conversely, these proteins decorate the outer membrane surface of intracellular synaptic vesicles (47). Likewise, the concentration of SNAP25 and synaptophysin in the vesicle fraction militates against the possibility that the vesicles harvested by our technique represent synaptic vesicles released during tissue processing. Furthermore, the KEGG terms “phagosome,” “cytoplasmic vesicle,” and “rheumatoid arthritis” were statistically overrepresented term, compared with the mouse genome, was “extracellular.”

The BDEX had 340 proteins in common with human CSF extracellular vesicles (Fig. 2F). SH-SY5Y cells are an immortalized human neuroblastoma cell line and are often used as neuronal surrogates in vitro. A proteomic description of exosomes secreted by SH-SY5Y cells (50) had 413 proteins in common with the mouse BDEX proteome (Fig. 2F). Together, the high percentage of overlap with previously published extracellular vesicle proteomes provides additional support for characterization of the vesicles collected form whole brains as extracellular.

Additionally, when we performed a gene ontology analysis of the BDEX proteome and surface proteome, the most overrepresented term, compared with the mouse genome, was “extracellular exosome” (Fig. 3). Many terms were found to be overrepresented (Tables S8 and S9), including RNA binding, ribosome, neuron part, and synapse (Fig. 3). The proteomic analysis suggested that these vesicles are similar to previously described exosomes, that they are of a neural origin, and that the expression of human SOD1G93A had a minimal impact on the wider vesicle proteome.

Brain- and spinal cord–derived extracellular vesicles are primarily of astrocyte and neuronal origin

One of the goals of these studies was to determine the relative proportion of BDEXs that were derived from various CNS cell types (i.e. neurons, astrocytes, microglia, and oligodendrocytes). Due to differences in published proteome depth and limited information from primary cells, the proteomic comparisons did not provide significant insight into this question. Flow cytometry is often used to differentiate individual cell type contributions to a mixed cell population, such as whole blood. Whereas exosomes, at ~100 nm in diameter, are below the lowest level of detection by a forward scatter laser for the currently available flow cytometers, fluorescent labeling of smaller particles is detectable (51, 52). We examined the proportion of vesicles that were positive for various CNS cell type markers. We used the astrocyte marker GLAST, also known as excitatory amino acid transporter 2 (EAAT2), the neuron marker NCAM/CD56, the microglia marker CD11b, the exosome marker CD81, and the synaptic protein SNAP25. Fig. 4A includes forward (FSC) and side scatter (SSC) plots illustrating the noise and how the BDEX and BDMV populations were affected by lysis; the left panels show 0.1-μm-filtered PBS, the middle panels show unlabelled vesicles purified after 100,000 × g (EX) or 15,000 × g (MV) centrifugation; and the right panels show the same after the vesicles had been lysed with 1.0% Nonidet P-40. After lysis, the events from side scatter 10^4-10^5 essentially disappeared, suggesting that these are true vesicles (Fig. 4A).

Similar results are seen with spinal cord EVs (Fig. S2A). However, events that disappear with lysis appear as low as 10^3 in SSC; these events cannot be isolated from noise by gating on the scatter plot. As such, for calculation of the proportion of positive events for each marker, first we analyzed all of the events, including noise, and subtracted the events that were positive even after lysis. We performed additional confirmation that the markers were associated with bona fide vesicles by immunofluorescence (Fig. S2B). We found vesicular structures labeled with the canonical vesicle markers CD63 and CD81, the astrocyte marker GLAST, and the synaptic markers SNAP25 and VGAT (Fig. S2B).

When analyzing the EVs for cell type marker expression by flow cytometry, we saw little difference in the marker percent-
ages between SOD1<sup>G93A</sup> and NTg BDEVs (Fig. 4, B and C). Moreover, no significant effect of genotype was found for vesicles harvested from spinal cords (Fig. 4, E and F). Within each vesicle population, the majority of markers existed at about the same level in the population (15–40% EX, 40–65% MV), with CD11b as the exception and at a much lower expression level (2–5%; Fig. 4, B–G). CD11b is a marker of microglia, and its low representation suggests that the brain and spinal cord EV populations, under these specific conditions, are primarily derived from neurons and astrocytes. The contribution of oligodendrocytes is yet to be determined.

A comparison of marker expression between MV and EX samples and between brain and spinal cord samples generated some interesting observations. We observed a statistically significant reduction in positive events in brain-derived EX samples compared with MV samples (Fig. 4D), likely corresponding to the smaller size of the EX compared with MVs. The larger MVs will have a greater number of fluorochrome-conjugated...
antibodies bound and as such will have a greater signal. The spinal cord EVs showed a slightly different pattern; only CD81 had a significant decrease in EX samples, and SNAP25 demonstrated a significant increase in the smaller EXs (Fig. 4G).

A similar pattern was observed when we compared brain and spinal cord MVs or EXs. For MVs, the brain-derived vesicles had significantly higher marker expression across all measured markers, excepting CD11b (Fig. 4H). In contrast, the EX vesicles from brains and spinal cords had equal levels of CD81 and CD56/NCAM, whereas spinal cord EXs had less GLAST expression and more SNAP25 expression than the brain EXs (Fig. 4I). The equivalent levels of CD81 suggested that the total number of vesicles is the same in brain and spinal cord EX samples. The reduction in GLAST and increase in SNAP25 suggests that fewer of the vesicles are derived from astrocytes and perhaps more are derived from neurons.

**Mouse BDEVs reflect neurodegeneration occurring over time**

The data presented thus far were derived from animals sacrificed at 3 months of age, the age of disease onset in human SOD1<sup>G93A</sup>-expressing mice. We hypothesized that as the animal ages and the disease progressed, the vesicle population would change along with the tissue. However, we did not observe clear evidence of this in the brain-derived EVs. We examined brain MVs from animals sacrificed at 2 and 5 months of age to determine whether the cell type marker phenotype is age-dependent. We found no effect of age on MVs from NTg animals within this range (Fig. 5, A–D). However, the GLAST MV population from SOD1<sup>G93A</sup> animals did have a significant decrease at 5 months, and CD11b was increased from 2 to 5 months of age (Fig. 5, A and B). The percentage of CD11b-positive vesicles remained below 5%, so the impact of this increase was minimal; however, the data do indicate that the course of the disease, the MV population may be modified.

**Brain and spinal cord EVs from mice overexpressing human SOD1<sup>G93A</sup> contain and are decorated by misfolded SOD1**

We have previously shown misfolded SOD1 to be involved in intermolecular and intercellular prion-like template-directed misfolding (3, 6). As part of that investigation, we showed that exosomes secreted by a mouse motor neuron–like cell (NSC34) after transfection with various human SOD1 mutations were surface-decorated with misfolded SOD1 (6). The CNS–tissue vesicle isolations allowed us to determine ex vivo whether misfolded SOD1 associates with extracellular vesicles within neural tissues of ALS model mice.

We utilized antibodies specifically designed to bind only misfolded SOD1 (3, 53) to assay the association of misfolded SOD1 with tissue-derived EVs. Whole EXs harvested from brains and spinal cords of SOD1<sup>G93A</sup> and NTg mice were immunoprecipitated with the SOD1-misfolded antibodies 3H1 and 10C12 (Fig. 6A). VESs from both brains and (Fig. 6B) spinal cords (Fig. 6C) of SOD1<sup>G93A</sup> mice had significantly more misfolded SOD1 on the vesicle surface than NTg littermate mice. This finding is in agreement with our findings from exosomes secreted by cells in culture. Misfolded SOD1 on vesicle surfaces is surprising, considering the cytosolic nature of SOD1 and the established topology of EVs, which recapitulate the cell of origin, with cytosolic components in the vesicle lumen and plasma membrane outer leaflet components exposed on the outer leaf of the vesicle membrane (19). Moving from immunoprecipitation to ELISA, we found that lyses of brain- and spinal cord–derived EXs from SOD1<sup>G93A</sup> mice were significantly enriched in misfolded SOD1, compared with vesicles from NTg animals (Fig. 6D). Moreover, we found that brain and spinal cord MVs (Fig. 6D) displayed a similar pattern, although they appeared to have lower total levels of misfolded SOD1 than the smaller EXs. No
difference in misfolded SOD1 load is seen in the vesicles harvested from brains compared with spinal cords.

Vesicles from human spinal cord tissues from SOD1 FALS patients and control patients showed a similar pattern. When vesicles were immunoprecipitated, both misfolded SOD1-specific antibodies (3H1 and 10C12) had an increased signal from FALS patient vesicles compared with controls (Fig. 6E, 3H1 \( p = 0.0496 \) and 10C12 \( p = 0.0219 \)). Similar results were observed when vesicle lysates were subjected to ELISA (Fig. 6F); unfortunately, access to patient spinal cord tissue was limited, and only two samples were obtained, resulting in a clear trend toward increased misfolded SOD1 in the vesicles, but lacking in statistical significance \( (p = 0.1003) \).

**Differential aggregation profiles of mutant SOD1 in mouse and human CNS EVs**

We have previously demonstrated the prion-like capability of mutant human SOD1\(_{G127X}\) to convert human WT SOD1 (HuWT SOD1) into a misfolded conformation and the subsequent spreading of this misfolded conformation from cell culture to cell culture successively \((3, 6)\). SOD1-induced motor neuron disease has also been demonstrated in vivo using the...
spinal cord homogenate from mutant SOD1G93A as the seed (5). Under native cytosolic conditions, HuWT-SOD1 forms a stable homodimer that does not aggregate. HuSOD1G93A is thought to be indistinguishable from HuWT-SOD1 until late in disease progression (>120 days in mice), at which point aggregates (54) and oligomers (55) have been observed. Conversely, the molecular state of SOD1G127X in cytosol is unclear, considering 1) the SOD1G127X truncation mutation, which results in the loss of β strand 8, a critical portion of the homodimer interface; 2) lack of Cys-146, which forms a monomer-stabilizing disulfide bond with Cys-57 required for native homodimer formation; and 3) lack of stoichiometric metal binding, which contributes significantly to stabilization. HuSOD1G127X has been shown to form intracellular aggregates (56), likely due to non-native interchain disulfide bonds (57).

To identify the quaternary structure of the propagation-competent species of HuSOD1G127X and HuSOD1G93A in the CNS EVs, brain MVs from NTg mice with endogenous mouse SOD1 (mSOD1), HuSOD1G127X transgenic mice, SOD1 knockout mice (mSOD1/−/−), mSOD1/−/−/HuSOD1G127X crosses and HuSOD1G93A transgenic mice were subjected to SDS-PAGE under reducing and nonreducing conditions with and without iodoacetamide to block artificial formation of disulfide bonds (Fig. 7A). HuSOD1G127X is a truncation mutation, and the truncation results in the loss of epitope for the misfolded SOD1-specific antibody used in the immunoprecipitations and ELISAs in Fig. 6 (3, 58). For this reason, nonreducing PAGE could produce the most information regarding the aggregation profile of mutant SOD1 in EVs.

The vesicles were collected at symptom onset, 90 days for HuSOD1G93A and 150 days for HuSOD1G127X. Under reducing conditions, all species of SOD1, mouse WT and human G127X and G93A, appeared as monomer bands around 17 kDa (Fig. 7A). However, under nonreducing conditions, where disulfide bonds were maintained, higher-order aggregates of HuSOD1G93A and, more robustly, HuSOD1G127X appeared in the very top of the gel, which included the stacking gel (Fig. 7A). Artificial aggregation was observed in HuSOD1G93A without iodoacetamide. The bona fide disulfide-mediated aggregates were restricted to the expression of HuSOD1, as no such aggregates were observed in the NTg and no SOD1 bands were detected in the mSOD1/−/− vesicles (Fig. 7A). Moreover, HuSOD1G127X expression was found to be relatively low; twice as much protein per well was required from the mSOD1/−/−/HuSOD1G127X sample to visualize a clear signal (Fig. 7A). Volumetric quantitation of the SOD1 monomer band (Fig. 7B) and aggregate bands/smears (Fig. 7C) from replicate immunoblot analyses confirmed that EV SOD1G93A is predominantly monomeric under nonreducing conditions, in contrast to SOD1G127X, which takes the form of insoluble aggregates under these conditions. We can conclude that SOD1G127X mouse CNS EVs contain large insoluble aggregates of SOD1G127X as well as native mSOD1. In contrast, SOD1G93A mice at 3 months of age have fewer and lower-order aggregates of SOD1 in brain-derived EVs.

We confirmed these findings with EVs harvested from human spinal cords. Under reducing conditions, SOD1 signal was restricted to the monomeric range, as well as some lower-molecular weight degradation products, in EVs from control and FALS patients (Fig. 7D). However, FALS SOD1A4V patient-specific SOD1 aggregates at ~70 and ~140 kDa were observed under nonreducing conditions (Fig. 7D). After volumetric quantitation of replicate experiments with additional patient material, we observed that the monomeric SOD1 is equivalent in the control and FALS patients (Fig. 7E). Conversely, under nonreducing conditions, the FALS patient EVs show a concentration of aggregated SOD1 (Fig. 7F). In conclusion, both human ALS and mouse ALS-model CNS EVs contain aggregated SOD1 cargo.

**Discussion**

We developed methods to investigate the intra-organ extracellular vesicle population of the mouse brain and spinal cord (Figs. 1–7). At the onset of motor neuron disease in SOD1G93A mice, the vesicle proteome is largely unchanged compared with nontransgenic mice, except for the presence of human SOD1G93A on the surface and in the lumen of vesicles (Figs. 2–5). Microglia contribute little to the EV population, with the majority of vesicles likely being secreted by astrocytes and neurons (Figs. 4 and 5).

Understanding the cellular mechanisms of prion-like protein misfolding and neuroanatomical pathology propagation is essential for a comprehensive understanding of the etiology of neurodegenerative diseases and for effective therapeutic development. Using mouse models of disease, descriptions of brain EV seeding of Tau pathology in AD (31) and α-synuclein pathology in dementia with Lewy body disease (59) are demonstrating, *ex vivo*, a role for EVs in propagation of proteopathy. In this work, we show evidence that CNS-derived extracellular vesicles isolated from the SOD1G93A ALS mouse model and human SOD1-FALS neural tissues carry misfolded and aggregated SOD1 cargo. This finding indicates that mutant/misfolded SOD1 within EVs might be sufficient to induce misfolding of full-length WT protein in recipient cells, representing a potential mechanism for systematic spread of ALS pathogenesis. Follow-on functional studies are needed to fully elucidate the role of EVs in disease.

SOD1-induced motor neuron disease has been demonstrated *in vivo* using spinal cord homogenate from paralyzed SOD1G93A mice (5). We have demonstrated the ability of SOD1G127X to seed conversion of HuWT-SOD1 into a misfolded conformation and the subsequent spreading of this misfolded conformation in cell cultures (3, 6). It is thought that both vesicles and free aggregates of pathogenic proteins may be involved in releasing SOD1 “seeds” from affected cells (6), consistent with contiguous propagation of clinical symptoms. However, it remains to be determined which mechanism, if any, is responsible for the spread of proteopathy. Moreover, as both SOD1 and TDP43 mutant-associated ALS have been shown to be non-cell-autonomous (60–63), the question of which cell is releasing the misfolded protein species and which cell is the recipient remains unclear.

Our data suggesting an overrepresentation of astrocyte-derived EVs in the brain are evocative of the large body of literature describing a toxic role of astrocytes in the context of SOD1-ALS (63–65). Additionally, mutant SOD1-expressing
Acidozytes have been found to increase exosome release and lead to SOD1 pathology in naive recipient neurons (65). The lower levels of GLAST vesicles in the spinal cord compared with brain, and the increased SNAP25 surface decoration of EVs in the spinal cord, point to differing populations in these two tissues, the phenotypic effect of which is not yet understood.

In cell culture systems, we have previously reported that misfolded SOD1 aggregates are released from dead and dying cells, whereas EVs were released primarily from live cells early in the degenerating process (6). Here, we showed that mouse ALS-model CNS vesicles carry soluble mutant SOD1 as well as insoluble aggregates (Fig. 7, A–C) and confirmed those findings with

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**Figure 7.** Differential aggregation profiles of mutant SOD1 in mice and human brain EVs. The protein cargo of microvesicles harvested from NTg and multiple ALS model mice (A–C) or human spinal cord tissue (D–F) was analyzed by reducing and nonreducing SDS-PAGE followed by SOD1 immunoblot. For representative immunobLOTS (A and D), reducing lanes include β-mercaptoethanol (β-merc.). Iodoactamide (idoact.) was included to block artifactual disulfide bonds from forming during sample processing. -mon., monomer band (−17 kDa); *, aggregate band(s). B and C, quantitation of volume intensity of immunoblots from n = 2–4 brain MV samples per genotype normalized to the amount of protein analyzed. + β-merc./−idoact., reducing lanes; −β-merc./+idoact., nonreducing lanes. B, HuSOD1^G93A^ reported on right y axis; C, aggregates; * band at top of gel. Mean and S.E. (error bars) are shown. Differences were determined by two-way ANOVA with Sidak’s multiple-comparison test. B, p = 0.2667 (interaction), p ≤ 0.0001 (genotype), and p = 0.0140 (reduction). C, p = 0.2003 (interaction), p = 0.0031 (genotype), and p = 0.4553 (reduction). E and F, quantitation of volume intensity of immunoblots from n = 4 FALS A4V samples and n = 4 control samples, normalized to the amount of protein analyzed. Mean and S.E. are shown. Aggregate signal (F) is the sum of the volume intensities for bands marked with an asterisk in D. + β-merc./−idoact., reducing lanes; −β-merc./+idoact., nonreducing lanes. Differences were determined by two-way ANOVA with Sidak’s multiple-comparison test. E, p = 0.4012 (interaction), p = 0.5241 (disease status), and p = 0.0184 (reduction). F, p = 0.2204 (interaction), p = 0.0466 (disease status), and p = 0.0761 (reduction). Error bars, S.E.
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human spinal cord EVs (Fig. 7, D–F). The age of the animal may also be relevant to the packing of EVs with aggregated SOD1; aggregates of SOD1<sup>G93A</sup> have been previously observed in disease-affected animals of η120 days of age, whereas little aggregation was seen in younger animals, in agreement with our findings (54, 55). In the context of the literature, our results suggest that astrocytes are primarily secreting vesicles carrying toxic cargo, including aggregated and soluble mutant SOD1, whereas free protein aggregates are also released from dead and dying neurons. One might speculate that both mechanisms play significant roles during disease, perhaps at different times, or simultaneously but in dynamic ratios.

Recent work has suggested that, in the context of the synapse, the release of vesicles packed with misfolded proteins is a proteostatic protective cellular function mediated by neuron-specific cysteine string protein (CSPα, DnaJC5) (32, 66). We identified this protein in the mouse brain–derived EXs, and it appears in similar levels in the SOD1<sup>G93A</sup> and NTg samples. In light of the demonstrated role for DnaJC5 in vesicle secretion of unfolded proteins (32), the lack of concentration of DnaJC5 in the SOD1<sup>G93A</sup> vesicles, which we know to be enriched in the mutant and misfolded SOD1 (Figs. 1, 2, and 6), supports our conclusion that perhaps the majority of these vesicles are derived from astrocytes.

The role of protein supersaturation in triggering toxicity and perhaps propagated misfolding has been investigated (67). In this elegant computational study, SOD1 and TDP-43 are shown to have orders of magnitude higher supersaturation values in motor neurons, compared with other cellular proteins. The authors suggest that supersaturation is responsible for protein aggregation or co-aggregation and gain of toxic function. We compared the list of supersaturated proteins found in human and mouse SOD1 inclusions with our mouse brain EX proteome and found relatively little overlap: 23 of the 77 human SOD1 inclusion proteins and 12 of the 58 mouse SOD1 inclusion proteins. This indicates that key proteins may dominate disease progression, rather than co-aggregation of multiple proteins, most consistent with a prion-like propagated misfolding mechanism of spreading.

SOD1 is widely thought to play an important role only in familial cases of ALS where SOD1 mutations occur, less than 2% of all ALS cases (68), although some literature supports a role for WT human SOD1 in sporadic ALS (69). In this light, it is interesting that SOD1 is a common EV marker, and future CNS vesicle studies in sporadic ALS may reveal misfolded SOD1, perhaps concentrated as a protective proteostatic cellular function. Our current findings on the baseline phenotype of mouse brain and spinal cord EVs may have bearing on the wider spectrum of ALS (70). The apparent significant enrichment of ribosome-associated proteins on the surface of only the ALS mouse model BDEV is reminiscent of the emerging consensus that dysregulation of translation is a hallmark of ALS and other neurodegenerative diseases like FTD (71, 72). RBPs like TDP-43 and FUS, as well as a growing list of others, are recognized as pathogenic markers of disease, although the exact mechanisms of pathogenicity are under intense investigation (73). A growing body of work is linking RBP and EVs to disease (8, 32, 74), and RNA and RBP cargo of exosomes is a well-established phenomenon (23). What remains to be determined is the interplay between RBP, stress granules, protein aggregation, vesicle release, and their summated toxicity. Our work supports the conclusion that RBPs are constitutively secreted in association with brain and spinal cord vesicles but that the mouse RBPs are not recruited to the vesicles by mutant SOD1. Additionally, the enrichment of synaptic and synaptic vesicle proteins in the brain EVs suggests a role for these vesicles in regulation of synaptic transmission, supported by a small body of literature (75, 76).

Only one protein, besides human SOD1<sup>G93A</sup>, had significantly modified representation in the SOD1<sup>G93A</sup> BDEXs, MOG (Fig. 2A). Antibodies targeting MOG are thought to play a role in some demyelinating diseases (77). The biological significance of MOG’s reduced abundance in the SOD1<sup>G93A</sup> vesicles is unclear and may simply reflect the process of neurodegeneration occurring in the transgenic mice (78). However, it is tempting to speculate that effective therapies would protect against the loss of this protein, and as such, it may have value as a biomarker for therapeutic efficacy. The field of cancer diagnosis has turned to vesicles as potent and plentiful markers of migrating tumor cells and, by screening for multiple disease-associated proteins, has been able to drastically improve diagnostic potential for prostate and breast cancer (79). Our work has identified at least one attractive candidate protein (and, including protein-disulfide isomerase, perhaps two) for similar sorting of patients with early signs of ALS or neurologic dysfunction.

In conclusion, extracellular vesicles collected from whole tissues provide an <i>ex vivo</i> platform for the investigation of intraorgan intercellular communication. It is of great interest to perform additional characterization studies on vesicles from human tissue to assess the baseline phenotype of tissue EVs and the complex modifications that occur in disease.

**Experimental procedures**

**Animals**

Animals used in this study, C57 BL/6 and HuSOD1<sup>G93A</sup>, mSOD1<sup>+/−</sup> (strains: B6SJL, Cg-Tg(Sod1-G93A)1Gur/JB6, and B6;129S-Sod1tm1Leb/J purchased from Jackson Laboratories (Bar Harbor, ME)), and HuSOD1<sup>G127X</sup> mice (Tg(SOD1*G127X)716Mrk; a gift from Stefan Marklund) (80–82) were housed in accredited facilities in accordance with University of British Columbia and international animal ethics guidelines. All animals were sacrificed early in disease onset to avoid unnecessary suffering: Human WT SOD1 mice and G93A mice and WT littermates at 3 months of age, G127X mice and WT littermates at 5 months of age. The studies were reviewed and approved by the University of British Columbia Animal Care Committee.

**Human tissue**

The human tissues used in this study were obtained from brain banks affiliated with the University of British Columbia and the Target ALS–Johns Hopkins School of Medicine. Consent for autopsy was obtained from the legal representative in accordance with local institutional review boards. The studies were reviewed and approved by the University of British Columbia Ethics Board and are in accordance with the Decla-
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Vesicle isolation

The isolation of vesicles from whole tissues was adopted and modified from Ref. 30. Frozen tissue, brain, and spinal cord from SOD1-G93A and NTg littermates and human spinal cords were first weighed. Whole mouse brains (0.4–0.6 g) were used. Five murine spinal cords, ~100 mg/cord, were pooled for the isolation. 0.3–0.75 g of human spinal cord tissue was employed for each isolation. The tissue was sliced minimally in Hibernate A solution (Brain Bits, Springfield, IL) and treated with papain (20 units/ml) or collagenase III (75–125 units/ml) at 37 °C for 15 min to digest connective tissue. Tissue was further disassociated by up-and-down aspiration in 2 volumes of cold Hibernate A solution plus protease inhibitor (Roche, Mississauga, Canada). The disassociated tissue was then sequentially centrifuged at 2000 × g to separate cell debris and collect extracellular material and at 15,000 × g to collect large vesicles, such as microvesicles. EVs were collected by centrifugation at 100,000 × g for 1 h.

Sucrose gradients

The EV pellet was purified by density gradient centrifugation in a sucrose step gradient, with concentrations ranging from 2.0 M (~60% sucrose) to 0.25 M (~7.5% sucrose) in 0.35 M increments and centrifuged at 200,000 × g for 16 h at 4 °C in a Beckman L90 Ultracentrifuge (Beckman Coulter, Mississauga, Canada). Fractions of equal volume are collected, diluted in PBS, and centrifuged again at 100,000 × g for 70 min at 4 °C. Sucrose gradient fraction pellets were resuspended in PBS and stored at −80 °C. Where appropriate, protease inhibitors were added to storage buffer.

OptiPrep gradients

BDEVs were purified on OptiPrep™ density gradient following previously published protocols (35, 83). Briefly, a discontinuous iodixanol (OptiPrep™, MilliporeSigma, Oakville, Canada) gradient was prepared by diluting stock solutions of OptiPrep™ (60% (w/v) aqueous iodixanol) with 0.25 M sucrose, 10 mM Tris, pH 7.5. The 100,000 × g pellet was overlaid onto the top of the gradient and centrifuged at 100,000 × g for 18 h at 4 °C. 0.6-ml gradient fractions were collected manually from the top (with increasing density). Fractions were diluted with PBS and centrifuged at 100,000 × g for 3 h at 4 °C, washed in PBS, and resuspended in PBS and stored at −80 °C until further use.

Sucrose cushion

For quantitative proteomics and flow cytometric studies, BDEVs were purified using a sucrose cushion system. A cushion of 0.5 ml of 2 M sucrose overlaid with 0.25 ml of 1 M sucrose followed by 0.6 ml of PBS was constructed in 1.5-ml ultracentrifuge tubes. Resuspended EV pellets (0.2–0.4 ml) were layered on top of the gradient followed by ultracentrifugation at 150,000 × g at 4 °C for 2 h in a Hitachi CX 150NS Micro Ultracentrifuge. 0.7 ml was removed from the top of the gradient and discarded. 0.4 ml, including the ~1 M EV fraction was removed and resuspended in PBS followed by additional ultracentrifugation, 100,000 × g for 1 h. Pellets were resuspended in PBS and stored at −80 °C until further use.

Immunoblotting

Equal volumes of either sucrose or OptiPrep fraction samples were boiled in SDS sample buffer containing 1% β-mercaptoethanol and subjected to SDS-PAGE, followed by immunodetection with a pan-SOD1 rabbit polyclonal antibody (SOD100; ENZO Life Sciences, Plymouth Meeting, PA), prion protein (PrP 6D11; Santa Cruz Biotechnology, Inc., Dallas, TX), CD9 (clone EPR2949, Abcam Inc., Cambridge, MA), or GRP78/BiP (clone 40/BiP, BD Biosciences, Mississauga, Canada) to confirm the presence of exosome vesicles. Fractions were also blotted for markers of other organelar membranes, such as Bcl-2 (clone 7/Bcl-2) and GM-130 (clone 35/GM130), all purchased from BD Biosciences, to assess the purity of the EV pellets. Vesicles were also blotted for GLAST (clone ACSA-1, Miltenyi Biotech, Auburn, CA), SNAP25 (clone SMI 81, BioLegend, San Diego, CA), NeuN (clone A60, MilliporeSigma), GFAP (polyclonal, ab7260; Abcam), and synaptophysin (clone YE269; Abcam).

Electron microscopy

Transmission EM of sucrose-purified EVs was performed essentially as described (84). Briefly, EVs were fixed in 2% glutaraldehyde and 4% paraformaldehyde in 0.1 M cacodylate buffer for 2 h at room temperature. Vesicles were collected by ultracentrifugation and washed in 0.1 M cacodylate buffer. Fixed vesicles were absorbed onto Formvar-coated 200-mesh nickel grids (Electron Microscopy Sciences, Hatfield, PA). For immunoelectron microscopy, grids were blocked with 1% milk in PBS followed by probing with primary antibodies overnight in blocking buffer. After washing, grids were incubated with secondary antibodies in 1% milk and 0.5% PEG. All grids were negatively stained with 0.5% aqueous uranyl acetate for 30 s and viewed on a FEI Tecnai G2 Spirit transmission electron microscope.

Antibodies were purchased from the following sources: mouse anti-CD63 (clone H5C6) and mouse anti-SNAP25 both from BioLegend; mouse anti-GLAST (clone ACSA-1) and hamster anti-CD81-biotinylated (clone EAT2) both from Miltenyi; guinea pig anti-VGAT (Synaptic Systems, Goettingen Germany); all secondary antibodies (anti-mouse 15-nm gold, anti-biotin 25-nm gold) from Electron Microscopy Sciences, MilliporeSigma, Mississauga, Canada). The isolation of vesicles from whole tissues was adopted and modified from Ref. 30. Frozen tissue, brain, and spinal cord from SOD1-G93A and NTg littermates and human spinal cords were first weighed. Whole mouse brains (0.4–0.6 g) were used. Five murine spinal cords, ~100 mg/cord, were pooled for the isolation. 0.3–0.75 g of human spinal cord tissue was employed for each isolation. The tissue was sliced minimally in Hibernate A solution (Brain Bits, Springfield, IL) and treated with papain (20 units/ml) or collagenase III (75–125 units/ml) at 37 °C for 15 min to digest connective tissue. Tissue was further disassociated by up-and-down aspiration in 2 volumes of cold Hibernate A solution plus protease inhibitor (Roche, Mississauga, Canada). The disassociated tissue was then sequentially centrifuged at 2000 × g to separate cell debris and collect extracellular material and at 15,000 × g to collect large vesicles, such as microvesicles. EVs were collected by centrifugation at 100,000 × g for 1 h.

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Immunoprecipitation

Quantitative immunoprecipitation of misfolded SOD1 was performed as described previously (3, 6) using the resuspended pellet fractions in PBS alone so as not to disrupt vesicles; pull-down of misfolded SOD1 therefore only occurred if it presented on the vesicle surface. The following antibodies were coupled to magnetic Dynabeads (Invitrogen) following the manufacturer’s instructions: mouse IgG2A isotype control (Abcam Inc.), SOD100, and misfolded SOD1-specific antibodies 3H1 and 3H2.
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An equal amount of EV sample, 10–20 μg, was added to each antibody bead combination and incubated for 24 h at 4 °C with constant rotation, followed by three brief washes in PBS. Samples were boiled in SDS sample buffer containing 1% β-mercaptoethanol and subjected to SDS-PAGE, followed by immunodetection with a pan-SOD1 rabbit polyclonal antibody (SOD100).

ELISA

96-well Nunc ELISA plates (Thermo Fisher Scientific) were coated with 7.38 μg/well of the anti-misfolded SOD1 mAb 3H1 overnight at 4 °C. Plates were washed with TBS containing 0.1% Tween 20 and blocked with filtered PBS + 0.1% Tween 20 with 3% normal goat serum (MilliporeSigma) overnight at 4 °C and washed again. Samples were prepared by measuring total protein concentration using the Pierce BCA assay kit (Thermo Fisher Scientific). Equal protein amounts (MV and EV = 0.05 μg/μl) of EV lysates, lysed in 1% Triton X-100 for 20 min on ice, were incubated with the captured 3H1 antibody for 1 h at room temperature. Plates were washed and incubated with 2 μg/ml rabbit polyclonal SOD100 antibody at room temperature for 1 h. Finally, the plate was washed and incubated with HRP-linked anti-rabbit IgG (GE Healthcare) diluted 1:5000 for 1 h at room temperature, followed by five final washes. The plates were incubated with Supersignal™ ELISA Femto substrate (Thermo Fisher Scientific) for 5 min at room temperature. Total chemiluminescence at all wavelengths was read on a SpectraMax M2 Molecular Devices instrument.

Quantitative proteomics

When isolating BDEX for proteomics, TBS was substituted for PBS at all steps, and at the final pelleting step, vesicles were resuspended in TBS without protease inhibitor. Protein concentration was determined using a Pierce BCA kit (Thermo Fisher Scientific).

To remove surface proteins from BDEX for separate analysis, it was assumed that the surface protein content was 1% of total vesicle protein content, as is generally assumed for eukaryotic cells (48). Trypsin (Promega, Madison, WI) was added to BDEX at a 1:50 protein ratio in TBS and incubated at 37 °C for 4 h, after which vesicles were acidified to pH 2 with 10% acetic acid to kill trypsin activity. TBS was added to the trypsinized BDEX followed by ultracentrifugation at 100,000 × g at 4 °C for 1 h. Tryptic peptides from surface proteins remained in the supernatant while intact vesicles were pelleted and resuspended in 50 mM ammonium bicarbonate, 1% sodium deoxycholate and stored at −80 °C.

The resulting surface-depleted vesicle pellet and surface peptide preparations were reduced and alkylated as per (85) and further digested with trypsin at 37 °C overnight. Peptide samples were purified by solid-phase extraction on C-18 STop And Go Extraction (STAGE) tips (86), and each treatment was chemically labeled by reductive demethylation using formaldelyde isotopologues (87). The final product was purified again by C-18 STAGE tips and run on a Bruker Impact II Q-TOF coupled to an Easy-Nano LC 1000 HPLC (Thermo Fisher Scientific) (88).

MaxQuant was used to assign peptide and protein identifications and relative abundance ratios (89). Perseus was used to analyze relative abundance ratios and determine significantly enriched proteins (90). Ermine was used to determine statically enriched gene ontology (GO) annotations in the BDEX and surface proteomes compared with the entire mouse genome (91, 92).

Flow cytometry

Flow cytometry analysis of 15,000 × g and 100,000 × g pellets was performed with modifications following published protocols (51, 52, 93, 94). All buffers are passaged through a 0.1-μm polyvinylidene difluoride filter (Corning Life Sciences, Tewksbury MA) prior to use. BDEVs and BDMVs were resuspended in 0.4% BSA in PBS. EVs and MVs were probed with the following antibodies at the manufacturer’s recommended concentration. Anti-GLAST (phycocerythrin and allopbyocyanine, clone AC2A-1), anti-CD81 (phycocerythrin, clone EA12), and anti-CD11b (FITC, clone M1/70.15.11.5) were purchased from Miltenyi Biotec. Anti-CD56/NCAM (allophycocyanine, clone FAB7820A) was purchased from R&D Systems (Minneapolis, MN). All isotype control antibodies and anti-SNAP25 were purchased from Biolegend. Anti-mouse Alexa-488 secondary antibody was purchased from Life Technologies (Thermo Fisher Scientific). Antibodies were centrifuged at 20,000 × g for 20 min prior to incubation with vesicles for 1 h at room temperature in the dark. Unbound antibody was removed from vesicles after resuspension in 1 ml of PBS by ultracentrifugation at either 15,000 × g for 30 min or 100,000 × g for 1 h for BDMV and BDEX, respectively. Vescicle pellets were resuspended in 100–500 μl of PBS and then diluted further to 1:250 for BDMV and 1:10 for BDEV in PBS and PBS + 1.0% Nonidet P-40. Vesicles were analyzed on a MACSQuant analyzer (Miltenyi Biotec) after thorough washing of the machine with 1% bleach. Samples were collected on the lowest flow setting for 2 min each. Between each sample, PBS was run on the high flow setting. Data were analyzed with FlowJo (Ashland, OR). Statistical analysis was performed with GraphPad Prism versions 5–7.

Nonreducing SDS-PAGE

Purified vesicles were thawed on ice, and the total protein concentration was measured using the Pierce BCA assay kit. Reducing and nonreducing SDS-PAGE was carried out using 15 μg of protein from mouse MVs (30 μg for mSOD1−/−/HuSOD1G127X MVs) and 5 μg from human MVs, with and without the presence of 100 mM iodoacetamide. The vesicles were mixed with 5 μl of 1X NuPAGE sample buffer (Thermo Fisher Scientific) with one of four possible treatments and buffered with PBS (pH 7.4) to a total volume of 15 μl. The treatments were as follows: 2.5% β-mercaptoethanol, 2.5% β-mercaptoethanol with 100 mM iodoacetamide, 100 mM iodoacetamide, and no treatment. Samples were heated for 5 min at 95 °C and electrophoresed on NuPAGE 4–12% Bis-Tris gels (Thermo Fisher Scientific) before being transferred onto polyvinylidene difluoride membranes. After transfer, the membranes were probed with rabbit polyclonal SOD100 antibody followed by horseradish peroxidase anti-rabbit IgG (GE Healthcare). Membranes were exposed to Supersignal™ West
Femto substrate (Thermo Fisher Scientific), and the chemiluminescence was measured using a ChemiDoc system (Bio-Rad). The relative intensity of the bands was measured using ImageLab, and the percentage of aggregate signal compared with the total band signal was calculated.

Author contributions—J. M. S. proposed the hypothesis, designed and performed experiments, and drafted the manuscript. D. C., C. C. S., Y. B., S. F., Z. G., and L. M. contributed to optimization and performed vesicle collection and immunoblotting, proteomics, flow cytometric, and ELISA experiments. K. M. M. performed quantitative mass spectrometry, and R. G. S. assisted in quantitative proteomics analysis. L. I. G. contributed to experimental design and performed immunoprecipitation experiments. Z. G. and C. M. C. performed TEM experiments. I. R. M. contributed to experimental design and provided human ALS tissue. L. J. F. and N. R. C. contributed to the hypothesis, experimental design, and manuscript drafting.

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