Mutant SOD1 inhibits ER-Golgi transport in Amyotrophic Lateral Sclerosis

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Abbreviations used: ALS, Amytrophic Lateral Sclerosis; BDNF, brain-derived neurotrophic factor; CHOP, C/EBP homologous protein; COPII, coatamer coat protein II; EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum stress; hpt, hours post...
transfection; mSOD1, mutant SOD1; PDI, protein disulphide isomerase; SOD1, Cu/Zn-superoxide dismutase; UPR, Unfolded Protein Response

Abstract

Cu/Zn-superoxide dismutase (SOD1) is misfolded in familial and sporadic Amyotrophic Lateral Sclerosis (ALS), but it is not clear how this triggers endoplasmic reticulum (ER) stress or other pathogenic processes. Here we demonstrate that mutant SOD1 (mSOD1) is predominantly found in the cytoplasm in neuronal cells. Furthermore, we show that mSOD1 inhibits secretory protein transport from the ER to Golgi apparatus. ER-Golgi transport is linked to ER stress, Golgi fragmentation and axonal transport and we also show that inhibition of ER-Golgi trafficking preceded ER stress, Golgi fragmentation, protein aggregation and apoptosis in cells expressing mSOD1. Restoration of ER-Golgi transport by over-expression of coatamer coat protein II (COPII) subunit Sar1 protected against inclusion formation and apoptosis, thus linking dysfunction in ER-Golgi transport to cellular pathology. These findings thus link several cellular events in ALS into a single mechanism occurring early in mSOD1 expressing cells.

Running Title: ER-Golgi transport dysfunction in ALS

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder whose cellular pathophysiology is poorly understood. Nonetheless, ALS presents consistent clinical and pathological processes and endoplasmic reticulum (ER) stress is recognized as a key pathway to cell death (Atkin et al. 2008, Saxena et al. 2009, Atkin et al. 2006). However, it remains unclear how ER stress is linked to other pathogenic mechanisms in ALS, including fragmentation of the Golgi apparatus, disruption of axonal transport and misfolding/aggregation of proteins including Cu/Zn-superoxide dismutase (SOD1) which is misfolded in both sporadic and familial ALS (Rosen et al. 1993, Bosco et al. 2010, Forsberg et al. 2010).

Conventionally, ER stress is triggered when proteins accumulate within the ER lumen, triggering the unfolded protein response (UPR), which if unresolved, leads to apoptosis. However, most proteins linked to ALS aetiology do not possess a signal leader peptide and hence should not enter the ER. Although some reports have associated SOD1 with
microsomes, thus implying entry into the ER (Kikuchi et al. 2006, Urushitani et al. 2008), others argue that cytosolic SOD1 contaminated the microsome preparation and led to erroneous interpretations (Nishitoh et al. 2008). ER stress can also be triggered by other mechanisms such as inhibition of ER-Golgi transport, which results in accumulation of secretory proteins within the ER (Hoyer-Hansen & Jaattela 2007, Preston et al. 2009). mSOD1 can trigger ER stress by interacting with Derlin-1 (Nishitoh et al. 2008), but this mechanism has not been described early in pathology, suggesting that other processes also trigger ER stress in ALS.

One third of all cellular proteins transit the ER and Golgi compartments destined for transmembrane, ER or extracellular locations (Ghaemmaghami et al. 2003). The coat protein II complex (COPII) is central to this process. Vesicles containing secretory proteins are coated with COPII and bud from the ER at specific ER exit sites. COPII is a complex of five subunits: Sar1, Sec23, Sec24, Sec13 and Sec31 (Lee & Goldberg 2010). The GTPase Sar1 drives ER-Golgi trafficking by sequentially recruiting the other subunits from the cytoplasm to the outer ER membrane, thus initiating assembly of the complex (Long et al. 2010). The assembled COPII complex deforms the flat membrane of the ER, leading to vesicle release and rapid turnover of the subunits (Hammond & Glick 2000). Sec23 subsequently mediates vesicle binding to the dynein/dynactin motor complex for microtubule-based transport to the Golgi (Watson et al. 2005). ER and Golgi abnormalities (Mourelatos et al. 1990, Sasaki 2010), and dynein dysfunction (Puls et al. 2003, Lai et al. 2007, Laird et al. 2008) are well described in ALS, and ER-Golgi transport is linked to axonal transport (Reiterer et al. 2008, Hammerschlag et al. 1982).

We examined the possibility that ER-Golgi trafficking is disrupted in ALS, thus explaining the induction of UPR by non-ER proteins such as mSOD1. We found that ER-Golgi transport was impaired before the induction of ER stress, Golgi fragmentation, SOD1 aggregation and apoptosis, in motor neuronal cells expressing mSOD1. We could not find evidence of mSOD1 in the ER, indicating that it triggers ER stress primarily from the cytoplasm. Over-expression of COPII rescued inhibition of ER-Golgi transport, inclusion formation and apoptosis, thus linking ER-Golgi transport dysfunction to neurodegeneration. We conclude that mSOD1 disrupts ER-Golgi transport and we discuss how these findings link several previously unrelated cellular pathologies in ALS.
Materials and methods

Constructs

Wildtype and mSOD1 constructs encoding EGFP tagged human SOD1 at the C-terminus used were as previously described (Atkin et al. 2006, Turner et al. 2005). The pEF-BOS vectors containing human SOD1\textsuperscript{WT}, SOD1\textsuperscript{A4V} cDNAs were provided by Dr. David Borchelt (Johns Hopkins University, MD). A COPII construct based on pFLAG-CMV-2 (Sigma, Sydney, NSW, Australia) encoding Sar1 from Chinese hamster ovary cells tagged with a N-terminal FLAG epitope, was a kind gift from Professor Katsuko Tani, Tokyo University, Japan. VSVG-ts045 tagged with mCherry was a kind gift from Dr Jennifer Lippincott-Schwartz and Dr George Patterson, National Institutes of Health, Bethesda, USA. The PDI-DsRed construct was as previously described (Walker et al. 2010).

Cell culture and transfection

Mouse motor neuron-like NSC-34 cells were a generous gift of Professor Neil Cashman (University of Toronto, Ontario, Canada) and were maintained in DMEM with 10% (v/v) foetal calf serum (FCS) and 1% (v/v) penicillin-streptomycin (Invitrogen, Victoria, Australia). Cells were subcultured in 24-well plates at a density of 1 x 10\textsuperscript{5} cells per well and were transfected transiently with plasmids (1 μg DNA per well) using a 1:1 ratio of lipofection reagent (‘Transfast’, Promega) to DNA. Cells were examined with an inverted fluorescent microscope (Olympus, NSW, Australia) 72 h after transfection unless otherwise stated. Cells containing prominent inclusions were counted in at least 4 wells and expressed as a percentage of total EGFP-positive cell transfectants.

Animals

Transgenic mice carrying the human SOD1\textsuperscript{G93A} mutation were obtained from the TgN (SOD1-G93A) 1Gur line (Jackson Labs, Bar Harbor, ME, USA) and were bred on a B6SJL background. Controls were age matched non-transgenic littermates. Spinal cords from female SOD1\textsuperscript{G93A} transgenic mice and female control (non-transgenic) mice were used in this

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study. All methods conformed to the Australian National Health and Medical Research Council published code of practice for the use of animals in research, and was approved by the Florey Neuroscience Institute animal ethics committee.

Immunocytochemistry

Transfected NSC-34 cells were fixed in 4% (w/v) paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 5 min. Cells were washed three times with PBS after each step and then blocked with 1% BSA for at least 1 h at room temperature. Cells were then incubated with primary antibodies overnight: calreticulin (1:200, Abcam, Cambridge, UK), calnexin (1:200, Abcam, Cambridge, UK), GM130 (1:200, BD Transduction Labs, San Jose, CA, USA) Sec23 (1:100, Affinity Bioreagents, Golden, CO, USA), Sar1 (1:100, Upstate Bioreagents, USA), CHOP (1:50, Santa Cruz, CA, USA), Bax (clone 6A7, 1:200, BD Biosciences Pharmingen, San Jose, CA, USA), or GCC88 (1:1000, kind gift from Professor Paul Gleeson, University of Melbourne), and washed three times with PBS. Incubation with a suitable secondary antibody conjugated to Alexa Fluor secondary antibodies (BD Biosciences Pharmingen, San Jose, CA, USA) at 4°C overnight then followed. Nuclear morphology was also monitored by staining fixed cells with 0.5 µg/ml DAPI for 5 min at room temperature (Soo et al. 2009) and then cells were washed two times with PBS. Bax recruitment to mitochondria was determined by a loss of nuclear staining as used previously (Soo et al. 2009). Coverslips were mounted in Fluorescence mounting medium (DAKO Australia, Victoria, Australia). ER Tracker (Molecular Probes, Invitrogen, Victoria, Australia) was used to stain live cells. Cells were rinsed with Hank’s Balanced Salt Solution (HBSS) and ER Tracker added (1 μM) for 15-30 min at 37°C. Cells were imaged with a Fluoview 1000 inverted confocal laser scanning microscope equipped with an Argon/HeNe laser light source (FV1000 confocal system, Olympus, NSW, Australia) or LSM 510 inverted confocal microscope equipped with a Confocor 3 system containing an Avalanche PhotoDiode (APD) detector (Carl Zeiss, North Ryde, NSW, Australia). A Coolsnap-HQ camera (Photometrics, Australia) was used to acquire images. The Z-stack images were generated using a constant Z-stack interval. All images were obtained at room temperature. Images obtained using the FV1000 Confocal: Images were collected using a 100x/1.4NA oil immersion lens, green fluorescence was detected using an Argon laser, while red fluorescence was detected using the HeNe laser, and DAPI staining was detected using UV light. Images obtained using Zeiss Confocal: Images were collected using a 100x/1.4 Plan-
Apochrome oil objective lens, green fluorescence was detected using an Argon laser, red fluorescence was detected using a DPSS laser, and far red fluorescence was detected using a HeNe laser. In dual-channel imaging, photomultiplier sensitivities and offsets were set to a level at which bleed-through effects from one channel to another were negligible. All images were processed using Image J (http://rsbweb.nih.gov/ij/index.html), Fluoreview 1000, Zeiss and/or MetaMorph (Visitron Systems, Puchheim, Germany) software. For each cell type, greater than 200 cells were scored in all untransfected and dispersed SOD1 groups; for cells with SOD1 inclusions, greater than 60 cells were scored using confocal microscopy. All data are expressed as mean ± SEM from 3 independent experiments unless specified. A one way ANOVA or two-tailed student-t test was performed to detect significant differences between untransfected bystander cells and green fluorescent cells under various conditions. A p value less than 0.05 was considered significant.

Immunohistochemistry

**Mouse.** Paraffin-fixed spinal cord tissue was cut into 25 μm thick sections and washed twice in xylene, twice in 100% ethanol, once in 50% ethanol, once in H2O and once in PBS. Antigen retrieval was conducted by boiling the sections for 5 mins in sodium citrate buffer. Sections were blocked using 3% goat serum/0.3% Tween20 for 30 mins and then immunostained with Sec23 (1:300) and SMI-32 (1:800) antibodies and left overnight at 4°C. Sections were washed twice in PBS Tween 20 (0.1%) and secondary antibodies were added; anti-rabbit Alexa Fluor red (1:250, Molecular Probes, Invitrogen, Victoria, Australia), and anti-mouse Alex Fluor far-red (1:250) for 2 h at RT. Sections were again washed twice in PBS Tween 20 and then a cover slip was applied using Dako fluorescent mounting medium.

**Fluorescence protease protection assay**

The fluorescence protease protection assay was performed as described previously with minor modifications (Lorenz et al. 2006). Briefly, NSC-34 cells were transfected with the indicated plasmids as described previously 48 h prior to analysis. Cells were washed to remove culture medium three times with KHM buffer (110 mM potassium acetate, 20 mM HEPES, 2 mM MgCl2 in H2O) for 1 min each wash. Digitonin ( Sigma Australia, Sydney, NSW, Australia), at 55% purity, was dissolved in H2O by heating to 95-98°C for 10 mg/mL stock. KHM buffer was removed and ~ 1 mL KHM buffer with 60 μM digitonin (effectively ~30 μM) was added. Fluorescence images were captured at regular intervals with 1/4 s
exposure; fluorescence exposure outside of this capture period was kept to a minimum to prevent photobleaching. Buffer was removed 120 s after digitonin addition, and cells were washed briefly with KHM buffer. Proteinase-K (QIAGEN, Victoria, Australia, stock 20 mg/mL) at 50 μg/mL in KHM buffer was added to cells, and fluorescence images were captured at regular intervals using identical settings between samples.

**Protein extraction**

Cells were lysed in Tris-NaCl (TN) buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 0.1% (w/v) SDS, with 1% (v/v) protease inhibitor mixture, for 10 min on ice. Lysates were clarified by centrifugation at 15,800 x g for 10 min and were frozen at -20°C until required. The resulting pellets were resuspended in TN buffer with 1% (w/v) SDS and ultra-sonicated for 15 s to give the insoluble cell fraction.

**Immunoblotting**

Protein samples (20 μg) were electrophoresed through 10-12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Merck-Millipore, Billerica, MA, USA). Unless otherwise stated, reducing conditions were used by the addition of 5% β-mercaptoethanol (βME). Membranes were blocked with 5% (w/v) non-fat milk powder in Tris-buffered saline (TBS), pH 8.0 for 1 h. The appropriate primary antibodies were added as follows; SOD1 (1:1000, Calbiochem, La Jolla, CA, USA), BDNF (1:1000, Abcam, Cambridge, UK), Sec23 (1:500, Affinity Bioreagents), Flag antibody (1:1000, Sigma) and β–actin as a loading control (1:750, Sigma, Sydney, Australia). All antibodies were incubated with membranes for 1 h at room temperature (RT). Blots were probed with HRP-conjugated donkey anti-sheep, goat anti-rabbit, or goat anti-mouse antibodies at 1:2500 (Chemicon, Boronia, Victoria, Australia) for 1h at room temperature, and developed using enhanced chemiluminescence (ECL) reagents (Roche, Dee Why, NSW, Australia). Quantification of band intensities was performed by densitometric analysis following subtraction of background using Image J (National Institutes of Health, Bethesda, Maryland) and expressed relative to the corresponding signal for β-actin. Blots were stripped using Reblot Plus solution (Chemicon, Boronia, Victoria, Australia) for 15 min, and then reprobed as above.

**Glycosylation studies**

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The sequence S V N A Y T D I E M N, containing the glycosylation motif A-X-T, was added to the end of human-SOD1 EGFP and control constructs (described previously (Opat et al. 2000)) by two rounds of site directed mutagenesis (Quikchange, Stratagene, CA, USA) using the following primers:

SOD1-NgF15' GCTGTACAAGAGCGTGAACGCCACCTACTAAAGCGGCC-3';
SOD1-Ng R1  5'-GGCCGCTTTAGTAGGTGGCGTTCACGCTCTTGTACAGC-3';
SOD1-Ng F2  5'-CGCCACCTACACCAGACATCGAGATGAACTAAAGCGGCC-3';
SOD1-Ng R2  5'-GGCCGCTTTAGTTCATCTCGATGTCGGTGTAGGTGGCG-3'.

Cell lysate fractions were denatured, 1 µl βME was added, and boiled for 10 min, then treated with 1 µl Peptide N-glycosidase (PNGase) for one hour at 37ºC to deglycosylate the proteins.

**Immunoprecipitation**

Mouse spinal cord extracts (20 µg total protein) or whole transfected cell lysates (250 µl) were incubated with 1 µl precipitating antibodies (anti-Sec23, Affinity Bioreagents, Golden, CO, USA, or GFP-Trap, ChromoTek GmbH, Germany) and 30 µl of 50% (w/v) protein A-Sepharose CL-4B (Amersham Biosciences, NSW, Australia) in Tris buffer (50 mM Tris-HCl, pH 7.5, 0.02% (w/v) NaN₃) on a rotating wheel overnight at 4ºC. After centrifugation at 15,800 x g for 1 min, pellets were washed twice in Tris buffer for 10 min each. For GFP precipitations, GFP-Trap agarose beads (20 µl per reaction) were incubated with ice cold Tris buffer on a rotating wheel for 1 h at 4ºC then washed twice in Tris buffer. For Western blotting, immunoprecipitates were released by incubation in 2% (w/v) SDS sample loading buffer. The levels of BDNF were quantified by immunoprecipitating 500µl of conditioned medium or 250µl of cell lysate using an anti-BDNF (Abcam) antibody followed by immunoblotting using an anti-BDNF (Promega) antibody.

**VSVG-mCherry secretion assay**

NSC-34 cells were plated on 24-well plates with 13 mm coverslips. The following day, cells were co-transfected with SOD1 and VSVG-ts045 tagged with fluorescent mCherry for indicated time points. Cells were incubated at 40ºC directly after transfection except in the case of the 72 h transfection experiments, where cells were first incubated at 37ºC for 48 h. The temperature was then shifted to 40ºC for a further 24h after transfection to accumulate VSVG-ts045 in the ER. Cycloheximide (20 µg/ml) was added and cells were shifted to the
permissive temperature, 32°C for 0, 15 or 30 min. At each time interval, cells were washed with ice-cold PBS and fixed with 4% PFA. The ER and Golgi compartments were immunostained using rabbit polyclonal anti-calnexin antibody (1:200, Abcam, Cambridge, UK) and mouse monoclonal anti-GM130 antibody (1:200, BD Transduction Laboratories, North Ryde, NSW, Australia) respectively. Eighteen cells were scored in each experiment and all experiments were performed in triplicate. Image analysis was performed using Image J: only single cells expressing both SOD1-EGFP and VSVG-ts045-mCherry were selected for analysis. Plugins were used and the measuring areas (both mCherry and far red channels) were selected above a threshold against background staining. After analysis, the Mander’s coefficient (Manders 1993) was calculated to determine the degree of overlap between images.

Statistics
Data were tested using either one-way analysis of variance (ANOVA) with Tukey’s post-hoc test (with 0.05 significance), two-way ANOVA using Bonferroni’s post-test, or unpaired two-tailed t test with 95% CI. A p value of 0.05 or less was judged to be significant, and results were expressed as mean ± standard error of the mean (SEM).

Results

Mutant SOD1 triggers ER stress from the cytoplasm
The motor neuron-like cell line, NSC-34, transiently expressing human ALS-linked mSOD1 proteins tagged with enhanced green fluorescent protein (EGFP), was used to address the question of whether ER stress is triggered by mSOD1 from the ER or cytoplasm. Transfection of these cells with mSOD1, especially SOD1A4V, induces ER stress (Atkin et al. 2006, Turner et al. 2005) followed by prominent inclusions with apoptosis induced by ER stress at 72 h (demonstrated by nuclear immunoreactivity to the transcription factor C/EBP homologous protein, CHOP), (Walker et al. 2010).

Exclusion of mSOD1 from the ER and its localisation within the cytoplasm was demonstrated using several techniques. Confocal microscopy demonstrated that SOD1A4V did not co-localize with ER marker proteins calnexin in fixed cells or ER Tracker in live cells (Fig. 1a), and minimal co-localisation with calreticulin in fixed cells. This was confirmed using fluorescence protease protection (Lorenz et al. 2006), where digitonin treatment allows
proteinase-K to degrade proteins that are not protected by the ER/Golgi or other membranes. Both SOD1\textsuperscript{A4V} and wildtype SOD1 (SOD1\textsuperscript{WT}) fluorescence disappeared from cells following digitonin treatment (Fig. 1b). By comparison, in control cells the expression of DsRed-tagged protein disulfide isomerase (PDI), a chaperone located in the ER of unstressed cells (Yang et al. 2009), was unchanged after extended treatment.

N-linked glycosylation occurs exclusively within the ER and this was exploited to further examine the intracellular location of SOD1\textsuperscript{A4V}. A previously described N-linked glycosylation motif (Asn-Ala-Thr) was engineered into SOD1\textsuperscript{WT} and SOD1\textsuperscript{A4V} constructs at the C-terminus (SOD1-Ng; construct design in Fig. S1)(Opat et al. 2000). Hence, the presence of carbohydrate would indicate that SOD1 had previously entered the ER. Positive control constructs were created containing the human SOD3 signal peptide fused to SOD1 N-terminus (ER-SOD1) with the glycosylation motif (ER-SOD1-Ng). The presence of carbohydrate was demonstrated by differences in the mobility of these expressed proteins (ER-SOD1\textsuperscript{WT}-Ng and ER-SOD1\textsuperscript{A4V}-Ng) from ER-SOD1\textsuperscript{WT} and ER-SOD1\textsuperscript{A4V} (Fig. 1c). This was confirmed by Peptide N-glycosidase (PNG) treatment to remove the oligosaccharide: protein mobility altered to become equivalent to ER-SOD1\textsuperscript{WT} and ER-SOD1\textsuperscript{A4V}. Hence the glycosylation motif was authentic and both control proteins entered the ER. In contrast, the mobility of SOD1\textsuperscript{WT}-Ng or SOD1\textsuperscript{A4V}-Ng (lacking the signal sequence) was unchanged by PNG treatment, indicating the absence of oligosaccharide and, therefore, that SOD1\textsuperscript{WT} and SOD1\textsuperscript{A4V} are either not in the ER, or at levels that are below detection by immunoblotting. Hence, we conclude that mSOD1 triggers ER stress by mechanisms external to the ER.

**Mutant SOD1 disrupts trafficking of proteins from ER to Golgi apparatus**

The possibility that induction of ER stress occurs by impaired ER-Golgi transport in ALS was then examined in NSC-34 cells expressing SOD1\textsuperscript{WT}, or ALS-mutants SOD1\textsuperscript{A4V}, SOD1\textsuperscript{G85R}, and SOD1\textsuperscript{G93A}. Brain-derived neurotrophic factor (BDNF) was used as a marker of classical ER-Golgi protein secretion in neurons. BDNF levels were significantly higher in the conditioned medium of untransfected cells, and cells expressing SOD1\textsuperscript{WT} or EGFP controls (Fig. 2a), than in the medium of cells expressing mSOD1, particularly SOD1\textsuperscript{G85R} and SOD1\textsuperscript{G93A} (Fig. 2b, 3 fold; p < 0.05). This reflects impaired secretion rather than reduced synthesis of BDNF because intracellular BDNF levels were higher in cells expressing mSOD1 than in control cells. The proform of BDNF was detected, consistent with previous

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reports describing secretion of this form of BDNF from neuronal cells (Adachi 2008, Lu 2003). Hence BDNF secretion is inhibited in cells expressing mSOD1, implying that classical ER-Golgi protein secretion is inhibited by mSOD1. This finding is consistent with previous studies describing impaired secretion of BDNF in ALS (Nagahara & Tuszynski 2011).

Vesicular stomatitis virus glycoprotein ts045 (VSVG-ts045) is a widely used marker specific for ER-Golgi trafficking (Hirschberg et al. 1998). At 40°C, VSVG-ts045 misfolds and is retained in the ER, whereas at 32°C, it is transported to the Golgi. Retention of VSVG-ts045 is detected immunocytochemically by co-location with markers of ER (calnexin) and Golgi (GM130) and is quantified using Mander’s coefficient (Manders 1993). This coefficient calculates an overlap of the fluorescent signals and thus represents an accurate degree of colocalization (Zinchuk et al. 2007). At 32°C, VSVG-ts-045 was transported to the Golgi within 30 min in untransfected cells and in cells expressing SOD1WT or EGFP. However, in cells expressing SOD1A4V or SOD1G85R (Fig. 3a, b, complete images shown in Fig. S2), most VSVG-ts-045 remained in the ER and significantly less (~50%) was transported to the Golgi (p<0.001) (Fig. 3c). We also examined cell lines that stably express SOD1A4V or SOD1G85R. Unlike the transiently expressing cells used above, and presumably due to adaption to survive long-term in culture, the stable cells did not manifest ER stress (data not shown). ER-Golgi trafficking was inhibited in both stable lines in comparison to stable SOD1WT or control EGFP expressing cells (Fig. 3d), demonstrating that ER-Golgi transport inhibition by mSOD1 is not triggered by ER stress. The inhibition of transport could not be attributed to non-specific over-expression of protein because ER-Golgi trafficking was no greater in control cells expressing SOD1WT or EGFP than in untransfected cells. We also considered the possibility that tagging of SOD1 with EGFP may impact on ER-Golgi trafficking, but the ability of untagged mutant SOD1 to inhibit VSVG-ts045 transport was found to be similar to EGFP-tagged SOD1(Fig. 3e). Thus mSOD1 specifically inhibits protein transport from the ER to Golgi apparatus.

The Golgi apparatus fragments when ER-Golgi transport is blocked and has been previously described in ALS (Mourelatos et al. 1990). Immunocytochemistry using markers of the trans-Golgi (GCC88) confirmed that the Golgi was fragmented in mSOD1-expressing cells, but remained intact in SOD1WT cells, EGFP cells, untransfected and bystander cells (Fig. S3).
**ER-Golgi trafficking is disrupted early after mSOD1 expression**

If disruption of ER-Golgi trafficking is relevant to cellular pathology in mSOD1 expressing cells, then it should occur early after transfection of mSOD1 expressing cells. This possibility was examined in a time-course analysis whereby the time of onset of cellular events was established by examining for their presence every 2 h after transfection of NSC-34 cells. SOD1<sup>A4V</sup> expression was first detected by immunoblotting 10-12 h post transfection (hpt) and had increased significantly by 18 hpt (Fig. 4a). EGFP fluorescence was first observed microscopically at 14 hpt but inclusions were not present until 24 hpt (Fig. 4a). ER stress was first detected at 18 hpt using immunoblotting for immunoglobulin binding protein (BiP), the first marker of ER stress to be up-regulated in the UPR (Fig. 4b). This was confirmed by examining a later marker of ER stress, CHOP (Walker *et al.* 2010). Nuclear CHOP immunoreactivity in SOD1<sup>A4V</sup> cells was significantly greater than in untransfected or SOD1<sup>WT</sup> cells (3-fold, p<0.001; Fig. 4b). In contrast, inhibition of ER-Golgi transport was first detected in SOD1<sup>A4V</sup> and SOD1<sup>G85R</sup> cells at 16 hpt (Fig. 4c, 2-fold, p<0.001), increasing by 18 hpt in SOD1<sup>WT</sup> cells compared to mutant cells (p<0.001). Hence mSOD1 inhibits ER-Golgi transport as an early event after transfection, which we detected 2 h prior to the first observation of ER stress and 6 h prior to SOD1<sup>A4V</sup> aggregation and inclusion formation. These data suggest that inhibition of ER-Golgi transport contributes to ER stress in ALS. Fragmentation of the neuronal Golgi apparatus, examined by immunocytochemistry for GM130, was first observed at 18 hpt in a proportion of cells expressing SOD1<sup>A4V</sup> (Fig. 4d) and was not observed in SOD1<sup>WT</sup> and untransfected cells as expected. Hence inhibition of ER-Golgi transport was present 2 h prior to the first detection of Golgi fragmentation, suggesting that Golgi fragmentation is not the primary cause of ER-Golgi transport disruption. Apoptosis was also examined using two cellular markers. Bax is an early apoptotic protein recruited to mitochondria when apoptosis is initiated (Soo *et al.* 2009). Immunocytochemistry for Bax revealed recruitment to mitochondria at 20 hpt in SOD1<sup>A4V</sup> cells (Fig. 4e). However, condensed, apoptotic nuclei, indicative of late apoptosis, did not appear until 24 hpt. Hence together these data demonstrate that, at least subject to the limitations of the methods used, inhibition of ER-Golgi transport preceded ER stress, Golgi fragmentation, mSOD1 inclusion formation, and apoptosis, in neuronal cells expressing mSOD1.
Over-expression of COPII subunits rescues ER-Golgi trafficking and protects against mSOD1 inclusion formation and apoptosis

We next examined the effect of restoration of ER-Golgi transport on inclusion formation and apoptosis to determine if ER-Golgi transport is linked to neurodegeneration. Sar1 is the rate-limiting subunit which initiates assembly of the COPII complex (Long et al. 2010, Routledge et al. 2010). Hence over-expression of Sar1 should enhance COPII-mediated ER-Golgi vesicular transport, and rescue transport inhibition if the mSOD1 defect is linked to COPII. Hence Sar1 was co-expressed with SOD1\textsuperscript{A4V} in NSC-34 cells with an empty vector control (‘A4V’, Fig. 5a). Immunoblotting of cell lysates revealed that similar levels of SOD1\textsuperscript{A4V} was expressed in each case, demonstrating similar transfection efficiencies and that Sar1 does not alter the level of mSOD1 expression (Fig. 5a). ER-Golgi transport was then examined at 18 hpt, when ER-Golgi transport was maximally inhibited. ER-Golgi transport was inhibited in cells expressing SOD1\textsuperscript{A4V} alone, as expected (Fig. 5b, left). However in contrast, VSVG-ts045 was efficiently transported from ER to the Golgi apparatus when Sar1 and SOD1\textsuperscript{A4V} were co-expressed. The Mander’s coefficient was now similar to that of SOD1\textsuperscript{WT} and untransfected cells (Fig. 5b, right) demonstrating that Sar1 over-expression completely rescues inhibition of ER-Golgi transport by SOD1\textsuperscript{A4V}. There was no effect of Sar1 overexpression on transport of VSVG-ts045 in untransfected or SOD1\textsuperscript{WT} expressing cells, demonstrating that Sar1 could restore the transport defects induced specifically by mSOD1, as opposed to simply accelerating ER-Golgi transport. Hence these data imply that inhibition of ER-Golgi transport is linked to COPII vesicle formation and/or budding from the ER.

The effect of restoration of ER-Golgi transport on inclusion formation and apoptosis by Sar1 was then examined. A significantly smaller proportion of cells expressing Sar1 formed SOD1\textsuperscript{A4V} inclusions than did cells expressing the empty vector (Fig. 5c; p<0.001). Similarly, significantly fewer cells had apoptotic nuclei when co-expressed with Sar1 (p<0.05). The protection against these processes provided by increasing the expression of COPII subunits links ER-Golgi trafficking to neurodegeneration and implies an upstream role for ER-Golgi transport in cellular ALS pathology.

Mutant SOD1 co-localizes and interacts with ER-Golgi transport proteins

Mutant SOD1 proteins contain regions of unfolding (Shaw et al. 2006) which could easily lead to promiscuous interactions with other proteins, such as those necessary for ER-Golgi
transport, including COPII. NSC-34 cell lysates were therefore examined for an interaction between mSOD1 and COPII subunits using co-immunoprecipitation experiments. Using anti-Sec23 antibodies, a band of ~50 kDa (representing SOD1-EGFP) was immunoprecipitated from lysates of SOD1^{A4V}, SOD1^{G93A} and SOD1^{G85R} cells (Fig. 6a, top). There was little precipitate from SOD1^{WT} cells or control EGFP-only or untransfected cells. Similarly, immunoprecipitations using Sec23 with DMEM alone or isotype control antibodies were negative, demonstrating that the precipitating antibodies did not cross-react (Fig. 6a). The mSOD1-Sec23 interaction was confirmed by immunoprecipitations in the reverse orientation: Sec23 was precipitated from insoluble cell fractions using GFP-Trap to pull-down SOD1 (Fig. 6a, bottom). These data imply that COPII physically interacts with mSOD1. We also examined transgenic SOD1 mouse spinal cord lysates for an interaction between mSOD1 and COPII. Immunoprecipitation of tissue lysates from SOD1^{G93A} mice using anti-Sec23 antibodies precipitated a SOD1 band (Fig. 6b) from animals as young as post-natal age 10 days (p10), 20 days prior to UPR induction, Golgi fragmentation and axonal transport inhibition (Saxena et al. 2009, Mourelatos et al. 1996, Bilsland et al. 2010) and 80 days prior to symptom onset (Gurney et al. 1994). Hence, mSOD1 binds to COPII early in SOD1 animal models, implying an active role in pathophysiology.

The distribution of COPII in SOD1 expressing cells was examined further by immunocytochemistry. The immunoreactivity of COPII subunits Sar1 and Sec23 was greater in SOD1^{A4V}-expressing cells than in controls expressing SOD1^{WT} (Fig. 6c). Furthermore, the distribution of COPII was altered in SOD1^{A4V}-expressing cells. In SOD1^{WT} cells, Sec23 staining was diffuse and perinuclear (Fig. 6c). In contrast, in SOD1^{A4V} cells Sec23 was co-located with mSOD1 inclusions in every cell examined (>50, Fig. 6c), confirmed by serial z-stack analysis (Fig. S4). The distribution of Sec23 was examined further in transgenic SOD1^{G93A} mice. In control animals, Sec23 was expressed diffusely or fine-granular throughout the cytoplasm of motor neurons. In contrast, in transgenic SOD1^{G93A} mice, Sec23 formed aggregate-like structures (Fig. 6d) within most motor neurons, providing evidence for disruption of the ER-Golgi transport machinery in disease-relevant tissues. We conclude that mSOD1 inhibits the transport of secretory proteins from the ER to the Golgi. COPII over-expression restores this defect and reinstates ER-Golgi transport, leading to a reduction in inclusion formation, and apoptosis, thus linking ER-Golgi transport to cellular pathology. An aberrant interaction between mSOD1 and COPII provides a possible molecular explanation for transport inhibition, although further studies are required to identify the precise mechanisms involved.
Discussion

This study demonstrates that ALS-associated SOD1 mutants inhibit the transport of secretory proteins from the ER to the Golgi apparatus. Evidence of impaired ER-Golgi transport in cells expressing mSOD1 was provided using the VSVG-ts045 transport assay and by impaired BDNF secretion. BDNF levels were higher intracellularly but lower in the conditioned medium of mSOD1-expressing cells than in control cell lines. Transport of VSVG-ts045 was impaired in stable cell lines without ER stress, demonstrating that trafficking disruption is upstream and not due to ER stress. ER-Golgi transport is a fundamental cellular trafficking process, involving proteins that eventually reside in the plasma membrane, Golgi, lysosomal or endosomal compartments, as well as secretory proteins: in total, one third of all eukaryotic proteins (Ghaemmaghami et al. 2003). Hence, disruption to ER-Golgi transport could severely impact on many cellular functions and viability. Re-instatement of ER-Golgi transport in our study prevented inclusion formation and apoptosis, thus implying a functional relationship between ER-Golgi trafficking and cellular pathology in ALS.

SOD1\(^{WT}\) or SOD1\(^{A4V}\) was not detected in the ER by any of the methods used in this study, leading us to conclude that mSOD1 triggers ER stress largely from the cytoplasm. Inhibition of ER-Golgi transport provides a possible explanation for how cytoplasmic SOD1 triggers ER stress because impairment of ER-Golgi traffic could lead to accumulation of secretory proteins within the ER. SOD1 lacks a secretory leader peptide, and although there are reports that mSOD1 aberrantly enters and misfolds within the ER, it is argued by others that cytosolic SOD1 contaminates microsome preparations, leading to erroneous interpretations (Nishitoh et al. 2008). While there are reports that SOD1 is present in vesicles, they have not been confirmed using a protease to release SOD1 from within membrane-bound organelles. Mutant SOD1 interacts with BiP (Kikuchi et al. 2006) which is typically ER resident, but it can relocate to the cytoplasm and cell surface (Zhang et al. 2010). Similarly, mSOD1 interacts with PDI (Atkin et al. 2006), which is conventionally ER-resident but is also found on the cell surface, in the cytoplasm and nucleus (Turano et al. 2002), and it redistributes away from the ER in ALS (Yang et al. 2009). Hence the previously described interactions between mutant SOD1 and PDI or BiP may also occur in the cytoplasm in ALS. Furthermore, SOD1 was not found in the ER/Golgi by electron microscopy (Chang et al. 1988), despite the presence of ER abnormalities in ALS patients (Sasaki 2010). The proposal
that ER stress is triggered by SOD1 from the cytoplasm in ALS is consistent with cytoplasmic localisation of other proteins involved in ALS, including TDP-43 and FUS. Nevertheless, we cannot exclude the possibility that mSOD1 may be present in the ER at levels that are too low to be detected by Western blotting (Fig. 1). However, it is unnecessary to invoke induction of ER stress by mSOD1 within the ER when most mSOD1 is present in the cytoplasm and we have provided a mechanism for induction of UPR from the cytoplasm, which occurs prior to ER stress. Others have previously proposed that ER stress in ALS is triggered by cytoplasmic SOD1 aberrantly binding to Derlin-1 (Nishitoh et al. 2008). However this interaction was not detected until after symptom onset in SOD1<sup>G93A</sup> mice, whereas the UPR is induced at least 60 days prior to this in these animals (Saxena et al. 2009), demonstrating that other mechanisms in addition to binding to Derlin-1 must trigger ER stress.

The cascade of events that follow transfection suggests that early events trigger other cellular pathologies that eventually lead to cell death. ER stress is a precursor of cell death in ALS (Saxena et al. 2009, Atkin et al. 2006) and can be caused by failure of ER-Golgi trafficking (Preston et al. 2009). The intact Golgi architecture is maintained by both anterograde (ER-Golgi) and retrograde (Golgi-ER) traffic and an imbalance in these processes leads to fragmentation of the Golgi (Nakagomi et al. 2008). Hence impaired anterograde transport would also lead to fragmentation of the Golgi, and both ER stress and Golgi fragmentation occurred later than inhibition of VSVG-ts045 trafficking in the time course study. However, one caveat is that although we detected ER stress and Golgi fragmentation 2h later than ER-Golgi trafficking in our study, it is possible that there is some overlap in these events which could not be detected by the methods used here. Similarly, axonal transport is another ALS pathology linked to ER-Golgi trafficking. The relationship between ER-Golgi and axonal transport is poorly understood but these processes are clearly linked and COPII is implicated in both events (Reiterer et al. 2008, Hammerschlag et al. 1982, Ramirez & Couve 2011). Further studies are required to establish whether ER-Golgi disruption is an example of a more generic transport disruption in ALS. Future studies could establish whether ER-Golgi disruption precedes other forms of trafficking or occurs simultaneously.

The function of other proteins implicated in ALS suggests that a disturbance in intracellular transport, particularly ER-Golgi trafficking, is not unique to mSOD1. Mutations causing ALS have now been identified in numerous genes encoding intracellular transport.
proteins, including Alsin (Mimnaugh et al. 2006), vesicle-associated protein (VAPB) (Nishimura et al. 2004), dynactin (Puls et al. 2003), CHMP2B (Cox et al. 2010), optineurin (Maruyama et al. 2010), and valosin-containing protein (VCP) (Johnson et al. 2010). Some of these proteins are directly implicated in ER-Golgi transport: optineurin is Golgi-localized and involved in vesicle trafficking (Sahlender et al. 2005), knockdown of VCP disrupts ER-Golgi trafficking (Mimnaugh et al. 2006), and VAPB regulates ER-Golgi transport (Prosser et al. 2008, Peretti et al. 2008). Several ER-Golgi transport proteins are implicated in other motor neuron disorders, including atlastin (Botzolakis et al. 2011) and seipin (Ito & Suzuki 2007). Furthermore, disruption in ER-Golgi trafficking has also been described in spontaneous mouse mutants with motor phenotypes, pmn (Schaefer et al. 2007) and wobbler (Schmitt-John et al. 2005). A recent study also demonstrated mice with a deletion of Scy1, implicated in COPI mediated transport between the Golgi and ER, display a motor neuron degenerative phenotype (Pelletier et al. 2012). Interestingly, the uncharacterized gene C9ORF72, which contains a non-coding repeat expansion in the most common familial forms of ALS (DeJesus-Hernandez et al. 2011), is predicted to regulate membrane traffic in conjunction with Rab proteins (Levine et al. 2013).

Mutant SOD1 is expressed ubiquitously, yet motor neurons are preferentially affected in ALS. What might render these cells uniquely sensitive to inhibition of ER-Golgi transport? The ER in neurons is poorly characterised, but the protein synthetic machinery is abundant, extending throughout the axon and dendritic arbour (Ramirez & Couve 2011). Motor neurons also have complex morphologies with very long processes, and there may be specific functions of large ER-Golgi which render them uniquely vulnerable to abnormalities in these compartments. Alternatively, motor neurons may be more sensitive than other cell types to cellular transport defects. With unusually long axons and large soma, motor neurons are highly dependent on efficient axonal transport for maintenance and viability.

The molecular nature of mSOD1 which inhibits ER-Golgi transport remains unclear. In the time course analysis, mSOD1 inclusions were not detected until 24 hpt, but VSVG-ts045 transport was inhibited 6hr prior to this. Similarly, VSVG-ts045 transport was inhibited in two different mSOD1 stable cell lines, and fragmentation of the Golgi was detected in mSOD1D90A expressing cells (Fig. S3), neither of which formed inclusions. These data therefore suggest that small solubles forms of SOD1 inhibit transport, and whilst linked to apoptosis (Soo et al. 2009), inclusions form after apoptosis has commenced.

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We demonstrated previously that SOD1 is secreted and that the secretion of mSOD1 is impaired relative to SOD1^{WT} (Turner et al. 2005). The findings of the current study are consistent with the impairment of protein secretion in mSOD1 expressing cells. However, the secretion of mSOD1 occurs via a non-classical secretory route (Mondola et al. 2003, Mondola et al. 1996), unlike the classical ER-Golgi secreted proteins VSVG-ts045 and BDNF examined in this study. Other studies have reported that mSOD1 secretion is enhanced by an aberrant interaction with chromagranins (Urushitani et al. 2006). Hence it is probable that mSOD1 secretion is a distinct process from classical ER-Golgi protein secretion.

We detected an aberrant interaction between mSOD1 and COPII in this study. This would reduce the levels of COPII available for vesicle budding from the ER, which as a consequence could disrupt COPII vesicle formation. This mechanism therefore provides one explanation for the inhibition of transport from the ER, and it is consistent with increased levels of VSVG-ts045 within the ER detected in mSOD1 expressing cells. We also observed an increase in COPII immunoreactivity in mSOD1 expressing cells, which implies compensation for altered ER architecture and damaged intracellular traffic. Sar1 is a small Ras-like GTPase which initiates COPII vesicle assembly by recruiting the other COPII subunits (39). The restoration of ER-Golgi trafficking by Sar1 supports the view that COPII vesicle budding from the ER is an important target for mSOD1 in triggering ER-Golgi transport failure. The protection against toxicity by Sar1 also supports this proposed mechanism and links ER-Golgi trafficking to neurodegeneration and disease.

The link between COPII and axonal transport suggests that if mSOD1 binds aberrantly to COPII, this may impact on axonal transport. The correct sorting of proteins to the axon is dependent on ER export via Sec24 (Reiterer et al. 2008). Axon growth and elongation is also Sar1-dependent (Aridor & Fish 2009) and mutations in Sec23 and Sar1 inhibit dendritic arborization (Ye et al. 2007). We detected mSOD1-COPII interaction in SOD1^{G93A} transgenic animals as young as p10, prior to Golgi fragmentation, ER stress and axonal transport inhibition in these mice, and well before inclusion formation, cell loss and symptom onset at p90-120 in these animals (Saxena et al. 2009, Mourelatos et al. 1996). However, although our results fit the current model, we cannot rule out other possibilities. Dynein is also a potential target for aberrant mSOD1 interactions, and this would also disrupt ER-Golgi transport. Dynein has multiple roles in ER-Golgi transport, including maintenance of the architecture of the ER and Golgi, recruitment of COPII, and ER exit (Palmer et al. 2009).

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Mutant SOD1 has been linked to ALS and shown previously to interact with dynein (Zhang et al. 2007), although this has been disputed (El-Kadi et al. 2010). Hence it is possible that dynein dysfunction may also inhibit ER-Golgi transport either directly, by disruption of COPII transport along microtubules, or by recruitment of COPII via dynactin (Fromme et al. 2008). Alternatively, mSOD1-dynein interaction may exacerbate trafficking disruption already initiated by another upstream target. Hence, further studies are required to determine the precise molecular events responsible for the inhibition of ER-Golgi transport.

We conclude that ALS-associated mSOD1 impairs the early secretory pathway by inhibition of protein transport from the ER to Golgi. Our data suggest that impairment of ER-Golgi transport represents an early cellular disturbance and with clear links to other previously described cellular events in ALS, these findings, summarised in Fig. 7 draw together multiple pathological events into a possible single mechanism.

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**Figure Legends**

**Fig. 1** Mutant SOD1A4V is not present in the ER of NSC-34 cells (a) Confocal fluorescent microscopy of NSC34 cells expressing EGFP-tagged mSOD1A4V at 72 h post-transfection. SOD1A4V does not co-localize with ER markers, either calreticulin or calnexin in fixed cells or ER Tracker in live cells. Arrows indicate SOD1A4V which is excluded from regions with

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immunoreactivity to ER markers. Scale bar 10 μm. (b) SOD1A4V is not found within membranes: fluorescence protease protection assay. SOD1A4V and SOD1WT are released after digitonin addition, indicating cytoplasmic localisation. Proteinase-K eliminates fluorescence, indicating SOD1A4V is located outside of membrane-bound organelles. In contrast, digitonin/proteinase-K treatment has little influence on PDI indicating location in membrane-bound organelles. Scale bar 25 μm. (c) Glycosidase treatment does not alter the mobility of SOD1-Ng, indicating no transit through the ER. Immunoblotting of NSC-34 cell lysates with/without PNGase (P) treatment to remove oligosaccharide. Control SOD1WT and SOD1A4V containing signal peptide and Ng demonstrate a shift in mobility after glycosidase treatment unlike when the signal peptide is absent, revealing no oligosaccharide and exclusion from the ER.

**Fig. 2** ER-Golgi trafficking is inhibited in NSC-34 cells expressing mSOD1 at 72 h post transfection (a) Secretion of BDNF is reduced in cells expressing mSOD1. Immunoblotting of conditioned medium immunoprecipitated for SOD1 or cell lysates expressing EGFP alone, SOD1WT or mutants SOD1A4V, SOD1G93A, SOD1G85R, or untransfected cells (Untr). (b) Densitometric quantification of immunoblots in (A), mean ± SEM, *p<0.05 versus wildtype by one-way ANOVA, n=3.

**Fig. 3** Representative fluorescent images and z-stack orthogonal views of co-expressing cells with EGFP tagged SOD1WT, SOD1A4V, SOD1G85R and VSVGts045-mCherry at 72 h post-transfection stained with markers of (a) ER (calnexin), or (b) Golgi apparatus (GM130). VSVGts045-mCherry was trapped in the ER at 40°C for 24 hours. The temperature was then shifted to the permissive temperature (32°C) for 30 min and the cells were fixed with 4% PFA at 30 min at 32°C. Scale bar 10 μm. (c) The degree of co-localisation of VSVGts045-mCherry with calnexin (Cal) or GM130 (GM) was quantified using Mander’s coefficient. Data are presented as mean ± SEM, ***, indicates significant (p<0.001) difference with SOD1WT or control untransfected cells (Untr), tested with one-way ANOVA and Tukey’s post-test, n=3. (d) ER-Golgi transport of VSVGts045-mCherry is inhibited in NSC-34 cells stably expressing SOD1WT, SOD1A4V and SOD1G85R that do not have ER stress. Stably transfected cells were transfected with VSVGts045-mCherry for 48 h. Cells were then treated as above. Quantification of the co-localisation of VSVGts045 with calnexin (Cal) and GM130 (GM) using Mander’s coefficient is shown. Data are presented as mean ± SEM, ***, p<0.001, **, p<0.01, versus SOD1WT or GFP expressing cells (GFP) by one-way ANOVA.
with Tukey’s post-test, n=3. (e) NSC-34 cells expressing untagged mSOD1 have impaired ER-Golgi trafficking. Quantification of the co-localisation of VSVGts045 with calnexin (Cal) and GM130 (GM) in cells expressing untagged SOD1\(^{WT}\) and SOD1\(^{A4V}\) expressed in pEF-BOS, and control cells. Mander’s coefficient is shown. Data are presented as mean ± SEM, ***, p<0.001, versus SOD1\(^{WT}\) by one-way ANOVA with Bonferroni’s post-test, n=3.

**Fig. 4** Temporal analysis shows ER-Golgi trafficking is inhibited prior to ER stress, Golgi fragmentation, inclusion formation and apoptosis. NSC-34 cells were transfected with SOD1\(^{WT}\) or SOD1\(^{A4V}\) at indicated time points. (a) SOD1-EGFP expression is detected 10-12 hpt but increases at 18 hpt by immunoblotting; endogenous mouse SOD1 (mSOD1) shown for comparison. SOD1\(^{A4V}\) is expressed diffusely until 24 hpt when inclusions form. (b) Induction of ER stress in cells expressing SOD1\(^{A4V}\) occurs at 18 hpt. Transfected cell lysates were collected at the indicated time (hpt) and subjected to western blotting using an anti-BiP antibody (early ER stress marker). Western blot with β-actin antibody was used as a loading control. Transfected cells were also fixed with 4% PFA at the indicated time and subjected to immunocytochemistry using anti-CHOP antibodies. Cells were then counterstained with DAPI to visualize the nucleus and identify nuclear CHOP immunoreactivity (white arrow). Quantification of data are presented as mean ± SEM, ***,**, indicates significant (p<0.001, p<0.01) difference with SOD1\(^{WT}\) from 100+ cells per group, tested with one-way ANOVA and Tukey’s post-test, n=3. (c) Quantification of VSVGts045-mCherry transport using Mander’s coefficient, presented as mean ± SEM, ***, p<0.001, versus SOD1\(^{WT}\) by one-way ANOVA with Bonferroni’s post-test, n=3. All scale bars 10 μm. ER-Golgi trafficking inhibition at 14, 16, 18 and 72 hpt is shown. (d) Golgi fragmentation, identified by immunocytochemistry using anti-GM130 antibodies, presented as mean ± SEM, *, p<0.05, versus SOD1\(^{WT}\) by one-way ANOVA with Bonferroni’s post-test, n=3. (e) Bax is recruited to mitochondria and depleted from nucleus in SOD1\(^{A4V}\) but not SOD1\(^{WT}\) cells, presented as mean ± SEM, ***, p<0.001, **, p<0.01, *, p<0.05, versus SOD1\(^{WT}\) by one-way ANOVA with Bonferroni’s post-test, n=3.

**Fig. 5** Over-expression of COPII ameliorates SOD1\(^{A4V}\) inclusions, apoptosis, and inhibition of ER-Golgi transport. NSC-34 cells were transiently co-transfected with SOD1-EGFP plus empty pCMV-2 vector (‘A4V’), or SOD1-EGFP plus pCMV-2 construct encoding COPII Sar1. (a) Immunoblotting of soluble cell lysates reveals similar transfection efficiencies of SOD1 and expression of Flag-Sar1. (b) Quantification of transport of VSVG-ts045-mCherry
from ER-Golgi in cells transiently co-expressing SOD1 and VSVG-ts045-mCherry with/without Sar1 at 18 hpt using Mander’s coefficient; calnexin (Cal) and GM130 (GM). Data are presented as mean ± SEM, ***, p<0.001, versus SOD1<sup>WT</sup> by one-way ANOVA with Tukey’s post-test, n=3. (c) Overexpression of Sar1 reduces the percentage of cells forming inclusions and apoptosis in SOD1<sup>A4V</sup> expressing cells. Left panel; the percentage of transfected cells bearing fluorescent inclusions, calculated from 100+ cells/group; Right panel; Quantification of condensed, apoptotic nuclei, showing the percentage of transfected cells with condensed/fragmented nuclei, 100+ cells/group were analysed. Data are presented as mean ± SEM, ***, p<0.001, *, p<0.05 versus A4V by one-way ANOVA with Tukey’s post-test, n=3.

**Fig. 6** mSOD1 interacts with COPII. (a) Co-immunoprecipitation of mSOD1 and Sec23 using cell lysates from transient transfections at 72 hpt, n=3. Top panel; Mutants SOD1<sup>A4V</sup>, and SOD1<sup>G85R</sup> but little SOD1<sup>WT</sup> are precipitated by anti-Sec23 antibodies. Input control (2%), representing cell lysate used for immunoprecipitations. The anti-SOD1 and anti-Sec23 antibodies do not cross-react with other proteins because no precipitate is found in controls with GFP only (GFP) or untransfected cells (Un), DMEM only (Buffer) or isotype-matched IgG control antibody (IgG). Bottom panel; The reverse IP using GFP trap followed by immunoblotting using anti-Sec23 antibodies confirms the interaction, (b) SOD1 is precipitated by anti-Sec23 antibodies from transgenic SOD1<sup>G93A</sup> mice spinal cord lysates at 5 postnatal ages, p10, p30, p60, p90, p120 days: 2% input is shown. (c) The immunoreactivity of COPII subunits is altered in NSC-34 cells. Confocal fluorescent images of transfected NSC-34 cells using immunocytochemistry with anti-COPII antibodies shows altered distribution and co-localisation of Sar1 and Sec23 in SOD1<sup>A4V</sup> expressing cells (right) at 48 hpt in comparison to SOD1<sup>WT</sup> expressing cells (left). Scale bar 10μm. (d) The immunoreactivity of COPII subunits is altered in motor neurons of SOD1<sup>G93A</sup> mice. Confocal fluorescent images of lumbar spinal cord motor neuron sections (20μm) from SOD1<sup>G93A</sup> mice at postnatal age 120 days. Sections were immunostained with SMI32 (first column) antibodies to locate the motor neurons and Sec 23 (second column) antibodies. Sec23 staining was reduced in SOD1<sup>G93A</sup> motor neurons compared to SOD1<sup>WT</sup> mice and appeared to be confined to aggregate-like structures (white arrow). Scale bar 10μm.

**Fig. 7** Diagram summarising the findings of this study. Mutant SOD1 located primarily in the cytoplasm inhibits ER-Golgi transport, which triggers ER stress, fragmentation of the Golgi apparatus and eventually apoptosis.
Fig. 1

(a) 

(b) Proteinase K

Digitonin

Time

0 s 60 s 120 s 135 s 240 s 420 s

SOD1\textsuperscript{Mv}

PDI

SOD1\textsuperscript{WT}

(c) 

Ng - + + - + +
P - - + - - +
ER - - - + + +

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Fig. 2

(a) Untr EGFP WT A4V G93A G85R kDa

Medium

Lysates

(b) Medium

Densitometry units

Untr EGFP WT A4V G93A G85R

Lysates

Densitometry units

Untr EGFP WT A4V G93A G85R

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**Fig. 3**

(a) EGFP, VSVG/Calnexin, Z-stack

(b) EGFP, VSVG/GM130, Z-stack

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Fig. 3

(c) ER and Golgi

(d) ER and Golgi

(e) ER and Golgi

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Fig. 4

(a) SOD1\(^{AAV}\) expression in different time points (10-24 hours).

(b) BiP expression at 16 and 18 hours under WT and AAV conditions.

Graph showing the percentage of cells with nuclear CHOP over time (16-22 hours) for different treatments (Uninfected WT, Uninfected AAV, Infected WT, Infected AAV) with error bars indicating standard deviation.

Images showing EGFP, CHOP, DAPI, and Merge channels for 16 and 18 hours post-transfection with WT and AAV conditions.

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Fig. 4

(c) ER-Golgi transport

(d) Golgi fragmentation

(e) Bax recruitment

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Fig. 5

(a) kDa Un A4V Sar 1
50 → SOD1-EGFP
25 → Flag-Sar1

(b) VSVG No Sar1
VSVG With Sar1

(c) Inclusions
Apoptosis

% Transfected cells with inclusions
% Transfected cells with apoptotic nuclei

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