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Targeting Tau Mitigates Mitochondrial Fragmentation and Oxidative Stress in Amyotrophic Lateral Sclerosis

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1 **Targeting tau mitigates mitochondrial fragmentation and oxidative stress**
2 **in amyotrophic lateral sclerosis**

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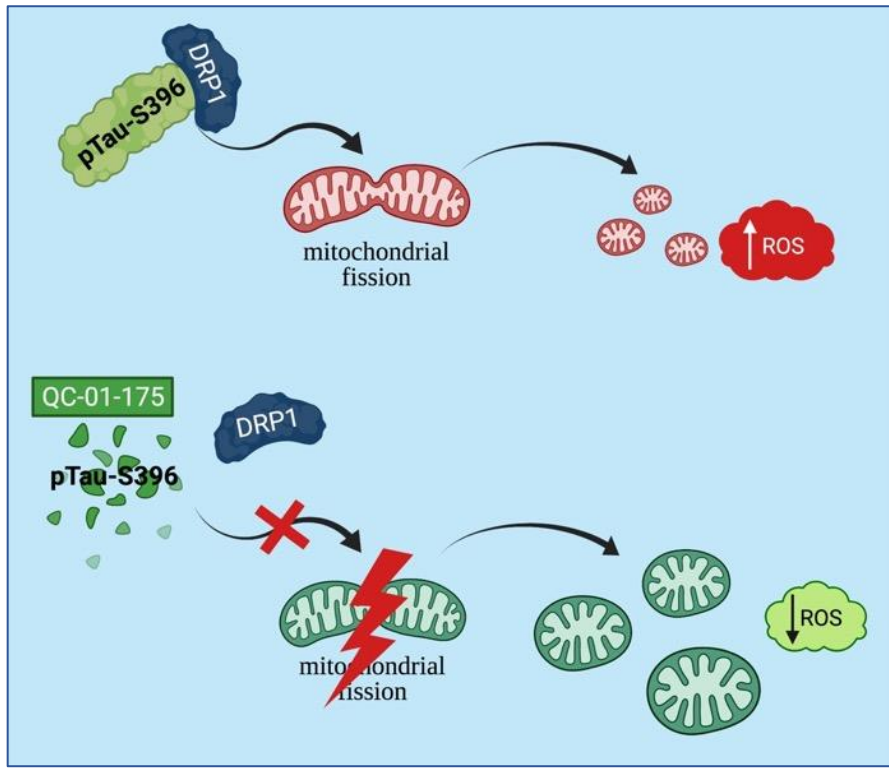
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50
51 **Running Title:** Targeting tau in ALS

1 **Graphical abstract**



- 3
- pTau-S396 mis-localizes to synapses in ALS.
 - ALS synaptoneuroosomes (SNs), enriched in pTau-S396, increase oxidative stress and induce mitochondrial fragmentation *in vitro*.
 - pTau-S396 interacts with the pro-fission GTPase DRP1 in ALS.
 - Reducing tau with a specific degrader, QC-01-175, mitigates ALS SNs-induced mitochondrial fragmentation and increases in oxidative stress *in vitro*.
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1 **Abstract**

2
3 Understanding the mechanisms underlying amyotrophic lateral sclerosis (ALS) is crucial for the
4 development of new therapies. Previous studies have demonstrated that mitochondrial dysfunction is
5 a key pathogenetic event in ALS. Interestingly, studies in Alzheimer's disease (AD) post-mortem brain
6 and animal models link alterations in mitochondrial function to interactions between
7 hyperphosphorylated tau and dynamin-related protein 1 (DRP1), the GTPase involved in mitochondrial
8 fission. Given that recent evidence suggest that tau may be involved in ALS pathogenesis, we sought
9 to determine whether hyperphosphorylated tau may lead to mitochondrial fragmentation and
10 dysfunction in ALS and whether reducing tau may provide a novel therapeutic approach. Our findings
11 demonstrated that pTau-S396 is mis-localized to synapses in post-mortem motor cortex (mCTX) across
12 ALS subtypes. Additionally, the treatment with ALS synaptoneurosomes (SNs), enriched in pTau-S396,
13 increased oxidative stress, induced mitochondrial fragmentation, and altered mitochondrial connectivity
14 without affecting cell survival *in vitro*. Furthermore, pTau-S396 interacted with DRP1, and similar to
15 pTau-S396, DRP1 accumulated in SNs across ALS subtypes, suggesting increases in mitochondrial
16 fragmentation in ALS. As previously reported, electron microscopy revealed a significant decrease in
17 mitochondria density and length in ALS mCTX. Lastly, reducing tau levels with QC-01-175 prevented
18 ALS SNs-induced mitochondrial fragmentation and oxidative stress *in vitro*. Collectively, our findings
19 suggest that increases in pTau-S396 may lead to mitochondrial fragmentation and oxidative stress in
20 ALS and decreasing tau may provide a novel strategy to mitigate mitochondrial dysfunction in ALS.

21
22 **Key words:** hyperphosphorylated tau, mitochondrial dynamics, mitochondrial dysfunction, tau
23 degrader, amyotrophic lateral sclerosis

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13 **Availability of data and materials:** The datasets used and analyzed during the current study are
14 available from the corresponding author on reasonable request.

15 **Conflicts of interest:** B.T.H. is a member of Novartis, Dewpoint, and Cell Signaling Scientific Advisory
16 Board (SAB), and of Biogen DMC, and acts as consultant for US DoJ, Takeda, Virgil, W20, and Seer;
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27 of the SAB and equity holder in Rodin Therapeutics, Psy Therapeutics, Frequency Therapeutics, and
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33 **Code availability:** Not applicable

34 **Authors' contributions:** T.P. and E.A.B. contributed to the study design, data collection, data analysis,
35 and drafting of the manuscript. A.N.M., S.E.K., E.S., B.A.D., A.A.O., S.M.K.F., A.C.A., S.D., P.M.D.
36 contributed to the data collection, data analysis, and editing of the manuscript. C.H., D.H.O., A.N.,
37 B.T.H., T.S.J., S.D.B., K.V., M.E.C., J.D.B., M.D., M.C.S., S.J.H. contributed to the study design, and
38 editing of the manuscript. G.S.V. contributed to the study design, data analysis, drafting of the
39 manuscript. All authors read and approved the final manuscript.

40 **Ethics approval:** The study was approved by the Mass General Brigham Healthcare Institutional
41 Review Board (IRB).

42 **Consent to participate:** Written informed consent was obtained from all participants prior to study
43 enrollment. Post-mortem consent was obtained from the appropriate representative (next of kin or
44 health care proxy) prior to autopsy.

45 **Consent for publication:** Not applicable

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1 **Background**

2 Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease that primarily affects cortical
3 and spinal motor neurons [1]. Although 90% of ALS cases are sporadic, 10% are inherited and due to
4 mutations in a number of genes such as superoxide dismutase 1 (*SOD1*), TAR DNA binding protein
5 (*TARDBP*), fused in sarcoma (*FUS*), and a hexanucleotide repeat expansion in *C9ORF72* to name a
6 few [2]. Despite our understanding of the disease-causing mutations, the exact molecular mechanisms
7 leading to motor neuron death remain unknown. Therefore, unraveling these mechanisms is crucial for
8 the development of new therapeutic approaches.

9
10 Tau protein, a member of the microtubule-associated protein (MAP) family, plays a critical role in
11 stabilizing microtubules, and is involved in several neuronal processes, including axonal transport,
12 mobility and intracellular organization and trafficking of organelles [3]. Recent studies have begun to
13 link alteration in tau phosphorylation to ALS pathogenesis in both sporadic and familial cases [4- 5], as
14 cytoplasmic inclusions of hyperphosphorylated tau have been described in post-mortem motor cortex
15 (mCTX) and spinal cord from ALS patients [6-8].

16
17 Tau is required for the trafficking of mitochondria across the axons to the synapses [9], a crucial event
18 to sustain the high energy requirement of neuronal cells. Hyperphosphorylation of key epitopes on tau
19 impairs this process and disrupts mitochondrial localization [10-13], thus contributing to axonal
20 dysfunction and synapse loss in Alzheimer's disease (AD) [9, 14-15] . Furthermore, tau over-expression
21 and mis-localization decreases ATP levels, impairs oxidative phosphorylation (OXPHOS), and
22 increases oxidative stress in AD and other neurodegenerative tauopathies [16-18]. Lastly, accumulating
23 evidence link hyperphosphorylated tau to impairment in mitochondrial dynamics [17-18], a physiological
24 process that allows mitochondria to change their dynamic networks based on the energy requirement
25 of the cell [19]. Specifically, it has been reported that an abnormal interaction between
26 hyperphosphorylated tau and dynamin-related protein 1 (DRP1), the GTPase involved in mitochondrial

1 fission, exacerbates mitochondrial dysfunction in AD [20-21]. Increases in oxidative stress and
2 impairments in mitochondrial dynamics are also pathogenetic features and early events in the disease
3 process in ALS [1, 22-23]. Specifically, decreases in bioenergetics, calcium buffering, and mitochondrial
4 transport as well as increases in oxidative stress have been described in animal and cellular models of
5 ALS [22-25]. Similarly, a decrease in cellular respiration, ATP production, and deficits in the
6 mitochondrial electron transport chain have been described in post-mortem ALS spinal cord [26-28].
7 Furthermore, mitochondria are structurally altered in ALS with the appearance of vacuolated and
8 swollen mitochondria in ALS skeletal muscle and mCTX [29-31]. Together these findings demonstrate
9 that mitochondrial function and dynamics are altered in ALS; however, the exact mechanisms involved
10 remain unknown. Here, we sought to determine whether tau contributes to alterations in mitochondrial
11 morphology and functions in ALS.

12 13 **Methods**

14 All methods were carried out in accordance with the guidelines and regulations of Massachusetts
15 General Hospital and approved by the Massachusetts General Hospital licensing committees.

16
17 *Human tissue samples.* Post-mortem mCTX from control and ALS patient brains were provided by the
18 Massachusetts Alzheimer's Disease Research Center (ADRC) with approval from the Massachusetts
19 General Hospital IRB (1999p009556) and by the University of Edinburgh MRC/Sudden Death Brain
20 Bank (under ethical approvals 15-HV-016; 11/ES/0022). ADRC ALS cases were tested for TDP-43
21 proteinopathy by immunohistochemistry on formalin-fixed paraffin-embedded (FFPE) motor cortex
22 using either total TDP-43 antibody (Proteintech, IL) or phospho-specific antibody (Cosmo Bio USA, CA)
23 before they were received by our laboratory. A total of 47 ALS and 25 non-neurological control mCTX
24 samples were used to perform the outlined experiments in this study which include fractionations,
25 western blots, and electron microscopy. The exact sample size used for each experiment is provided
26 in each figure legend. The mean age was 68.2 years (SD=15.7) for control subjects and 62 years

1 (SD=10.9) for ALS subjects. The samples consisted of 56% males in the control group and 53.2%
2 males in the ALS group. There were 20 limb onset and 14 bulbar onset ALS samples, and 3 were
3 diagnosed with ALS/FTD. Five of the ALS cases were positive for *C9ORF72* repeat expansion, while
4 a single case was positive for *SOD1* mutation. The genetic status of the other ALS samples was
5 unknown. Lastly, 24 of the ALS cases demonstrated TDP-43 proteinopathy. Post-mortem interval (PMI)
6 range was 2-104h for control and 4-102h for ALS (Suppl. Tables 1 and 2).

7
8 *Synaptoneurosomes (SNs) fractionation.* SNs fractionation was performed as previously reported [32]
9 with a few modifications. Human post-mortem mCTX (200 to 300mg) from ALS (n=36) and non-
10 neurological control cases (n=14) were homogenized in 500 μ L ice-cold Buffer A composed of 25mM
11 HEPES pH 7.5, 120mM NaCl, 5mM KCl, 1mM MgCl₂, 2mM CaCl₂, 2mM dithiothreitol (DTT), 1mM NaF
12 supplemented with phosphate and protease inhibitors cocktail. The homogenates were then passed
13 through two layers of 80 μ m nylon filters (Millipore, MA) to remove tissue debris. Seventy μ L aliquot
14 was saved, mixed with 70 μ L H₂O and 23 μ L 10% sodium dodecyl sulphate (SDS), passed through a
15 27-gauge needle to shear DNA, boiled for 5min, and centrifuged at 15,000g for 15min to prepare the
16 total extract. The remainder of the homogenates was passed through a 5 μ m Supor membrane Filter
17 (Pall Corp, Port Washington, NY) to remove organelles and nuclei, centrifuged at 1000g for 5min and
18 both pellet and supernatant were saved. The supernatants were collected in small crystal centrifuge
19 tubes and centrifuged at 100,000g for 45min at 4°C to obtain the cytosolic fractions. The pellets were
20 resuspended in Buffer B composed of 50mM Tris pH 7.5, 1.5% SDS and 2mM DTT, boiled for 5min,
21 and centrifuged at 15,000g for 15min to obtain SNs. Bradford assay was used to determine protein
22 concentration in total extract, cytosolic fraction, and SNs.

23
24 *Western blotting.* Western blots were performed using previously described protocols [33]. Briefly, 50 μ g
25 of proteins was resuspended in sample buffer and fractionated on either a 4-12% bis-tris gel or a 10-

1 20% tricine gel for 90min at 120V. Then, proteins were transferred to a PVDF membrane in an iBlot
2 Dry Blotting System (Invitrogen, Thermo Fisher, MA), and the membrane was blocked with 5% milk in
3 tris-buffered saline with Tween 20 (TBST) before immunodetection with the following primary
4 antibodies: tau (1:1000; #A0024, DAKO, Denmark), pTau-S396 (1:500; #ab109390, Abcam, MA),
5 DRP1 (1:500; #611113, BD Bioscience, CA), pDRP1-S616 (1:500; #4494S, Cell Signaling, MA), Mfn1
6 (1:500; #13798-1-AP, Proteintech, IL), Mfn2 (1:500; #ab46889, Abcam, MA), OPA1 (1:500; #612606,
7 BD Bioscience, CA), Tomm20 (1:500; #NBP2-67501, Novus Biological, CO), PSD-95 (1:500; #2507S,
8 Cell Signaling, MA), GAPDH (1:1000; #MAB374, Millipore Sigma, CA), and β -Actin (1:1000;
9 #mAb3700S, Cell Signaling, MA), overnight at 4°C. Primary antibody incubation was followed by
10 washes and incubation with secondary antibody for 1h (#111-035-144, HRP-conjugated goat anti-rabbit
11 IgG, and #715-035-151, HRP-conjugated goat anti-mouse; Jackson ImmunoResearch Laboratories,
12 West Grove, PA). Protein bands were visualized using the ECL detection system (Thermo Fisher
13 Scientific, MA) and analyzed in ImageJ 1.53a (National Institutes of Health, Bethesda, MD)

14
15 *Cell culture.* Human neuroblastoma SH-SY5Y cells (ATCC® CRL-2266) were grown in Dulbecco's
16 Modified Eagle Medium (DMEM)/Nutrient Mixture F-12 (1:1) supplemented with 10% inactivated fetal
17 bovine serum (FBS), 2mM L-glutamine, and 50 μ g/ml streptomycin and 50IU/ml penicillin. The cells
18 were kept at 5% CO₂ at 37°C. Before each experiment, SH-SY5Y cells were treated with 10ng/mL
19 human recombinant tau-2N4R (tau), and 10ng/mL SNs from control and ALS human post-mortem
20 mCTX for 24h.

21
22 *Human caspase-3 Enzyme-Linked Immunosorbent Assay (ELISA).* Cell viability was assessed using a
23 Human caspase-3 (active) ELISA kit (#KHO1091; Thermo Fisher Scientific, MA) following
24 manufacture's protocols. Briefly, SH-SY5Y cells were treated with tau and SNs from control (n=5) and
25 ALS mCTX (n=9) (10ng/mL/24h) and homogenized in the Cell Extraction Buffer provided by the kit.

1 Samples were incubated with a caspase-3 detection antibody for 1h at RT, washed (4x), and incubated
2 with a stabilized chromogen for 30min at RT in the dark. The reaction was stopped with a stop solution
3 and absorbance was read at 450nm. Caspase-3 levels were calculated based on a standard curve
4 after subtracting background absorbance.

5
6 *Aconitase activity assay.* Aconitase activity was measured using a commercially available kit
7 (#MAK051; Sigma-Aldrich, MO) following manufacturer's protocols. Briefly, SH-SY5Y cells were treated
8 with tau and SNs from control (n=4) and ALS mCTX (n=4) (10ng/mL/24h) and homogenized in ice-cold
9 Assay Buffer provided by the kit. Next, samples were centrifuged at 800g for 10min at 4°C to remove
10 insoluble material, and the supernatant was used to assess c-aconitase activity. To assess m-aconitase
11 activity the supernatant was centrifuged at 20,000g for 15min at 4°C. The pellets were collected,
12 dissolved in Assay Buffer, and sonicated for 20sec. After preparation, the samples were activated with
13 the Aconitase Activation Solution on ice for 1h and then incubated with the Reaction Mix for 1h at 25°C
14 in the dark. Lastly, the samples were incubated with the Developer Solution at 25°C for 10min and the
15 absorbance was measured at 450nm. C- and m-aconitase activity was calculated following the kit's
16 instruction after subtracting background.

17
18 *Immunostaining and Image Analysis.* SH-SY5Y cells plated on Millicell EZ SLIDE glass slides
19 (#PEZGS0816, Millipore Sigma, CA) were treated with tau, and SNs derived from control (n=3) and
20 ALS mCTX (n=3) (10ng/mL/24h). At the end of the treatment, cells were fixed with 4%
21 paraformaldehyde for 20min prior to immunostaining. Cells were permeabilized with 0.15% Triton-X in
22 PBS for 20min, washed in PBS (5x), and then blocked in 7.5% BSA in PBS-T for 45min. Cells were
23 then incubated for 90min in Tomm20 antibody (1:1000; #NBP2-67501, Novus Biologicals, CO),
24 followed by 5 washes in PBS. Cells were then incubated for 1h in goat-anti-rabbit AlexaFluor 488
25 secondary antibody (1:200; #A11034, ThermoFisher Scientific, MA), followed by 5x PBS washes. Cells
26 were then incubated for 30min in HCS CellMask Deep Red (2µg/mL; #H32721, ThermoFisher

1 Scientific, MA), washed in PBS (2x), followed by 10min incubation in Hoechst 33342 (1 μ g/mL; # H3570,
2 ThermoFisher Scientific, MA). Slides were then washed in PBS (3x), and coverslipped with Vectashield
3 Antifade Mounting Medium (#H-1000, Vector Labs, CA) prior to imaging. Z-stacks were acquired on a
4 Nikon A1SiR confocal microscope with 60x magnification and 0.3 μ m step size. At least 9 random
5 images were captured for each condition with a Nikon A1R inverted confocal microscope using a 60x
6 objective. Images were captured using DAPI (Hoechst), FITC (Tomm20), and Cy5 (HCS CellMask
7 Deep Red) filter sets. Automatic 3D Deconvolution was then performed within NIS-Elements 5.002.00
8 software. Background subtraction of generated .tiff files were performed in ImageJ 1.53h (National
9 Institutes of Health, Bethesda, MD). Automatic thresholding numbers to be used in subsequent
10 reconstruction were determined in ImageJ for each channel: Hoechst = Default; Tomm20 = Default;
11 HCS CellMask Deep Red = Renyi. These background-subtracted files were then uploaded into IMARIS
12 9.5 software (Bitplane, Zurich, Switzerland) to create volumetric reconstructions of each individual
13 channel. Surface reconstructions of each channel were generated using the Surface tool, with threshold
14 values as determined by ImageJ previously. Mitochondrial length was determined using the
15 BoundingBoxOO Length C settings, measuring the length of the longest principal axis of each individual
16 mitochondrion. Mitochondrial volume of each individual mitochondria was also determined from the
17 surface volumetric reconstructions. Length or volume of every mitochondria for each condition were
18 input into GraphPad Prism 9.0 software to generate cumulative frequency plots. The Data Analysis
19 Histogram function on Microsoft Excel was used to generate binned frequency plots of mitochondrial
20 length and volume.

21
22 *Electron microscopy.* mCTX blocks for electron microscopy were made as previously described [34].
23 Tissue samples were obtained from motor cortex at autopsy, cut into slices of no more than 1mm thick
24 and 1 mm wide containing all 6 layers of cortex, and fixed for 48h in 4% paraformaldehyde, 2.5%
25 glutaraldehyde in phosphate buffer (0.1M). Samples were post-fixed in osmium tetroxide (1%) for

1 30min, stained with uranyl acetate (1% in 70% ethanol), dehydrated, and embedded in Durcupan resin.
2 Ultrathin sections were cut on a Reichert-Jung Ultracut E. Lastly, sections were imaged on a JEM1011
3 electron microscope (JEOL) and images were acquired with a digital camera from Advanced
4 Microscopy Techniques, Inc (Danvers, MA) and processed using AMT V601 software. For mitochondria
5 analysis, an average of 15 images were taken/case at 25000x magnification in a systemic, random
6 fashion from control (n=4) and ALS (n=5) mCTX (Supp. Table 3). Mitochondria were identified by the
7 presence of internal cristae and defined outer membrane. Mitochondrial density was calculated from
8 generated .tiff files in ImageJ 1.53h (National Institutes of Health; Bethesda, MD) as average of at least
9 eight images/case. Mitochondrial density for each condition was input into GraphPad Prism 9.0
10 software to generate individual value scatter plots. Mitochondrial lengths were calculated from
11 generated .tiff files in ImageJ 1.53h and length of individual mitochondria for each condition were input
12 into GraphPad Prism 9.0 software to generate cumulative frequency plots. All mitochondria within at
13 least six images per subject were measured to obtain mitochondrial length. All individual mitochondrial
14 lengths were used to generate cumulative frequency plots, whereas mitochondrial lengths from every
15 case image were averaged for binned analyses. The Data Analysis Histogram function on Microsoft
16 Excel was used to generate binned frequency plots of mitochondrial length and volume.

17
18 *Co-immunoprecipitation (co-IP)*. The co-IP experiments were performed as previously reported [35].
19 Briefly, 75 μ g of proteins from control and ALS post-mortem mCTX homogenates was incubated with
20 5 μ L anti-DRP1 (BD Bioscience, CA), and GAL4 immunoprecipitation buffer, composed of 250mM NaCl,
21 5mM ethylenediaminetetraacetic acid (EDTA), 1% Nonidet P-40, 50mM Tris pH 7.5. After incubation at
22 4°C for 3.5h, magnetic protein A beads (Invitrogen, Thermo Fisher, MA) were added (20 μ L per sample)
23 and left in agitation overnight at 4°C. Next, samples were placed on a magnetic rack to remove the
24 supernatants and washed in GAL4 buffer (3x). Twenty μ L sample buffer was added in each sample
25 before boiling at 95°C for 5min. For the input samples, used as control, 40 μ g of proteins from the same
26 mCTX homogenates was resuspended in sample buffer and boiled at 95°C for 5min. The samples were

1 then loaded in a 4-20% glycine gel as above described for the western blot method. An antibody against
2 pTau-S396 (1:500; #ab109390, Abcam, MA) was used to immunoblot.

3
4 *Small interfering RNA (siRNA)*. DRP1 knock down was obtained with siGENOME Human DNMT1L
5 siRNA (siDRP1) (SMARTPool; #M-012092-01-0005; Horizon Discovery, GE Healthcare Dharmacon,
6 CO), using a siGENOME non-targeting control pool (SMARTPool; #D-001206-13-05, Horizon
7 Discovery, GE Healthcare Dharmacon, CO) as control. Transfection of SH-SY5Y cells was carried out
8 in Optimem medium (ThermoFisher Scientific, MA) using Lipofectamine 2000 (ThermoFisher Scientific,
9 MA) together with 10, 100, or 150nM siDRP1 for 24 and 48h. Western blot experiments were performed
10 to verify the efficiency of the transfection by assessing DRP1 levels.

11
12 *QC-01-175 treatment*. QC-01-175 was designed using a targeted protein degradation technology to
13 recognize both tau and Cereblon (CRBN), a substrate receptor for the E3-ubiquitin ligase in order to
14 specifically induce pathogenic tau ubiquitination and degradation [36]. SH-SY5Y cells were treated with
15 1 or 10 μ M QC-01-175 for 2, 4, and 24h following treatment with ALS SNs (10ng/mL/24h). Western blot
16 experiments were performed to verify the efficiency of QC-01-175 by assessing pTau-S396 levels.

17
18 *Treatment of SH-SY5Y cells.*

19 *siDRP1*. SH-SY5Y cells were transfected with siControl (150nM) and siDRP1 (150nM) for 24h, as
20 previously described, and then treated with SNs from ALS mCTX (n=4) (10ng/mL) for 24h. At the end
21 of the treatments, DRP1 levels were assessed western blots. Mitochondrial morphological parameters
22 were measured as above-reported in SH-SY5Y cells treated with either recombinant tau protein
23 (10ng/mL) or SNs derived from control (n=3) and ALS mCTX (n=3) for 24h following transfection with
24 siControl (150nM) and siDRP1 (150nM) for 24h.

1 QC-01-175. SH-SY5Y cells were treated with ALS SNs (n=4) (10ng/mL) for 24h followed by exposure
2 to QC-01-175 (10 μ M) for 4h. At the end of the treatments, pTau-S396 levels were assessed by western
3 blots. Mitochondrial morphological parameters were measured as previously described in SH-SY5Y
4 cells treated with either recombinant tau protein (10ng/mL) or SNs derived from control (n=3) and ALS
5 mCTX (n=3) for 24h followed by exposure to QC-01-175 (10 μ M) for 4h. Reactive oxygen species were
6 measured in SH-SY5Y cells treated with either tau, control (n=4) or ALS SNs (n=9) (10ng/mL/24h)
7 followed by treatment with QC-01-175 (10 μ M/4h).

8
9 *Oxidative stress detection.* Reactive oxygen levels were measured in SH-SY5Y cells using CellROX
10 Orange Reagent, a fluorogenic probe for measuring oxidative stress in live cells (#C10443; Thermo
11 Fisher Scientific, MA). Briefly, following specific treatments, the cells were incubated with 2.5 μ M
12 CellROX for 30min at 37°C after which culture media was replaced for imaging on a BioTek Cytation 5
13 imaging reader (BioTek, VT). Images were captured from 6 random areas of each well every 10min for
14 2h. To analyze data generated by Cytation, images were processed using a custom Fiji macro, which
15 opened each image and automatically calculated mean fluorescent intensity (MFI). The MFI values
16 were then grouped using a custom Python script. Slopes (MFI/min) of CellRox fluorescence were
17 determined and normalized to the slope of vehicle-treated cells for each run. Statistics and plots were
18 then created using Graphpad Prism 9.0. All code for processing is currently hosted and publicly
19 available at <https://github.com/bendevlin18/cytation-analysis-2020>.

20
21 *Statistics.* Normal distributions of data were not assumed regardless of sample size or variance.
22 Individual value plots with the central line representing the median and the whiskers representing the
23 interquartile range, box plots with the central line representing the median, the edges representing the
24 interquartile ranges, and the whiskers representing the minimum and maximum values, and scatter
25 plots with the central line representing the mean, and the whiskers representing the standard deviation

(SD) were used for graphical representation. Comparisons were performed using a non-parametric Mann-Whitney U test, a one-way ANOVA followed by Tukey's post-hoc test, and a two-way ANOVA followed by Tukey's post-hoc test at a significance level (α) of 0.05. Cumulative frequency distribution graphs were compared by Kolmogorov-Smirnov test (electron microscopy) or Kruskal-Wallis test (confocal microscopy). Exact p values (two-tailed) are reported.

Study approval. The study was approved by the Mass General Brigham Healthcare Institutional Review Board (IRB). Written informed consent was obtained from all participants prior to study enrollment. Post-mortem consent was obtained from the appropriate representative (next of kin or health care proxy) prior to autopsy.

Results

pTau-S396 is mis-localized to synapses in ALS independent of sex, region of onset, and genotype.

Given that hyperphosphorylated tau accumulates at the synaptic level in AD [32], we first assessed the levels of tau and pTau-S396 in three different mCTX fractions – total, cytosolic, and synaptoneuroosomes (SNs; the synaptic fraction) – derived from post-mortem ALS and controls by western blots. While the post-synaptic density protein 95 (PSD-95) was detected in the total and SNs fractions, it was absent in the cytosolic fraction derived from both ALS and control mCTX, demonstrating successful fractionation and purity of SNs. Furthermore, the results revealed that both tau and pTau-S396 mis-localized to SNs in ALS mCTX (Figure 1a). Specifically, while there was more tau in both control and ALS SNs compared to their cytosolic fractions (Figure 1b), pTau-S396 was only increased in ALS synapses (Figure 1c).

Next, we correlated increases in pTau-S396 in ALS SNs with the known patient clinicopathologic information. A significant shift in pTau-S396 from the cytosolic fraction to the SNs was observed in both

1 male and female ALS mCTX (Figure 1d-e). Furthermore, synaptic pTau-S396 levels were significantly
2 increased in both bulbar and limb onset ALS (Figure 1f-g). While there was no significant difference in
3 synaptic pTau-S396 in ALS/frontotemporal dementia (ALS/FTD), there was a significant increase in
4 SNs derived from ALS cases with TDP-43 proteinopathy (Figure 1h-i). There was a significant shift in
5 pTau-S396 from the cytosol to SNs in *C9ORF72*-ALS, and the same shift was observed in the single
6 *SOD1*-ALS case used in this study (Figure 1j-k).

8 **ALS SNs decrease cytosolic and mitochondrial aconitase activity without affecting cell survival.**

9 To verify whether ALS SNs, enriched in pTau-S396, may affect cell survival, human neuroblastoma
10 SH-SY5Y cells were treated with SNs derived from control and ALS mCTX (10ng/mL) and recombinant
11 tau protein as a positive control (10ng/mL) for 24h. Cell viability assessment by measuring active
12 caspase-3 levels by an ELISA assay revealed no differences in the levels of active caspase-3 following
13 treatment with either tau, control SNs, or ALS SNs (Figure 2a).

14
15 Given that hyperphosphorylated tau has been associated with increased oxidative stress [16-18], we
16 next assessed the activity of aconitase, a cellular enzyme that catalyzes the isomerization of citrate to
17 isocitrate and is reversibly inactivated by increases in oxidative stress. We assessed the activity of both
18 cytosolic and mitochondrial aconitase (c- and m-aconitase, respectively) in SH-SY5Y cells following
19 treatment with ALS and control SNs, recombinant tau, and antimycin A as positive control
20 (25 μ M/30min) using an aconitase activity assay kit. As expected, antimycin A, an inhibitor of
21 mitochondrial complex III that increases ROS production [37], decreased both c- and m-aconitase
22 activity (Suppl. Figure 1a-b). While there was no alteration in aconitase activity following treatment with
23 control SNs, there was a significant decrease in both c- and m-aconitase activity following treatment
24 with recombinant tau or ALS-derived SNs compared to vehicle-treated cells (Figure 2b-c), indicative of
25 increased oxidative stress.

1 **ALS SNs induce mitochondrial fragmentation.**

2 To determine whether ALS SNs may affect mitochondrial morphology, SH-SY5Y cells were treated
3 with SNs derived from control and ALS mCTX (10ng/mL/24h) as well as recombinant tau protein
4 (10ng/mL/24h) as positive control, and mitochondrial morphological parameters were measured by
5 immunofluorescence using Tomm20 staining followed by volumetric reconstruction (Figure 3a). As
6 expected, mitochondria within recombinant tau-treated cells had significantly decreased length and
7 volume compared to mitochondria from vehicle- and control SNs-treated cells (Figure 3b-c). Similar
8 decreases were observed in ALS SNs-treated cells compared to both vehicle- and control SNs-treated
9 cells (Figure 3b-c). Specifically, there was a significant increase in the frequency of smaller
10 mitochondria ($<2\ \mu\text{m}$ in length or $<2\ \mu\text{m}^3$ in volume) as well as a significant decrease in the frequency
11 of larger mitochondria ($>8\ \mu\text{m}$ in length or $>10\ \mu\text{m}^3$ in volume) in recombinant tau- or ALS SNs-treated
12 cells compared to vehicle- or control SNs-treated cells (Suppl. Figure 2a-b).

13
14 In addition to morphometric analyses of individual mitochondrial morphologies, we also examined the
15 ability of ALS SNs to affect connectivity of the mitochondrial networks. While treatment with either tau
16 or ALS SNs had no effect on the number of mitochondrial networks/cell (Figure 3d), there was a
17 significant decrease in large networks/cell in recombinant tau- or ALS SNs-treated cells compared to
18 vehicle-treated cells (Figure 3e). Consistent with individual mitochondrial analyses, there was also a
19 significant decrease in the mean mitochondrial branch length in recombinant tau-treated cells
20 compared to vehicle-treated cells as well as in ALS SNs-treated cells compared to both vehicle- and
21 control SNs-treated cells (Figure 3f).

22
23 **DRP1 interacts with pTau-S396 and its levels are increased in ALS SNs.**

24 Given that pTau-S396 has been shown to interact with the pro-mitochondrial fission GTPase DRP1
25 [20], and ALS SNs induce mitochondrial fragmentation *in vitro*, we hypothesized that increases in pTau-

1 S396 may trigger pathological mitochondrial fission in ALS by binding DRP1. To test this hypothesis,
2 we first assessed the possible interaction between pTau-S396 and DRP1 in control and ALS post-
3 mortem mCTX by co-immunoprecipitation. Our results revealed that pTau-S396 interacts with DRP1 in
4 mCTX, however there was no significant difference in this interaction between control and ALS (Figure
5 4a-b).

6
7 Given the interaction between pTau-S396 and DRP1 and the increase in synaptic pTau-S396 levels in
8 ALS, we next assessed the levels of mitochondrial fission and fusion markers in SNs derived from ALS
9 and control mCTX by western blots. The results revealed a significant increase in both DRP1 and its
10 active form pDRP1-S616 in ALS SNs compared to control SNs (Figure 4c-e), while there was no
11 significant difference in the levels of pro-fusion proteins, mitofusin 1 (Mfn1), mitofusin 2 (Mfn2), or
12 dominant optic atrophy (OPA1) (Suppl. Figure 3a-d), suggesting an increase in mitochondrial fission in
13 ALS.

14
15 As pTau-S396 mis-localization at the synaptic level occurs across subtypes of ALS, we correlated
16 increases in DRP1 in ALS SNs with the known patient clinicopathologic information. The results did not
17 reveal a significant change in DRP1 levels in SNs-derived from male ALS patients and a significant
18 increase in female patients (Figure 4f-g). Furthermore, synaptic DRP1 levels were significantly
19 increased in both bulbar and limb onset ALS (Figure 4h-i). While there were no alterations in synaptic
20 DRP1 levels in ALS/FTD, there was a significant increase in SNs from ALS cases with TDP-43
21 proteinopathy (Figure 4j-k). Lastly, there was a significant increase in synaptic DRP1 levels in
22 *C9ORF72*-ALS, and the same increase was observed in the single *SOD1*-ALS subject used in this
23 study (Figure 4l-m).

1 **Mitochondrial morphology is altered in ALS post-mortem mCTX.**

2 Given the significant increase in DRP1, we next assessed the levels of the mitochondrial outer
3 membrane marker Tomm20 by western blots in post-mortem ALS and control mCTX. The data revealed
4 a significant decrease in Tomm20 levels in ALS compared to control mCTX, suggesting alterations in
5 mitochondrial density, mass, or morphology (Figure 5a). Similarly, analysis of post-mortem mCTX
6 using electron microscopy revealed a significant decrease in the density as well as in the length of
7 mitochondria (Figure 5b, white arrow) in ALS compared to controls (Figure 5c-d). Furthermore, there
8 was a significant increase in the frequency of shorter mitochondria ($<0.50\mu\text{m}$) in ALS mCTX (Figure
9 5e), consistent with increased mitochondrial fragmentation in ALS.

11 **siDRP1 mitigates ALS SNs-induced alterations in mitochondrial morphology.**

12 Given that DRP1 inhibition prevents mitochondrial dysfunction in AD [21], we used a siRNA approach
13 to reduce *DRP1* levels in SH-SY5Y cells prior to treatment with control or ALS SNs. SH-SY5Y cells
14 were treated with 10, 100 or 150nM of siDRP1 for 24 and 48h to identify the effective dose and
15 treatment time for knocking down *DRP1*. Our results demonstrated a significant decrease in DRP1
16 levels following silencing, with no signal detected following exposure to 150nM siDRP1 for 24h or
17 following 48h treatment (Suppl. Figure 4a). Therefore, SH-SY5Y cells were transfected with 150nM of
18 siDRP1 for 24h and then treated with ALS SNs (10ng/mL/24h) before assessing DRP1 levels by
19 western blots. The results demonstrated a significant decrease in DRP1 levels in both siDRP1- and
20 siDRP1+ALS SNs-treated cells compared to both siControl- and siControl+ALS SNs-treated cells
21 (Suppl. Figure 4b).

22
23 For mitochondrial morphological assessments, SH-SY5Y cells were silenced with siDRP1 (150nM/24h)
24 followed by treatment with recombinant tau or SNs derived from control and ALS mCTX (10ng/mL/24h)
25 (Suppl. Figure 5a). As expected, treatment with both recombinant tau and ALS SNs in the presence of
26 siControl significantly reduced mitochondrial length and volume, as mitochondria were significantly

1 shorter in recombinant tau- and ALS SNs-treated cells compared to siControl- or siControl+control SNs-
2 treated cells (Suppl. Figure 5b-c). Furthermore, DRP1 knockdown prevented recombinant tau- and ALS
3 SNs-induced increases in smaller mitochondria and reductions in larger mitochondria. Specifically, the
4 treatment with ALS SNs resulted in fewer smaller mitochondria and more larger mitochondria in
5 siDRP1- compared to siControl-treated cells (Suppl. Figure 4b-c). Similarly, silencing DRP1 prevented
6 the increase in the frequency of smaller mitochondria ($<2\mu\text{m}$ in length and $<2\mu\text{m}^3$ in volume) as well as
7 the decrease in the frequency of larger mitochondria ($>8\mu\text{m}$ in length and $>10\mu\text{m}^3$ in volume) induced
8 by recombinant tau or ALS SNs (Suppl. Figure 6a-b).

9
10 Mitochondrial connectivity analyses led to similar observations, with no differences in the number of
11 overall networks/cell (Suppl. Figure 5d). Similar to length and volume measurements, knocking down
12 *DRP1* prevented recombinant tau- and ALS SNs-induced alterations in large networks/cell, as there
13 was a significant increase in the number of large networks/cell in siDRP1-exposed cells treated with
14 either recombinant tau or ALS SNs (Suppl. Figure 5e). Lastly, siDRP1 prevented ALS SNs-mediated
15 decrease in branch length, as siDRP1+ALS SNs-treated cells had longer branch length compared to
16 siControl+ALS SNs-treated cells (Suppl. Figure 5f).

18 **Tau degradation mitigates ALS SNs-induced alterations in mitochondrial morphology.**

19 To test the hypothesis that reducing tau may recapitulate the same effect induced by knocking down
20 *DRP1* on mitochondrial morphology, a novel bifunctional tau degrader, QC-01-175, which has been
21 shown to be capable of recruiting tau to the E3 ubiquitin ligase CRL4^{CRBN} resulting in its degradation
22 from the proteasome [36], was used to reduce pTau-S396 levels in SH-SY5Y cells following treatment
23 with control or ALS SNs. To identify the effective dose and treatment time for QC-01-175 able to reduce
24 pTau-S396 levels, SH-SY5Y cells were first treated with 1 or $10\mu\text{M}$ of QC-01-175 for 2, 4, and 24h
25 following treatment with ALS SNs (10ng/mL/24h). Our results revealed that while $1\mu\text{M}$ QC-01-175 had

1 no effect on pTau-S396 levels, 10 μ M of the degrader reduced pTau-S396 in ALS SNs-treated cells
2 compared to vehicle-treated cells following 4h and that this effect was absent following 24h at the same
3 concentration (Figure 6a-b). Therefore, SH-SY5Y cells were treated with ALS SNs (10ng/mL/24h)
4 followed by QC-01-175 (10 μ M) for 4h before assessing pTau-S396 levels by western blots. The results
5 revealed a significant decrease in pTau-S396 levels in cells treated with QC-01-175 following exposure
6 to ALS SNs compared to ALS SNs-treated cells (Figure 6c).

7
8 For mitochondrial morphological assessments, SH-SY5Y cells were treated with either recombinant tau
9 or SNs derived from control and ALS mCTX (10ng/mL/24h) followed by treatment with QC-01-175
10 (10 μ M/4h) before assessing mitochondrial morphological parameters (Figure 7a). As in previous
11 experiments, recombinant tau and ALS SNs treatment significantly shortened mitochondrial length and
12 volume compared to vehicle- and control SNs-treated cells. Degrading tau with QC-01-175 reverted
13 recombinant tau and ALS SNs-induced increases in smaller mitochondria and decreases in larger
14 mitochondria. Specifically, there were fewer smaller mitochondria and more larger mitochondria in QC-
15 01-175+ALS SNs-treated cells compared to ALS SNs-treated cells (Figure 7b-c). Furthermore,
16 selective degradation of tau using QC-01-175 prevented recombinant tau- and ALS SNs-induced
17 mitochondrial length and volume alterations, as treatment with tau or ALS SNs resulted in fewer smaller
18 mitochondria (<2 μ m in length and <2 μ m³ in volume) following QC-01-175 treatment (Suppl. Figure 7a-
19 b). Lastly, there were more larger mitochondria (>8 μ m³ in volume) in ALS SNs+QC-01-175-treated cells
20 compared to ALS SNs-treated cells (Suppl. Figure 7b).

21
22 Mitochondrial network analyses revealed no alterations in the number of total networks/cell, similar to
23 previous experiments (Figure 7d). There were also no alterations in the number of large networks or
24 mitochondrial branch length following treatment with QC-01-175 (Figure 7e-f). Although QC-01-175

1 treatment more than doubled the number of large networks compared to ALS SNs treatment, this
2 protection did not rise to statistical significance.

3 **Tau degradation prevents the increase in reactive oxygen species induced by ALS SNs.**

4 Given that treatment with ALS SNs increases oxidative stress, and QC-01-175 ameliorates
5 mitochondrial morphology, we next verified whether QC-01-175 was also able to mitigate oxidative
6 stress induced by ALS SNs. Therefore, SH-SY5Y cells were treated with either recombinant tau or SNs
7 derived from control and ALS mCTX (10ng/mL/24h) followed by treatment with QC-01-175 (10 μ M/4h)
8 before assessing ROS levels by using CellROX, a fluorogenic probe for measuring oxidative stress in
9 live cells (Figure 8a). Antimycin A (25 μ M/30min) was used as positive control (Suppl. Figure 8).
10 According to previous results, the treatment with both recombinant tau and ALS SNs induced an
11 increase in ROS levels compared to both vehicle- and control SNs-treated cells (Figure 8b). Importantly,
12 ROS levels were significantly decreased in both recombinant tau+QC-01-175- and ALS SNs+QC-01-
13 175-treated cells compared to recombinant tau- and ALS SNs-treated cells (Figure 8b), indicating a
14 rescue of oxidative stress by tau-specific clearance.

16 **Discussion**

17 In this study we reported for the first time mis-localization of hyperphosphorylated tau at S396 to
18 synapses across subtypes of ALS in a large cohort of human post-mortem ALS mCTX. We also
19 demonstrated that ALS SNs, enriched in pTau-S396, increase oxidative stress *in vitro* without affecting
20 cell survival. Treatment with ALS SNs was also sufficient to alter mitochondrial length, volume, and
21 connectivity *in vitro*, thus indicating increases in mitochondrial fragmentation. Although pTau-S396 was
22 found to interact with DRP1 in both control and ALS mCTX, there was only a significant increase in
23 DRP1 levels in ALS SNs that was observed across subtypes similar to pTau-S396. Our results also
24 revealed a decrease in mitochondrial density and length in ALS mCTX, supporting previous findings
25 [22-23]. Importantly, we demonstrated that reducing tau with the specific tau degrader, QC-01-175 [36],

1 mitigated alterations in mitochondrial morphology, similar to knocking down *DRP1*, and decreased ROS
2 levels, thus suggesting a role for tau in inducing mitochondrial fragmentation and oxidative stress in
3 ALS.

4
5 Recently, alterations in tau phosphorylation have been reported in both sporadic and familial cases of
6 ALS [4-5]. Specifically, cytoplasmic inclusions of hyperphosphorylated tau have been described in ALS
7 post-mortem mCTX and spinal cord [6-7]. Here, our results expand on these findings and demonstrate
8 a significant mis-localization of tau and pTau-S396 from the cytosol to synapses, reminiscent of AD
9 [32]. Furthermore, this synaptic mis-localization occurred across ALS subtypes, suggesting that
10 hyperphosphorylation of tau may represent a common mechanism underlying ALS pathogenesis;
11 however, further investigation is required to determine the pathology of genetic subtypes with a larger
12 sample size. Additional studies assessing tau phosphorylation at different epitopes are also necessary
13 to better characterize the involvement of tau hyperphosphorylation, mis-localization and toxicity in ALS
14 pathogenesis.

15
16 Although the exact molecular mechanisms underlying tau toxicity are not yet fully understood, several
17 studies suggest that tau may trigger mitochondrial dysfunction in neurodegenerative diseases, such as
18 AD [17-18]. Mitochondrial loss and dysfunction lead to tau hyperphosphorylation and aggregation,
19 suggesting a deleterious interplay between tau and mitochondria that may contribute to alterations in
20 neuronal and synaptic function in neurodegeneration [17]. Specifically, tau hyperphosphorylation has
21 been shown to alter mitochondrial localization at the neuronal level [10-13, 15], and, in turn, axonal
22 mitochondrial loss further exacerbates tau hyperphosphorylation and its disassembly from the
23 microtubules within the axons [17, 38]. Mitochondria are mis-localized in both animal and cellular
24 models of ALS carrying mutations in *SOD1* and *TARDBP* [39-43] and have been shown to accumulate
25 in the soma of spinal motor neurons [28]. Ongoing and future studies will extend our findings and assess
26 alterations in the distribution and trafficking of mitochondria.

1

2 Besides alterations in mitochondrial trafficking, mitochondrial dysfunction has been widely described in
3 spinal cord from ALS patients [26-28] as well as in animal and cellular models of ALS [23-26]. Our
4 results here build on these findings and demonstrate that alterations in tau phosphorylation may
5 underlie mitochondrial dysfunction in ALS, given that ALS SNs enriched in pTau-S396 increased
6 oxidative stress, reduced mitochondrial length and volume, and altered mitochondrial connectivity *in*
7 *vitro*. Consistent with these *in vitro* findings we also found a significant decrease in mitochondrial density
8 and length in post-mortem ALS mCTX, suggesting an increase in mitochondrial fragmentation as
9 reported previously [23-24]. Our findings of decreased mitochondrial density and length in ALS post-
10 mortem mCTX together with an increase in synaptic DRP1 levels further supports the notion of
11 increased mitochondrial fission in ALS. Hyperphosphorylated tau has also been shown to impair
12 mitochondrial dynamics through an abnormal interaction with the pro-mitochondrial fission GTPase
13 DRP1 [20-21]. Therefore, the concomitant increase in synaptic pTau-S396 and DRP1 occurring across
14 ALS subtypes further suggests that these alterations may be a common pathogenetic mechanism in
15 ALS. Lastly, the involvement of hyperphosphorylated tau in inducing mitochondrial fission in ALS was
16 further confirmed by our *in vitro* studies, wherein we demonstrated alterations in mitochondrial length,
17 volume and connectivity following treatment with ALS SNs, enriched in both pTau-S396 and DRP1.

18

19 Studies utilizing Drp1 heterozygous mice ($Drp1^{+/-}$) with impartial DRP1 depletion or treated
20 pharmacologically with mdivi-1, a DRP1 inhibitor, demonstrate that DRP1 inhibition can prevent
21 mitochondrial dysfunction and synapse loss in AD [21, 44]. Similarly, we reported that silencing *DRP1*
22 prevented ALS SNs-induced decreases in mitochondrial length and volume in SH-SY5Y cells.
23 Importantly, we recapitulated the same mitochondrial morphologic protection by reducing tau levels
24 with the specific tau degrader QC-01-175, which is able to degrade disease-relevant forms of tau and
25 p-Tau, including pTau-S396, in neuronal cells derived from FTD patients [36]. Here, we further confirm
26 the therapeutic potential of QC-01-175, as we reported a significant decrease in pTau-S396 levels

1 following QC-01-175 treatment in SH-SY5Y cells treated with ALS SNs. Additionally, we demonstrated
2 that reducing pTau-S396 with QC-01-175 mitigates mitochondrial fragmentations and prevents
3 increases in ROS levels induced by ALS SNs, thus further supporting the involvement of tau in
4 mitochondrial dysfunction in ALS and, importantly, identifying a novel potential therapeutic target for
5 ALS.

6
7 Targeting mitochondrial proteins, such as DRP1, is a great challenge given that mitochondria are
8 involved in several vital cellular functions thus increasing the possibility of deleterious side-effects such
9 as the ones reported following treatment with the putative DRP1 inhibitor mdivi-1 [45-46]. Furthermore,
10 the clinical application of siRNA is limited by both their poor pharmacokinetic properties and the possible
11 induction of off-target effects [47]. Moreover, the Food and Drug Administration (FDA) has only
12 approved two RNA interference (RNAi)-based therapies in the last 20 years [48-49]. Therefore,
13 targeting tau to improve the status of mitochondria may represent a more viable therapeutic strategy.
14 Our findings suggest that reducing pTau-S396 levels lead to an improvement in mitochondrial dynamics
15 and, in turn, mitochondrial function, providing a new approach to mitigate mitochondrial dysfunction.
16 Given that tau mainly localizes at the neuronal level [50], targeting tau to improve the balance in
17 mitochondrial dynamics may be associated with less deleterious off-target effects. Future studies are
18 planned to further investigate the neuroprotective effects of QC-01-175 in animal models of ALS to
19 determine its therapeutic potential.

20
21 Although our results suggest that interactions between hyperphosphorylated tau and DRP1 may
22 underlie mitochondrial dysfunction across subtypes of ALS, further investigation is required to confirm
23 these findings. Specifically, future studies in a larger cohort of post-mortem mCTX derived from ALS
24 patients with known genotypes will clarify whether pTau and DRP1 alterations are involved in familial
25 ALS. Similarly, our findings in SH-SY5Y cells demonstrating that degrading tau mitigates mitochondrial
26 fragmentation and oxidative stress lay the groundwork for assessing tau and DRP1 in ALS. While SH-

1 SY5Y cells are extensively used by the field [51-54] to investigate molecular pathways and their
2 functional consequences, they are immortalized and not a representative cellular model for ALS.
3 Therefore, future studies will assess the effects of tau degradation in a more suitable *in vitro* model
4 such as iPSC-derived motor neurons from patients diagnosed with sporadic and familial ALS.

5 6 **Conclusions**

7 Collectively, our results in a large cohort of human post-mortem mCTX suggest that
8 hyperphosphorylated tau at S396 may underlie mitochondrial fragmentation in ALS by interacting with
9 the pro-fission GTPase DRP1. Lastly, our data provide the groundwork to assess QC-01-175 as a novel
10 potential therapeutic strategy to improve mitochondrial morphology and function, and, in turn, motor
11 neuron survival in ALS.

1 List of abbreviations

<u>Abbreviation</u>	<u>Definition</u>
ALS	Amyotrophic lateral sclerosis
AD	Alzheimer's disease
DRP1	Dynamin-related protein 1
SNs	Synaptoneuroosomes
mCTX	Motor cortex
pTau-S396	Phosphorylated tau at S396
ROS	Reactive oxygen species
SOD1	Superoxide dismutase 1
TARDBP	TAR DNA binding protein
FUS	Fused in sarcoma
MAP	Microtubule-associated protein
OXPHOS	Oxidative phosphorylation
ALS/FTD	Amyotrophic lateral sclerosis/Frontotemporal dementia
DTT	Dithiothreitol
SDS	Sodium dodecyl sulphate
TBST	Tris-buffered saline with Tween 20
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal bovine serum
ELISA	Enzyme-Linked Immunosorbent Assay
coIP	Co-immunoprecipitation
EDTA	Ethylene Diamine Tetraacetic Acid
PSD-95	Post-synaptic density protein 95
siRNA	Small Interference RNA

Mfn1	Mitofusin 1
Mfn2	Mitofusin 2
OPA1	Dominant Optic Atrophy 1
FDA	Food and Drug Administration
RNAi	RNA interference

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Figure legends

Figure 1. Synaptic pTau-S396 levels are increased independent of sex, region of onset, and genotype.

(a) Representative western blot images of tau, pTau-S396 and PSD-95 in total, cytosolic and SNs fraction from ALS and control mCTX. (b) There was a significant effect of cellular fractions (two-way ANOVA, [F(2,137)=15.03], $p < 0.0001$) on tau levels. Tau levels were increased in control (n=14) and ALS SNs (n=36) compared to their cytosolic fractions (Tukey's test, $p = 0.0059$, and $p = 0.0006$, respectively). There was an increase in tau levels in SNs compared to total extract in ALS (Tukey's test, $p = 0.0489$). Data are represented by box plots with the central line representing the median, the edge representing the interquartile range and the whiskers representing the minimum and maximum values. (c) There was a significant effect of cellular fractions (two-way ANOVA, [F(2,133)=4.106], $p = 0.0186$) on pTau-S396 levels together with a significant shift from ALS cytosolic fraction to the SNs (Tukey's test, $p = 0.0024$). Data are represented as box plots with the central line representing the median, the edge representing the interquartile range and the whiskers representing the minimum and maximum values. There was an increase in synaptic pTau-S396 levels in (d) male (n=20; Mann-Whitney U test=49, $p = 0.0004$) and (e) female ALS patients (n=16; Mann-Whitney U test=42, $p = 0.0026$). Synaptic pTau-S396 levels were increased in (f) bulbar onset (n=13; Mann-Whitney U test=21, $p = 0.0023$) and (g) limb onset ALS subjects (n=19; Mann-Whitney U test= 58, $p = 0.0077$). (h) There was no change in pTau-S396 levels in ALS/FTD (n=3; Mann-Whitney U test=0, $p = 0.1000$). (i) There was an increase in pTau-S396 in ALS with TDP-43 proteinopathy (n=18; Mann-Whitney U test=46,

1 p=0.0008). (j) There was an increase in pTau-S396 in *C9ORF72*-ALS (n=5; Mann-Whitney U test=1,
2 p=0.0159). (k) There was an increase in pTau-S396 in a single *SOD1*-ALS. Data in **d-k** are represented
3 as individual value plots with the central line representing the median and the whiskers representing
4 the interquartile range. *p<0.05; **p<0.01; ***p<0.001.

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6
7 **Figure 2. ALS SNs increase oxidative stress without affecting cell survival.**

8 (a) There was no effect of treatment on caspase-3 levels in SH-SY5Y cells (one-way ANOVA,
9 [F(3,20)=0.1535], p=0.9262). Similarly, there were no change in caspase-3 levels in SH-SY5Y cells
10 treated with tau (Tukey's test, p=0.9203), control SNs (n=5; Tukey's test, p=0.9999), or ALS SNs (n=9;
11 Tukey's test, p=0.999) compared to vehicle-treated cells. Data are represented as scatter plots with
12 bar indicating mean±SD. (b) There was a significant effect of treatment on c-aconitase activity in SH-
13 SY5Y cells (one-way ANOVA, [F(3,12)=4.306], p=0.0280). c-aconitase activity was significantly
14 decreased in tau- (Tukey's test, p=0.0207) and ALS SNs-treated cells (n=4, Tukey's test, p=0.0425)
15 compared to vehicle-treated cells. Data are represented as scatter plots with bar indicating mean±SD.
16 (c) There was a significant effect of treatment on m-aconitase activity in SH-SY5Y cells (one-way
17 ANOVA, [F(3,12)=5.269], p=0.0150). m-aconitase activity was significantly decreased in tau- (Tukey's
18 test, p=0.0106) and ALS SNs-treated cells (n=4, Tukey's test, p=0.0245) compared to vehicle-treated
19 cells. Data are represented as scatter plots with bar indicating mean±SD. *p<0.05.

20
21 **Figure 3. ALS SNs induce mitochondrial fragmentation.**

22 (a) Representative images of SH-SY5Y cells treated with vehicle, recombinant tau, control and ALS
23 SNs stained with Hoechst (blue), CellMask (white), and Tomm20 (red). (b) Cumulative frequency graph
24 indicated an effect of treatment on length (Kruskal-Wallis, H=55.78, p<0.0001) with smaller
25 mitochondria in recombinant tau- and ALS SNs- (n=3) compared to vehicle- (p<0.0001, and p<0.0001,

1 respectively) and control SNs (n=3)-treated cells (p=0.0001, and p<0.0001, respectively). (c)
2 Cumulative frequency graph demonstrated an effect of treatment on volume (Kruskal-Wallis,
3 H=51.2941, p<0.0001) with smaller mitochondria in recombinant tau- and ALS SNs- compared to
4 vehicle- (p<0.0001, and p<0.0001, respectively) and control SNs-treated cells (p=0.0001, and
5 p<0.0001, respectively). (d) Treatment with either recombinant tau or ALS SNs had no effect on
6 mitochondrial networks/cell (one-way ANOVA, [F(3,36)=1.157], p=0.3396). Data are represented as
7 scatter plots with bar indicating mean±SD. (e) There was an effect of treatment on large networks/cell
8 (one-way ANOVA, [F(3,36)=5.809], p=0.0024). Tukey's test demonstrated a decrease in recombinant
9 tau- or ALS SNs- compared to vehicle-treated cells (p=0.0082, and p=0.0105, respectively). Data are
10 represented as scatter plots with bar indicating mean±SD. (f) There was an effect of treatment on the
11 mean branch length of mitochondria within networks (one-way ANOVA, [F(3,36)=6.901], p=0.0009).
12 Tukey's test revealed a decrease in recombinant tau- compared to vehicle-treated cells (p=0.0124) and
13 in ALS SNs- compared to vehicle- and control SNs-treated cells (p=0.0032, and p=0.0184,
14 respectively). Data are represented as scatter plots with bar indicating mean±SD. See also Figure S1.
15 *p<0.05; **p<0.01; ***p<0.001, ****p<0.0001.

16
17 **Figure 4. DRP1 interacts with pTau-S396 and its levels are increased in ALS SNs.**

18 (a) Representative western blot images of pTau-S396 and DRP1 interaction in control and ALS mCTX.
19 (b) There was a significant effect of cellular fractions in pTau-S396 and DRP1 interaction (two-way
20 ANOVA, [F(1,27)=92.60], p<0.0001) with no significant changes in this interaction between control
21 (n=8) and ALS mCTX (n=8) (Tukey's test, p=0.6958). Data are represented by box plots with the central
22 line representing the median, the edge representing the interquartile range and the whiskers
23 representing the minimum and maximum values. (c) Representative western blot images of DRP1 and
24 pDRP1-S616 in ALS and control SNs. There was a significant increase in (d) DRP1 (Mann-Whitney U
25 test=41, p=0.0002) and (e) pDRP1-S616 levels (Mann-Whitney U test=103.5, p=0.0429) in ALS SNs

1 (n=32) compared to controls (n=12). (f) There was a trend towards a significant increase in synaptic
2 DRP1 levels in male (n=16) ALS patients compared to controls (n=3) (Mann-Whitney U test=12,
3 p=0.0800). (g) Synaptic DRP1 levels were increased in female ALS patients (n=15) compared to
4 controls (n=5) (Mann-Whitney U test=6, p=0.0050). Synaptic DRP1 levels were increased in (h) bulbar
5 onset (n=13; Mann-Whitney U test=9, p=0.0015) and (i) limb onset ALS (n=15; Mann-Whitney U
6 test=10, p=0.0009) compared to controls (n=9). (j) There was no change in DRP1 levels in ALS/FTD
7 (n=3; Mann-Whitney U test=6, p=0.2788). (k) There was a significant increase in DRP1 in ALS with
8 TDP-43 proteinopathy (n=18; Mann-Whitney U test=10, p=0.0002). (l) There was a significant increase
9 in pTau-S396 in *C9ORF72*-ALS (n=5; Mann-Whitney U test=3, p=0.0109). (m) There was a significant
10 increase in pTau-S396 in a single *SOD1*-ALS. Data in **d-m** are represented as individual value plots
11 with the central line representing the median and the whiskers representing the interquartile range. See
12 also Figure S2. *p<0.05; **p<0.01; ***p<0.001.

13
14 **Figure 5. Mitochondrial mass, density and length are decreased in ALS mCTX.**

15 (a) There was a significant decrease in Tomm20 levels in ALS mCTX (n=30) compared to controls
16 (n=10) (Mann-Whitney U test=60.50, p=0.0495). Data are represented as individual value plots with
17 the central line representing the median and the whiskers representing the interquartile range. (b)
18 Representative EM images from control and ALS mCTX. There were fewer and smaller mitochondria
19 (white arrows) in ALS compared to control mCTX. (c) There was a significant decrease in the density
20 of mitochondria in ALS mCTX (n=5) compared to controls (n=4) (Mann-Whitney U test=1, p=0.0317).
21 Data are represented as scatter plots with bar indicating mean±SD. (d) Cumulative frequency graph
22 demonstrated a significant decrease in the length of mitochondria in ALS mCTX compared to controls
23 (Kolmogorov-Smirnov test, p<0.0001). (e) There was a significant effect of mitochondrial length (two-
24 way ANOVA, [F(3,32)=30.08], p<0.0001) and length X disease interaction (two-way ANOVA,
25 [F(3,32)=3.314], p=0.0032) in mCTX. Furthermore, there was a significant increase in the frequency of
26 smaller mitochondria (<0.50um) in ALS mCTX compared to controls, while there was no change in the

1 frequency of larger mitochondria (Tukey's test, $p=0.0432$ and $p>0.9999$, respectively). Data are
2 represented as scatter plots with bar indicating mean \pm SD. Scale bar: 500nm. * $p<0.05$.

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6 **Figure 6. QC-01-175 decreases pTau-S396 levels in ALS SNs-treated cells.**

7 (a) Representative western blot images of pTau-S396 in vehicle- and ALS SNs-treated SH-SY5Y cells
8 following treatment with QC-01-175 (1 μ M) for 2, 4 or 24h. (b) Representative western blot images of
9 pTau-S396 in vehicle- and ALS SNs-treated SH-SY5Y cells treated with QC-01-175 (10 μ M) for 2, 4 or
10 24h. pTau-S396 levels were decreased following treatment with QC-01-175 (10 μ M) for 24h. (c) There
11 was a significant effect of treatment on pTau-S396 levels in SH-SY5Y cells (one-way ANOVA,
12 $[F(3,12)=3.699]$, $p=0.0429$) with a significant decrease in pTau-S396 levels in ALS SNs+QC-01-175-
13 treated cells compared to ALS SNs-treated cells (Tukey's test, $p=0.0328$). * $p<0.05$.

14
15 **Figure 7. Degrading tau mitigates ALS SNs-induced mitochondrial fragmentation.**

16 (a) Representative image of SH-SY5Y cells treated with vehicle, recombinant tau, control and ALS SNs
17 in the absence or presence of QC-01-175 stained with Hoechst (blue), CellMask (white), and Tomm20
18 (red). (b) Cumulative frequency graph indicated an effect of treatment on length (Kruskal-Wallis,
19 $H=113.3$, $p<0.0001$) with smaller mitochondria in tau- and ALS SNs- ($n=3$) compared to vehicle-
20 ($p<0.0001$, and $p<0.0001$, respectively) and control SNs ($n=3$)-treated cells ($p<0.0001$, and $p=0.0015$,
21 respectively). QC-01-175 prevented decrease in mitochondrial length induced by both tau ($p<0.0001$)
22 and ALS SNs ($p=0.0170$). (c) Cumulative frequency graph indicated an effect of treatment on volume
23 (Kruskal-Wallis, $H=39.11$, $p<0.0001$) with smaller mitochondria in tau- and ALS SNs- compared to
24 vehicle- ($p=0.0081$, and $p=0.0039$, respectively) and control SNs-treated cells ($p=0.0112$, and
25 $p=0.0024$, respectively). QC-01-175 prevented decrease in mitochondrial volume induced by ALS SNs

1 (p=0.0020). **(d)** There was no effect of treatment on total networks/cell following treatment with tau,
2 control and ALS SNs (two-way ANOVA, [F(1,44)=0.07953], p=0.7793). Data are represented as scatter
3 plots with bar indicating mean±SD. **(e)** There was an effect of treatment (two-way ANOVA,
4 [F(3,43)=4.792] p=0.0057) and treatment X tau degradation (two-way ANOVA [F(3,43)=3.121],
5 p=0.0357) in SH-SY5Y cells with a significant decrease in ALS SNs- compared to vehicle- or control
6 SNs-treated cells (Tukey's test, p=0.0052, and p=0.0307, respectively). Data are represented as scatter
7 plots with bar indicating mean±SD. **(f)** There was an effect of treatment (two-way ANOVA,
8 [F(3,44)=5.770], p=0.0020) on mean mitochondrial branch length within networks with a decrease in
9 ALS SNs- compared to vehicle- or control SNs-treated cells (Tukey's test, p=0.0028, and p=0.0182,
10 respectively). Data are represented as scatter plots with bar indicating mean±SD. See also Figures S4-
11 S7. *p<0.05; **p<0.01; ****p<0.0001.

12
13 **Figure 8. QC-01-175 prevents ALS SNs-induced increases in ROS levels.**

14 **(a)** Representative traces of CellRox mean fluorescence intensity (MFI) measured over time in SH-
15 SY5Y cells treated with recombinant tau, control and ALS SNs (10ng/mL/24h) followed by exposure to
16 QC-01-175 (10µM/4h) and incubated with CellRox (2.5µM/30min). **(b)** There was a significant effect of
17 treatment (two-way ANOVA, [F(3,64)=7.616], p=0.0002), QC-01-175 (two-way ANOVA,
18 [F(3,64)=22.27], p<0.0001), and treatment X QC-01-175 (two-way ANOVA, [F(3,64)=5.801], p=0.0014)
19 on ROS levels. There was an increase in ROS levels in recombinant tau- and ALS SNs- (n=9)
20 compared to vehicle-(Tukey's test, p<0.0001, and p=0.0036, respectively) and control SNs (n=4)-
21 treated cells (Tukey's test, p=0.0005, and p=0.0398, respectively). QC-01-175 prevented increase in
22 ROS levels induced by recombinant tau and ALS SNs (Tukey's test, p<0.0001, and p=0.0213,
23 respectively). Data are represented as scatter plots with bar indicating mean±SD.. *p<0.05; **p<0.01;
24 ***p<0.001; ****p<0.0001.

1 **Supplementary Figure 1. Antimycin A decrease c- and m-aconitase activity.**

2 (a) Antimycin A reduced (a) c-aconitase and (b) m-aconitase activity in SH-SY5Y cells (Mann-Whitney
3 U test=0, p=0.0286, and Mann-Whitney U test=0, p=0.0286, respectively). *p<0.05.
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6 **Supplementary Figure 2. ALS SNs induce mitochondrial fragmentation.**

7 (a) Two-way ANOVA revealed an effect of length ([F(4,234)=1148], p<0.0001) and length X treatment
8 interaction ([F(12,234)=9.629], p<0.0001) in SH-SY5Y cells. Tukey's test revealed an increase in the
9 frequency of smaller mitochondria (<2 μ m) in recombinant tau- and ALS SNs-treated cells compared to
10 vehicle- (p<0.0001, and p<0.0001, respectively) and control SNs-treated cells (p<0.0001, and
11 p<0.0001, respectively) as well as a decrease in the frequency of larger mitochondria (>8 μ m) in
12 recombinant tau- and ALS SNs-treated cells compared to control SNs-treated cells (p=0.0041, and
13 p=0.0003, respectively). Data are represented as scatter plots with bar indicating mean \pm SD. (b) Two-
14 way ANOVA demonstrated an effect of volume ([F(5,266)=1878], p<0.0001) and volume X treatment
15 interaction ([F(15,266)=5.943], p<0.0001) in SH-SY5Y cells. Tukey's test revealed an increase in the
16 frequency of smaller mitochondria (<2 μ m³) in recombinant tau- and ALS SNs- compared to vehicle-
17 (p<0.0001, and p<0.0001, respectively) and control SNs-treated cells (p=0.0028, and p<0.0001,
18 respectively) as well as a decrease in the frequency of larger mitochondria (>10 μ m³) in ALS SNs-
19 compared to vehicle-treated cells (p=0.0178) and in recombinant tau- and ALS SNs- compared to
20 control SNs-treated cells (p=0.0418, and p<0.0001, respectively). Data are represented as scatter
21 plots with bar indicating mean \pm SD. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.
22

23 **Supplementary Figure 3. Pro-fusion proteins are not altered in ALS SNs**

24 (a) Representative western blot images of Mfn1, Mfn2 and OPA1 in control and ALS SNs. (b) There
25 was no significant change in Mfn1 (Mann-Whitney U test=144, p=0.8597) (c) Mfn2 (Mann-Whitney U

1 test=61, $p=0.9321$) or **(d)** OPA1 levels (Mann-Whitney U test=135, $p=0.3994$) in ALS SNs ($n=32$)
2 compared to controls ($n=12$). Data are represented as individual value plots with the central line
3 representing the median and the whiskers representing the interquartile range.

4
5
6 **Supplementary Figure 4. siDRP1 decreases DRP1 levels in ALS SNs-treated cells.**

7 **(a)** Representative western blot images of DRP1 in SH-SY5Y cells following siDRP1. DRP1 levels were
8 reduced following transfection with 150nM siDRP1 for 24h and following 48h treatment. **(b)** There was
9 a significant effect of treatment on DRP1 levels in SH-SY5Y cells (one-way ANOVA, $[F(3,12)=18.52]$,
10 $p<0.0001$) with a significant decrease in DRP1 levels in siDRP1- and siDRP1+ALS SNs-treated cells
11 compared to siControl- (Tukey's test, $p=0.0023$, and $p=0.0033$, respectively) and siControl+ALS SNs-
12 treated cells (Tukey's test, $p=0.00004$, and $p=0.0005$, respectively). Data are represented as scatter
13 plots with bar indicating mean \pm SD. ** $p<0.01$; *** $p<0.001$.

14
15 **Supplementary Figure 5. siDRP1 mitigates ALS SNs-induced alteration in mitochondrial**
16 **connectivity.**

17 **(a)** Representative images of siControl- and siDRP1-transfected SH-SY5Y cells treated with vehicle,
18 recombinant tau, control and ALS SNs stained with Hoechst (blue), CellMask (white), and Tomm20
19 (red). **(b)** Cumulative frequency graph indicated an effect of treatment on length (Kruskal-Wallis,
20 $H=33.14$, $p<0.0001$) with smaller mitochondria in siControl+tau- and siControl+ALS SNs-treated ($n=3$)
21 compared to siControl- ($p=0.0023$, and $p=0.0477$, respectively) and siControl+control SNs-treated
22 ($n=3$) cells ($p=0.0008$, and $p=0.0377$, respectively). **(c)** Cumulative frequency graph indicated an effect
23 of treatment on volume (Kruskal-Wallis, $H=40.04$, $p<0.0001$) with smaller mitochondria in
24 siControl+tau- and siControl+ALS SNs- compared to siControl+control SNs-treated cells ($p<0.00001$,
25 and $p<0.0001$, respectively). **(d)** There was no effect of treatment on networks/cell following treatments
26 (two-way ANOVA, $[F(3,38)=0.9047]$, $p=0.4479$). Data are represented as scatter plots with bar

1 indicating mean±SD. (e) Two-way ANOVA revealed an effect of treatment ([F(3,39)=3.953], p=0.0149),
2 siDRP1 ([F(1,39)=12.80], p=0.0009), and treatment X siDRP1 interaction ([F(3,39)=5.659], p=0.0026)
3 on large networks/cell in SH-SY5Y cells. Tukey's test revealed a decrease in tau- and ALS SNs-
4 compared to vehicle- (p=0.0056, and p=0.0374, respectively) or control SNs-treated cells (p=0.0064,
5 and p=0.0416, respectively). siDRP1 prevented tau- and ALS SNs-induced decrease in large
6 networks/cell (Tukey's test, p=0.0137, and p=0.0082, respectively). Data are represented as scatter
7 plots with bar indicating mean±SD. (f) Two-way ANOVA demonstrated an effect of treatment
8 ([F(3,39)=4.034], p=0.0136) and treatment X siDRP1 interaction ([F(3,39)=6.527], p=0.0011) on mean
9 branch length. Tukey's test demonstrated a decrease in siControl+tau and siControl+ALS SNs-
10 compared to siControl- (p=0.0068, and p=0.0195, respectively) or siControl+control SNs-treated cells
11 (p=0.0153, and p=0.0398, respectively). siDRP1 prevented ALS SNs-mediated branch length
12 alterations (p=0.0398). Data are represented as scatter plots with bar indicating mean±SD. *p<0.05;
13 **p<0.01; ***p<0.001; ****p<0.0001.

14
15 **Supplementary Figure 6. siDRP1 prevents ALS SNs-induced mitochondrial fragmentation.**

16 (a) There was a significant effect of length (two-way ANOVA, [F(4,200)=1502], p<0.0001) and length X
17 treatment interaction (two-way ANOVA, [F(28,200)=3.621], p<0.0001) in SH-SY5Y cells. Tukey's test
18 revealed a significant increase in the frequency of smaller mitochondria (<2µm) in siControl+tau- and
19 siControl+ALS SNs- (n=3) compared to siControl (p<0.0001, and p=0.0002, respectively) and
20 siControl+control SNs-treated (n=3) cells (p<0.0001 and p=0.0002, respectively). Similarly, Tukey's
21 test demonstrated a significant decrease in the frequency of larger mitochondria (>8µm) in recombinant
22 tau- and ALS SNs- compared to siControl-treated cells (p=0.0032 and p=0.0024, respectively) as well
23 as in ALS SNs- compared to control SNs-treated cells (p=0.0433). siDRP1 prevented ALS SNs-induced
24 increases in smaller mitochondria (p=0.0015) and reductions in larger mitochondria (p=0.0433). Data
25 are represented as scatter plots with bar indicating mean±SD. (b) There was a significant effect of both

1 mitochondrial volume (two-way ANOVA, [F(5,237)=3239], $p<0.0001$) and volume X treatment
2 interaction (two-way ANOVA, [F(35,237)=3.702], $p<0.0001$) in SH-SY5Y cells. Tukey's test revealed a
3 significant increase in the frequency of smaller mitochondria ($<2\mu\text{m}^3$) in siControl+tau- and
4 siControl+ALS SNs compared to siControl ($p<0.0001$, and $p<0.0001$, respectively) and control SNs-
5 treated cells ($p<0.0001$ and $p=0.0007$, respectively). Similarly, Tukey's test demonstrated a significant
6 decrease in the frequency of larger mitochondria ($>10\mu\text{m}^3$) in recombinant tau- and ALS SNs-treated
7 cells compared to siControl ($p<0.0001$) as well as compared to siControl+control SNs-treated cells
8 ($p=0.0203$ and $p<0.0001$, respectively). siDRP1 prevented ALS SNs-induced increases in smaller
9 mitochondria ($p=0.0289$) and reductions in larger mitochondria ($p<0.0001$). Data are represented as
10 scatter plots with bar indicating mean \pm SD. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

11
12 ***Supplementary Figure 7. QC-01-175 prevents ALS SNs-induced mitochondrial fragmentation.***

13 (a) There was a significant effect of length (two-way ANOVA, [F(4,215)=955.7], $p<0.0001$), and length
14 X treatment interaction (two-way ANOVA, [F(28,215)=4.756], $p<0.0001$). Tukey's test revealed an
15 increase in the frequency of smaller mitochondria ($<2\mu\text{m}$) in recombinant tau- or ALS SNs- ($n=3$)
16 compared to vehicle- ($p=0.0021$, and 0.0235 , respectively) and control SNs-treated ($n=3$) cells
17 ($p=0.0005$, and $p=0.0068$, respectively). QC-01-175 prevented recombinant tau- and ALS SNs-induced
18 increase in the frequency of smaller mitochondria ($p=0.0014$ and $p<0.0001$, respectively). Data are
19 represented as scatter plots with bar indicating mean \pm SD. (b) There was a significant effect of volume
20 (two-way ANOVA, [F(5,264)=1510], $p<0.0001$), and volume X treatment interaction (two-way ANOVA,
21 [F(35,264)=2.864], $p<0.0001$) in SH-SY5Y cells. Tukey's test revealed an increase in the frequency of
22 smaller mitochondria ($<2\mu\text{m}^3$) in recombinant tau- and ALS SNs- compared to vehicle- ($p=0.0034$,
23 $p=0.0027$, respectively) and control SNs-treated cells ($p=0.0449$, and $p=0.0373$, respectively). QC-01-
24 175 prevented recombinant tau- and ALS SNs-induced increase in the frequency of smaller
25 mitochondria ($p=0.0179$ and $p<0.0001$, respectively) as well as ALS SNs-induced decrease in the

1 frequency of larger mitochondria ($p=0.0470$). Data are represented as scatter plots with bar indicating
2 mean \pm SD. * $p<0.05$; ** $p<0.01$; **** $p<0.0001$.

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4

5 ***Supplementary Figure 8. Antimycin A decreases ROS levels.***

6 Antimycin A decreased ROS levels in SH-SY5Y cells (Mann-Whitney U test=7, $p=0.0037$). ** $p<0.01$.

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