Axonal TDP-43 Drives NMJ Disruption through Inhibition of Local Protein Synthesis

Supplementary Figure Legends

Supplementary figure 1 – TDP-43 mis-localizes in TDP∆NLS mice in-vivo and in-vitro: **A)** Representative images of in-vivo SC MNs from $ChAT^{tdTomato}$ littermate or TDP∆NLS stained with tTDP-43 antibody (green) and DAPI (blue). ChAT signal appears in red. Scale bar = 10µm. **B)** Representative images of TDP∆NLS primary cultured MNs cell bodies with (upper panel-control) or without dox (middle and lower panel), stained with TDP-43 antibody (green) and DAPI (blue). Scale bar = 10µm. **C)** Western blot and **D)** PCR analysis of axons extracted from radial MFCs. **In C**, MN axons and soma were blotted for MAP-2 (upper panel) to mark dendrites and TAU-5 (middle panel) to mark axons. Tubulin (lower panel) was used as a loading control. **In D**, Qualitative RT-PCR was performed for cell body-specific Polymerase B (Pol-B) as a control for fraction purity, together with Beta-Actin as a positive control. **E)** Western blot analysis of purified TDP∆NLS MN axons and soma extracted from radial MFCs, with or without dox, blotted for human TDP-43 (hTDP43, upper panel) and total TDP-43 (tTDP-43, middle panel). ERK 1/2 was used as a loading control (lower panel). This is the full blot of what appears in Fig.1F.

Supplementary figure 2 - Axonal TDP-43 increased levels result in co-localization with RNP granule marker G3BP1: **A-B**) Quantification of TDP-G3BP1 colocalization of TDP∆NLS MN axons with (control) or without dox (∆NLS), immunostained for TDP-43 and G3BP1. In **A**, the % of TDP-G3BP1 colocalized area was compared to the total G3BP1 axonal area, measuring how many of G3BP1 puncta were in colocalization with TDP-43. In **B**, the % of TDP-G3BP1 colocalized area was compared to the total TDP-43 axonal area, measuring how many of TDP-43 puncta were in colocalization with G3BP1. n=22 and 26 axons for control and ∆NLS, respectively. Unpaired t-test, ***p<0.001, ****p<0.0001.

Supplementary figure 3 – Axonal RNP granule induction leads to reduction of local protein synthesis in MN axons and NMJs.

A-B) Representative images displaying the effect of RNP granule induction elicited by $NaAsO_2$ application, leading to G3BP1 aggregation in **A)** MN cell bodies and **B)** MN axons**.** Scale bar = 10µm and 5µm, respectively. **C**) Representative images and **D)** Quantification of OPP puncta in axons without (control) or with application of $NaAsO_2$ application. Scale bar = 10µm. n=110, 137 axons for control and $NaAsO_2$ conditions, respectively. Data is shown as the mean OPP intensity per µm ± S.D. One-way Anova with Holm-Sidak correction, ****p<0.0001. **E**) Representative images **F**) and quantification of OPP puncta in axons without (control) or with

application of protein synthesis inhibitors Cycloheximide (CHX) and Anisomycin (Aniso). An additional control, with only color labeling but no puromycin (no OPP) was included. Scale bar = 10µm. n=102, 63, 73, 6 axons for control, CHX and Aniso conditions, respectively. One-way Anova with Holm-Sidak correction, ****p<0.0001. **G)** Representative images and **H)** quantification of OPP density and **I)** OPP puncta fluorescent intensity in distal axons in MFC labeled with OPP (20µM) for different time periods (1 min, 5 min, 30 min). Scale bar = 10µm. Data is shown as the mean of OPP puncta/µm or OPP intensity per µm ± S.D. Puncta/µm n=70, 92, 110 axons from 3 independent repeats. Puncta intensity n=472, 741, 819 puncta from 3 independent repeats. One-way ANOVA with Holm-Sidak correction, ****p<0.0001. **J)** Representative images and **K)** quantification of puromycin labeling in control, versus puromycin resistant muscles (expressing PQCXIP-mCherry empty vector with PAC gene) demonstrating the ability of PAC to prevent puromycin ability to label newly synthesized peptides. Scale bar = 20µm. n=14, 14 muscles. Student's t-test ***p<0.001. **L)** Representative images of puromycin-resistant muscles stable morphology following 16h and 24hr incubation with 100µg/mL puromycin compared to muscles that were not exposed to puromycin. **M)** Representative images and **N)** quantification of OPP puncta density of *in-vitro* NMJs in the presence of absence of distal $NaAsO_2$. Scale bar = 5µm. n=42, 44 NMJs from 3 independent repeats. Data is shown as the mean OPP intensity per µm ± S.D. One-way ANOVA with Holm-Sidak correction, ***p<0.001.

Supplementary Figure 4 - TDP-43 axonal accumulation leads to profound reduction in MN axonal local protein synthesis. **A)** Representative images of OPP labeling versus only color labeling but no puromycin (no OPP) in SN sections. HB9 indicates MN axons. Scale bar = 10µm. **B)** Additional unmasked OPP images and overlay images of SN sections obtained from TDP∆NLS and LM mice. Scale bar = 10µm.

Supplementary Figure 5 - Mitochondria Activity and Local Protein Synthesis are Vital for NMJ Function and Their Inhibition Leads to Neurodegeneration. **A**) Representative images and **B**) quantification of the percent of degenerating HB9::GFP MN axons in the distal compartment of MFC following 24h axonal incubation with protein synthesis inhibitors Puromycin. DMSO was used as a control for anisomycin treatment. Scale bar = 100µm. n=3,3,3,3 (three independent repeats). Data is shown as the mean percent of degenerated axons per MFC ± S.E. Unpaired two-tailed student's t-test, **p<0.01.

Supplementary Figure 6 – Dox re-application restores TDP-43 localization: **A)** Western-blots for total-TDP-43 (tTDP-43; 43kDa) and human-TDP-43 (hTDP-43; 43kDa) in GC muscles,

spinal cords (SC) and SNs of LM, ΔNLS, and recovery mice. Tubulin (55kDa) or tERK (42-44kDa) were used as loading controls. **B)** Representative images and **C)** quantification of the percent of spinal cord MNs (indicated by ChAT-red) with nuclear-localized TDP-43 in recovered mice compared to TDPΔNLS mice with no recovery. Nuclear localization was marked by colocalization of DAPI (blue) staining with TDP-43 (green). Data is shown as the mean percent of TDP-43 positive nuclei within $ChAT^{tdTomato}$ motor neurons ± S.E. Scale bar = 5μm. n=3 mice from each group, one-way ANOVA with Holm-Sidak correction, ***p<0.001.

Supplementary Figure 7 – Dox re-application restores TDP-43 mediated local synthesis inhibition: **A)** Representative images and **B)** quantification of OPP signal (green) within $ChAT^{tdTomato}$ axons (red) in SNs from recovered mice compared to LM and ΔNLS mice. Scale bar = 10μm. Data is shown as the mean OPP signal in MN axons ± S.E. n=3 the averages of 3 independent repeats from 3 mice in each condition, one-way ANOVA with Holm-Sidak correction, *p<0.05. **C)** Representative co-localization images of ATP5A1 (green) with pre-synaptic ChAT (red) and OPP labeling (gray) in ΔNLS mice compared with LM and recovered mice. Scale bar = 10μm. **D)** Representative histogram of ATP5A1 (red) and OPP (green) signals within NMJ pre-synapse. **E)** Quantitative colocalization analysis of the percent of ATP5A1 area within the pre-synapse area ($ChAT^{tdTomato}$) in ΔNLS mice compared with LM and recovered mice. Data is shown as the mean percent of ATP5A1-ChAT colocalization ± S.D. n=20, 13, 12 NMJs. 3 mice from each group. One-way ANOVA with Holm-Sidak correction, **p<0.01, *p<0.05. **F)** Quantitative analysis of ATP5A1 colocalization with OPP in the pre-synaptic NMJ axon ($ChAT^{tdTomato}$) from ΔNLS mice compared with LM and recovered mice. Data is shown as the mean percent of ATP5A1-OPP colocalization ± S.D. n=20, 13, 12 NMJs, from 3 mice of each group. One-way ANOVA with Holm-Sidak correction, ***p<0.001, **p<0.01.

Supplementary Figure 8 – Dox re-application restores NMJ innervation, increase post synaptic size and rescues mice loss of weight **A)** Representative images of NMJ innervation from ΔNLS mice compared with LM and recovered mice. Scale bar = 100μm. **B)** Representative images and **C)** quantitative analysis of BTX (green) post synaptic area cluster size in GC muscle NMJs in recovered mice as compared with ΔNLS and LM mice. ChAT signal (red) marks pre-synaptic innervation. Data is shown as the mean ± SD. n=202, 263, 318 NMJs from 3 mice of each group, one-way ANOVA with Holm-Sidak correction *p<0.05, **p<0.01. **D)** TDPΔNLS, LM and recovered mice weight measurements after dox retraction. Dox was introduced back to recovered mice at week 3. Data is shown as the mean percent of weight in each time point compared to the initial weight ± SE. n =18, 15, 9 mice for LM, TDPΔNLS and recovery groups.

Supplementary Movie 1 – OPP labeling in muscles identifies local protein synthesis in the pre-synaptic MN axon at the NMJ: 3D reconstitution of NMJ from EDL muscle demonstrating labeling of newly synthesized proteins in the pre-synaptic axon. ChAT is indicated by red color. OPP is indicated by green color. Colocalization of ChAT and OPP is indicated by yellow color.

Supplementary Movies 2-3 – Irradiating pre-synaptic mitochondria inhibits NMJ function: Pre-synaptic MNs were infected with lentivirus encoding mito-killer-red (MKR) protein, and irradiated specifically at the muscle contact sites, the NMJs. Muscle calcium transients, indicating muscle activity, were imaged prior (pre-Sup. Movie 2) and 30 minutes after (post-Sup. Movie 3) MKR irradiation. 500-frame movies were acquired at 60 millisecond intervals for a total of 30 seconds.

Supplementary Movie 4 - Normal NMJ activity in control neuromuscular cultures: Neuromuscular co-cultures at 10 DIC labeled with OGB for visualizing axonal and muscular calcium transients. Arrowheads indicate presynaptic axon and muscle simultaneously become active, demonstrating undisrupted neuromuscular transmission. Scale bar = 20μm.

Supplementary Movie 5 - Dysfunctional NMJ activity in puromycin treated neuromuscular cultures: Neuromuscular co-cultures at 10 DIC labeled with OGB, 16 hours after puromycin (100μg/mL) was added exclusively to the distal/NMJ compartment of MFC. Arrowheads indicated calcium transient in presynaptic axon and unresponsive (inactive) muscle. Scale bar = 20μm.

Supplementary Movie 6 – Cox4i Enrichment in pre-synaptic axon at NMJs: 3D reconstitution of NMJ from EDL muscle demonstrates Cox4i enrichment in the presynaptic axon at the NMJ. ChAT is indicated by red color. Cox4i is indicated by green color. Colocalization of ChAT and Cox4i is indicated by yellow color.