Supplementary Material

Analyzing the ER stress response in ALS patient derived motor neurons identifies druggable neuroprotective targets

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**Supplementary Figure S1. Extended analyses of iPSC-derived MN cultures and response to proteostatic stressors.** (A) MN and non-MN viability from 1016 healthy control cultures at low density (25K/96 well or 12.5K/96 well) or with maturation (14 days in vitro (DIV) at 50K/96well) after 48hrs exposure to increasing doses of ER stressors. Nb=3, nt=2, two-way ANOVA; 25K thapsigargin- p = 7.67x10-6, 12.5K thapsigargin- p = 0.000258, 2week thapsigargin- p = 0.000691, 25K tunicamycin- p = 0.008. 12.5K tunicamycin- p = 2.63x10-7, 2week tunicamycin- p = 0.002. For simplicity, stars indicating significance are shown for the condition with the least significance. (B) Quantification of MN and non-MN viability 48hrs after treatment with increasing concentrations of MG132 (1016 healthy control line). Nb=3, nt=12, two-way ANOVA, p = 0.394. (C) Quantification of MN and non-MN viability after treatment with 1μM MG132 for various lengths of time (1016 healthy control line). (D) MN and non-MN viability from low density cultures (25K/96 well or 12.5K/96 well) or mature cultures (14 days in vitro (DIV) at 50K/96well) after 48hrs exposure to increasing doses of MG132 (1016 healthy control line). Nb=3, nt=2, two-way ANOVA; 25K- p = 0.894. 12.5K- p = 0.947. 2week- p = 0.284. (E) Western blot quantification of phosphorylated eIF2α with increasing time of DMSO, 1μM thapsigargin or 1μM tunicamycin (1016 healthy control line); Nb = 3, Nt=1, two tailed students t.test to DMSO control p<0.05 = \*, p<0.01 = \*\*. (F) Quantification of spliced XBP1 template, normalized to unspliced XBP1 template after 2 and 4hrs of DMSO, 1μM thapsigargin or 1μM tunicamycin treatment (1016 healthy control line); Nb = 3, Nt=1, two tailed students t.test to DMSO ctrl p<0.05 = \*, p<0.01 = \*\*. (G) Western blot quantification of BiP at 8 and 24hrs of DMSO, 1μM thapsigargin or 1μM tunicamycin treatment (1016 healthy control line); Nb = 3, Nt=1, two tailed students t.test to DMSO control p<0.05 = \*, p<0.01 = \*\*. (H) Western blot quantification of CHOP at 8 and 24hrs of DMSO, 1μM thapsigargin or 1μM tunicamycin treatment (1016 healthy control line); Nb = 3, Nt=1, two tailed students t.test to DMSO control p<0.05 = \*, p<0.01 = \*\*. (I) Western blot quantification of cleaved caspase 3 at 8 and 24hrs of DMSO, 1μM thapsigargin or 1μM tunicamycin treatment (1016 healthy control line); Nb = 3, Nt=1, two tailed students t.test to DMSO control p<0.05 = \*, p<0.01 = \*\*. (J) Healthy control 1016A, SOD1, and TDP43 iPSCs were differentiated into MN cultures (containing MN and non-MN cell populations) and exposed to 1 μM thapsigargin for 16 hours. qRT-PCR revealed no differences in response to thapsigargin exposure across healthy control and ALS MNs by expression of UPR-associated genes ATF6, IRE1, CHOP, and BiP. Nb = 2, Nt = 3.Biological replicate experiments denoted as Nb, each with technical replicate experiments nt. Data are mean value +/- SEM. p<0.05 was considered statistically significant and denoted in graphs with a \*, p<0.01 \*\*, p<0.001 \*\*\*, and P<0.0001 \*\*\*\*.

**Supplementary Figure S2. Overview of global phosphoproteomics experiment.** (A) Schematic of the ER stress and protection assay and the subsequent quantitative proteomics analysis pipeline. (B) Individual, separated proteomic volcano plots for all treatment conditions. -Log10(p-value) is graphed on the Y-axis, Log2(fold change) graphed on the X-axis for all plots. (C) Individual, separated phosphoproteomic volcano plots for all treatment conditions. -Log10(p-value) is graphed on the Y-axis, Log2(fold change) graphed on the X-axis for all plots.

**Supplementary Figure S3. Approach to identifying viable MNs.** (A) Example nuclear size exclusion parameters and Hoechst intensity thresholding used to identify the viable cell population. Histograms to right of selection script and input image demonstrate 2 distinct cell populations, live or dead, with live cells demonstrating a nuclear area >~37-55μm2 and Hoechst intensities lower than the threshold brightness of pyknotic nuclei (18,000 in this example). (B) Viable cell script accuracy confirmed with LIVE/DEAD Viability/Cytotoxicity Kit, for mammalian cells (Life Technologies L3224). (C) Example Isl1/2 intensity thresholding used to identify the viable MN population. Histogram to right of selection script and input image demonstrate that selected Isl1/2+ cell populations must have an Isl1/2 intensity greater than the basal intensity (>8000 in this example).

**Supplementary Figure S4. Neurite tracing of β-Tubulin III staining**. Representative image analysis pipeline to track neurites using B-Tubulin III staining.

**Supplementary Data Set 1. ER stress and protection proteomics and phosphoproteomics dataset.** Quantified proteins and phosphoproteins are displayed with corresponding log2foldchange with each treatment, compared to indicated control.

**Supplementary Video Files**. Automated live cell imaging of 1016A healthy control iPSC-derived MN cultures treated with DMSO (1), 1µM thapsigargin (2), or 1µM tunicamycin (3). Images were taken every 6hrs for 48hrs.

**Supplementary Table S1. Overview of quantified phosphopeptides and peptides**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Data Set** | **# Peptides (Set 1)** | **# Peptides (Set 2)** | **# Unique Proteins** | **# Unique Phosphorylation Sites** |
| Stress+Protection (Protein) | 67,357 | 59,708 | 6,697 |  |
| Stress+Protection (Phospho-protein) | 28,399 | 28,973 | 2,764 | 7,999 |

**Supplementary Table S2. Protective processes implicated by proteomic analyses.**

|  |  |  |
| --- | --- | --- |
| **Protective Compound** | **Implicated Protective Process** | **Specific Implicated Targets** |
| Kenpaullone | Microtubule Dynamics | Phospho-CLASP2, Phospho-DPYSL2, MAP1B, MAPT, TPP, Kif11 |
| Kenpaullone + MAP4K4i | Signaling | GSK3β, PKC, JNK, c-JUN, mTOR/S6K, RAF, PIM |
| Kenpaullone + MAP4K4i | Metabolism | ACSL4, HMG-CoA Reductase, SCD, ODC, CHDH |
| (Fatty Acid, Lipid, Cholesterol) |
| Kenpaullone + MAP4K4i | Receptors | Insulin, GABA |
| Kenpaullone + MAP4K4i | Cell Cycle Proteins | Cdc42, CDK3/5 |
| Kenpaullone + MAP4K4i | Calcium Dynamics | Cam2K, CSNK2 |
| Kenpaullone + MAP4K4i | ER-Golgi Anterograde Transport | GBF1, Phospho-EXOC1 |
| MAP4K4i | HDACs | HDAC1, 2, 5 |