**Expanded View Figures**

**Figure EV1.** ALS<sup>C9orf72</sup> MN display pathological accumulation of aggregates and autophagy blockade similar to ALS<sup>C9orf72</sup> cells.

A  List of the hiPSC lines used in this study.
B  RNA foci were detected in confocal microscopy using a fluorescent GGGGCC probe in ALS<sup>C9orf72</sup> neurons. Scale bar: 5 µm. Dashed line represents the cell soma.
C  ALS<sup>C9orf72</sup> MN accumulate aberrant perinuclear SQSTM1/p62<sup>+</sup> aggresomes. Scale bar: 5 µm. Dashed line represents the cell soma.
D  The levels of SQSTM1/p62 are significantly higher in ALS<sup>C9orf72</sup> than in Healthy cells (Welch’s t-test). n = 3 independent cultures for each hiPSC line. Scale bar: 10 µm.
E  ALS<sup>C9orf72</sup> MN display an autophagy blockade, as shown by the reduced levels LC3 II (Mann–Whitney test). n = 4 independent cultures, with the lines ALS<sup>C9orf72</sup> I and Healthy I used as representative of the two genotypes.
F  Representative TEM images showing cytosolic aggresomes in ALS<sup>C9orf72</sup> (DIV 21) and ALS<sup>TRX1</sup> (DIV 14) MN. Scale bar: 1 µm.
G  Aberrant aggresomes in ALS MN are also enriched with the proteasome 20 s alpha subunit. Scale bar: 5 µm. Dashed line represents the cell soma.

Data information: *P < 0.05. Error bars represent SEM. Arrows indicate the structures displayed at higher magnification. Exact P-values are reported in Appendix Table S1.

Source data are available online for this figure.
A

| hiPSC line   | Gender | Age at sampling | Mutated gene | Mutation          |
|--------------|--------|-----------------|--------------|-------------------|
| Healthy I    | Female | 45              | NA           | NA                |
| Healthy II   | Male   | 64              | NA           | NA                |
| Corrected\(\text{C9orf72}\) I | Male | 46              | NA           | NA                |
| ALS\(\text{C9orf72}\) II | Male | 60              | C9orf72      | \((G_{C})_{3.1}\)ub |
| ALS\(\text{C9orf72}\) III | Male | 46              | C9orf72      | \((G_{C})_{3.6}\)ub |
| ALS\(\text{TBK1}\) | Female | 51              | C9orf72      | \((G_{C})_{2.7}\)ub |

B

![Image of cell culture](B.png)

C

![Image of cellular fluorescence](C.png)

D

![Image of cellular fluorescence](D.png)

E

![Image of western blot](E.png)

F

![Image of electron microscopy](F.png)

G

![Image of cellular fluorescence](G.png)

Figure EV1.
Figure EV2. Progressive loss of synapses in ALS.

A. ALS MN have reduced Shank2:Bassoon synapses compared to Healthy controls (Welch’s t-test). \( n = 3 \) independent cultures for each hiPSC line. Scale bar: 10 \( \mu \)m.

B, C. Shank2^{+} dendritic clusters are significantly larger in ALS MN, while Bassoon puncta are comparable between genotypes (Welch’s t-test). \( n = 3 \) independent cultures for each hiPSC line.

D. Time course analysis of synaptophysin protein levels in human MN. \( n = 3 \) independent cultures for each hiPSC line (two-way ANOVA).

Data information: *\( P < 0.05 \). Error bars represent SEM. Exact P-values are reported in Appendix Table S1.
ALS\textsuperscript{TBK1} MNs are characterized by altered CREB activation and reduced synaptic contacts.

A. CBP is sequestered within the aggresomes accumulating in ALS\textsuperscript{TBK1} MN. Scale bar: 5 \( \mu \)m. Dashed line represents the cell soma.

B. DIV 21 ALS\textsuperscript{TBK1} MN have significantly higher nuclear levels of pCREB\textsuperscript{133} than an aged-matched Healthy control (Mann–Whitney test). \( n = 3 \) independent cultures. Scale bar: 25 \( \mu \)m.

C. Similar to ALS\textsuperscript{Sentr2} cultures, TBK1-mutant MN also show significantly lower phosphorylation of CREB than Healthy neurons at DIV 70 (Mann–Whitney test). \( n = 3 \) independent cultures. Scale bar: 25 \( \mu \)m.

D. The expression of the CREB-dependent post-synaptic gene HOMER1 is significantly lower in ALS\textsuperscript{TBK1} than Healthy I MN (Welch’s t-test). \( n = 3 \) independent cultures. Scale bar: 5 \( \mu \)m.

E. In agreement with the reduced activation of CREB, ALS\textsuperscript{TBK1} MN also show a significantly reduced number of excitatory synapses (Welch’s t-test). \( n = 3 \) independent cultures. Scale bar: 5 \( \mu \)m.

Data information: * \( P < 0.05 \), ** \( P < 0.01 \), and *** \( P < 0.001 \). Error bars represent SEM. Arrow indicates the structure displayed at higher magnification. Exact \( P \)-values are reported in Appendix Table S1.
Figure EV4. Optogenetic enhancement of MN activity reduces the accumulation of SQSTM1 aggregates.

A 1 Hz optogenetic stimulation reduces the accumulation of aggregated SQSTM1/p62 in human ALS<sup>CHChR2</sup> MN (Welch’s t-test). n = 18 MN analysed from three independent experiments. Scale bar: 10 µm. Dashed line represents the cell soma.

B The intensity of nuclear pCREB<sup>133</sup> signal is significantly higher in stimulated ALS<sup>CHChR2</sup> MN than unstimulated ones. n = 21 MN analysed from three independent experiments (Welch’s t-test). Scale bar: 5 µm. Dashed line represents the cell soma.

Data information: *P < 0.05 and **P < 0.01. Error bars represent SEM. Exact P-values are reported in Appendix Table S1.
Figure EV5. Apamin and XE991 exert a neuroprotective effect also in ALS^{TBK1} MN.

A Apamin and XE991 increase the neurite length of ALS^{TBK1} MN (one-way ANOVA followed by Dunnett’s multiple comparison test). \( n = 3 \) independent treatments. Scale bar: 50 \( \mu \)m.

B Both \( K^+ \) channel blockers reduce the accumulation of aggregated SQSTM1 in TBK1-mutant cells (one-way ANOVA followed by Dunnett’s multiple comparison test). \( n = 3 \) independent treatments. Scale bar: 10 \( \mu \)m.

C \( K^+ \) channel blockade reduces also the size of cytotoxic aggresomes (Kruskal–Wallis test). \( n = 3 \) independent treatments. Scale bar: 5 \( \mu \)m.

D In Apamin- and XE991-treated cultures, the levels of phosphorylated CREB are significantly higher than in vehicle-treated ones (one-way ANOVA followed by Dunnett’s multiple comparison test). \( n = 3 \) independent treatments. Scale bar: 10 \( \mu \)m.

E Apamin and XE991 rescue the loss of excitatory synapses also in TBK-mutant MN (Kruskal–Wallis test). \( n = 3 \) independent treatments. Scale bar: 5 \( \mu \)m.

Data information: *\( P < 0.05 \), **\( P < 0.01 \), and ***\( P < 0.001 \). Error bars represent SEM. Exact \( P \)-values are reported in Appendix Table S1.