Title: Human iPSC-derived astrocytes from ALS patients with mutated C9ORF72 show increased oxidative stress and neurotoxicity

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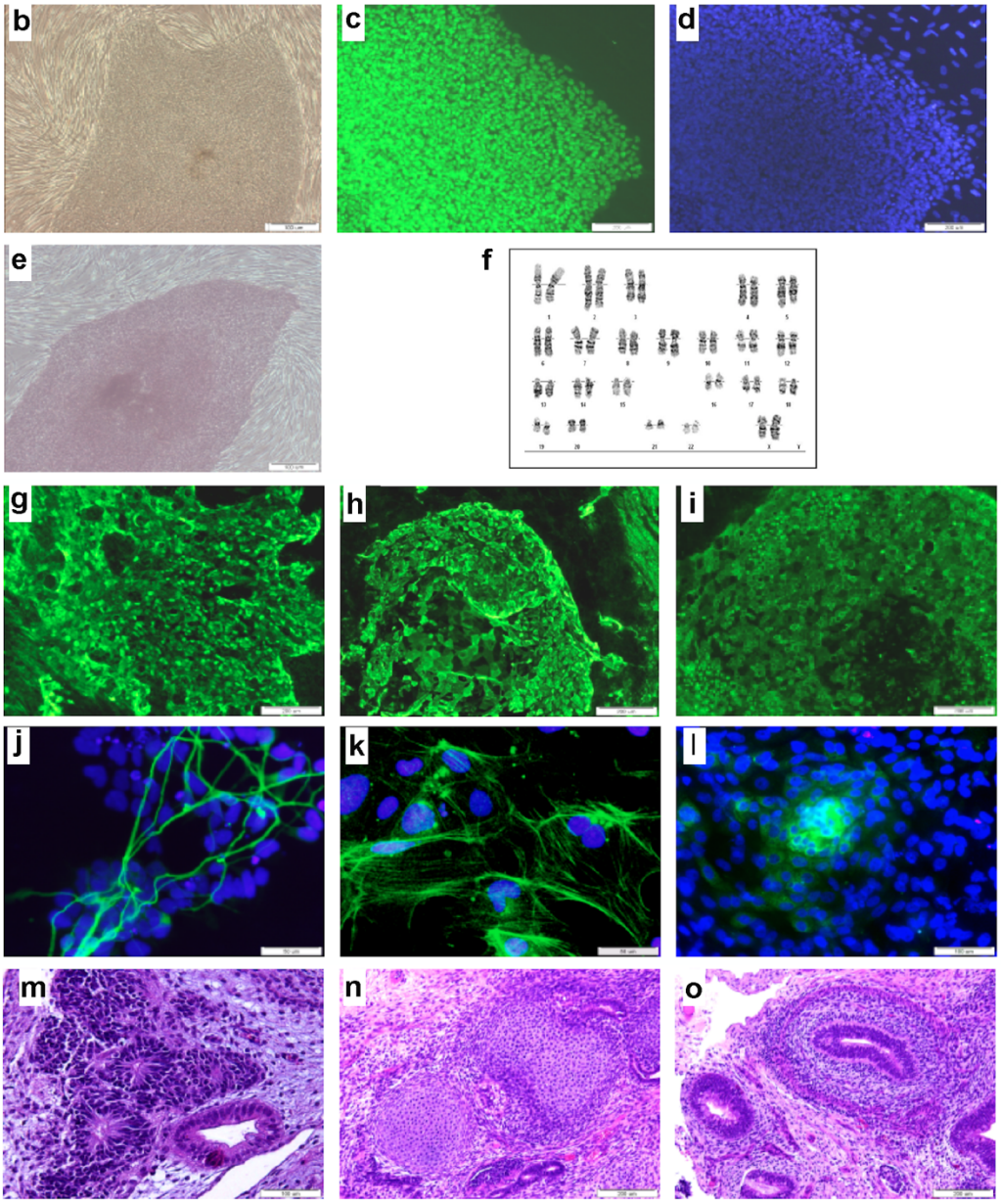
Supplementary information

Supplemental Fig1.

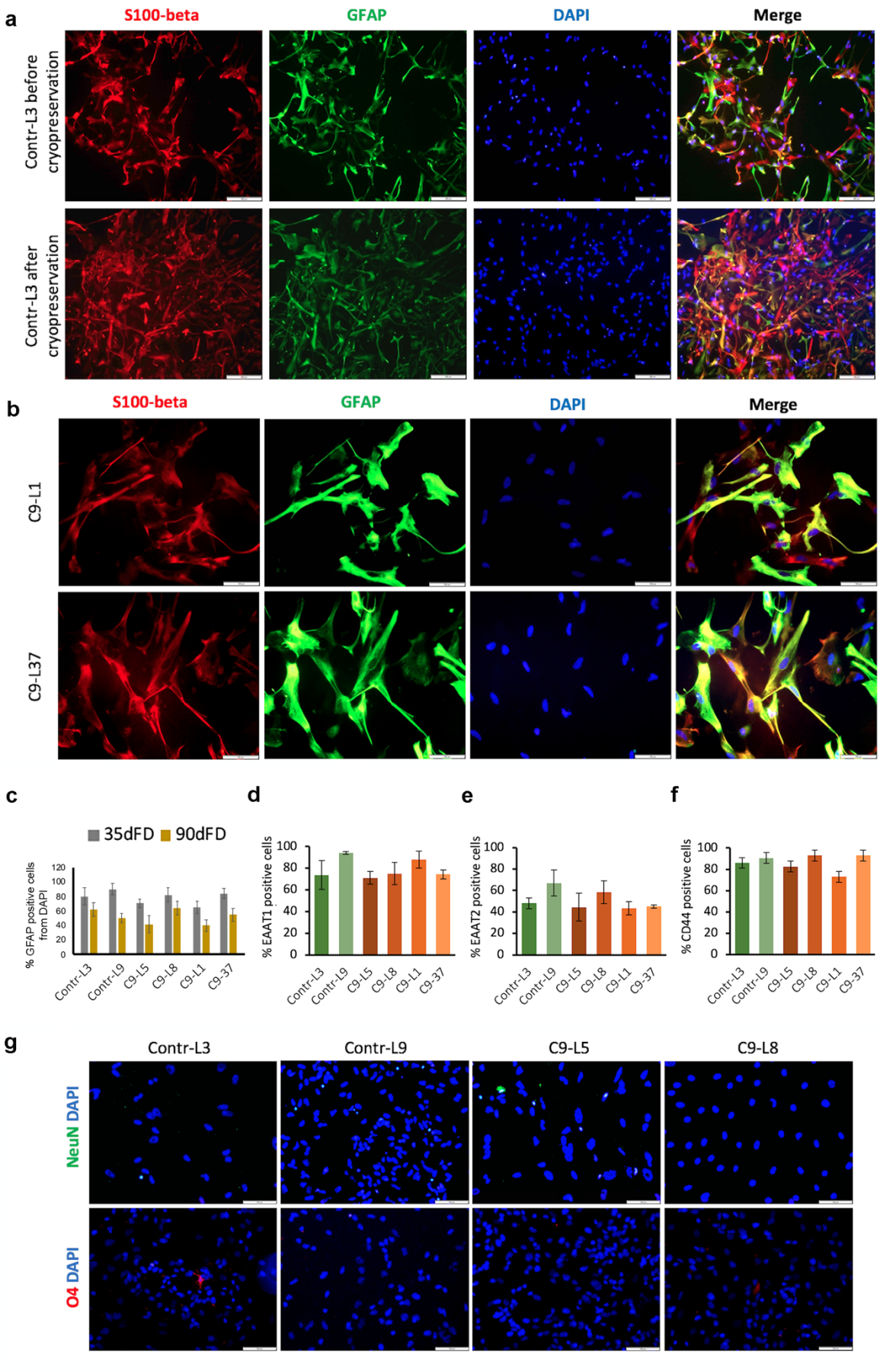
a

iPSCs ID	Age	Origin	Sex	Healthy or ALS
Contr-L3	61	European	male	healthy
Contr-L9	51	European	female	healthy
C9-L5	51	European	female	C9orf72
C9-L8	61	European	male	C9orf72
C9-L37	55	European	male	C9orf72
C9-L1	67	European	female	C9orf72



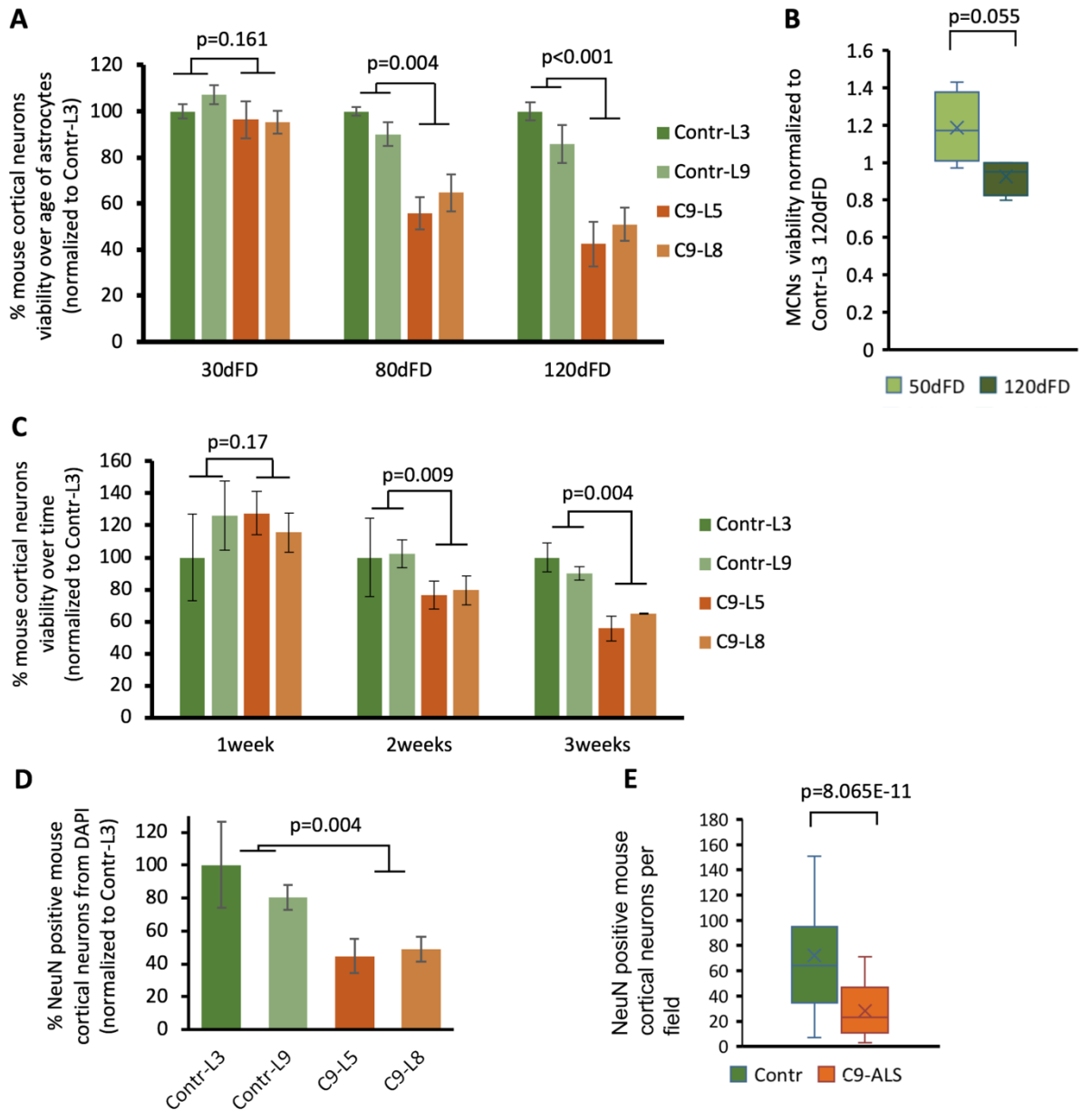
a. The age, gender and origin of C9orf72 ALS patients and healthy individuals that donated fibroblasts for the derivation of the indicated iPSC lines. b-o. Characterization of the phenotype and differentiation potential of a representative C9-ALS iPSC line (iPSC line 5 (C9-L5)). All other lines were similarly characterized and showed the same properties. The iPSC line formed colonies with typical embryonic stem cell morphology when cultured on foreskin feeder layer (b; phase contrast image) and expressed alkaline phosphatase (e). Immunofluorescence studies show Oct4 expression (c; green) and nuclear counterstained with DAPI (d; blue). The iPSCs express TRA-1-60, TRA-1-81 and SSEA-4 (g, h and i, respectively; green) and have a normal karyotype (f; 46XX). The pluripotent potential of the iPSCs was demonstrated by differentiation *in vitro* as embryoid bodies (EBs). Immunofluorescence staining of cells within the outgrowth from plated EBs shows cells stained for class III β-tubulin (Tuj1, pan-neuronal and ectodermal marker, j), muscle actin (mesoderm, k) and α-fetoprotein (endoderm, l). For demonstrating pluripotency potential *in vivo*, undifferentiated cells were inoculated subcutaneously into NOD/SCID mice and teratoma tumors developed. Hematoxylin-Eosin stained sections of the teratomas show neural rosettes (ectoderm, m), cartilage and bone (mesoderm, N) and columnar glandular epithelium (endoderm, o). Scale bars represent 50 μm for i and j; 100 μm for k and m; 200 μm for all other images.

Supplemental Fig2.


a
S100-beta
GFAP
DAPI
Merge
Contr-L3 before cryopreservation
Contr-L3 after cryopreservation
b
S100-beta
GFAP
DAPI
Merge
C9-L1
C9-L37
c
35dFD
90dFD
% GFAP positive cells from DAPI
Contr-L3
Contr-L9
C9-L5
C9-L8
C9-L1
C9-37
d
% EAAT1 positive cells
e
% EAAT2 positive cells
f
% CD44 positive cells
g
Contr-L3
Contr-L9
C9-L5
C9-L8
NeuN DAPI
O4 DAPI

a. Representative immunofluorescence images of non-mutated astrocytes (Contr-L3) before and after 1 week of cryopreservation expressing the glial markers GFAP (green) and S100b (red) at day 30 of FD. Nuclei are counterstained with DAPI (blue). Scale bars: 200 μm. These experiments were repeated 3 independent times with similar results for all astrocyte lines. b. Representative immunofluorescence images of C9-mutated astrocytes of two lines (C9-L1, C9-L37) expressing the glial markers: GFAP (green) and S100b (green) at day 30 of FD. Nuclei are counterstained with DAPI (blue). Scale bars: 100 μm. These experiments were repeated 3 independent times with similar results. c. Histogram presentation of the number of GFAP positive out of the DAPI positive cells. Data is represented as mean ± SEM of 3 independent experiments. d–f. Histogram presentation of FACS analysis of non-mutated (Contr-L3, Contr-L9, green bars) and mutated astrocyte cultures (C9-L5, C9-L8, C9-L1, C9-L37 orange bars) for the expression of the astrocyte markers EAAT1, EAAT2 and CD44. 10.000 PI-negative cells were analyzed for each marker. Data is represented as mean ± SEM of 3 independent experiments with astrocytes at 30-40 dFD. g. Immunostaining of C9-mutated (C9-L5, C9-L8) and control astrocytes (Contr-L3, Contr-L9) for the neuronal marker NeuN (green) and oligodendrocyte precursors marker O4 (red). Nuclei are counterstained with DAPI (blue). Scale bars: 100 μm. These experiments were repeated 3 independent times with similar results.

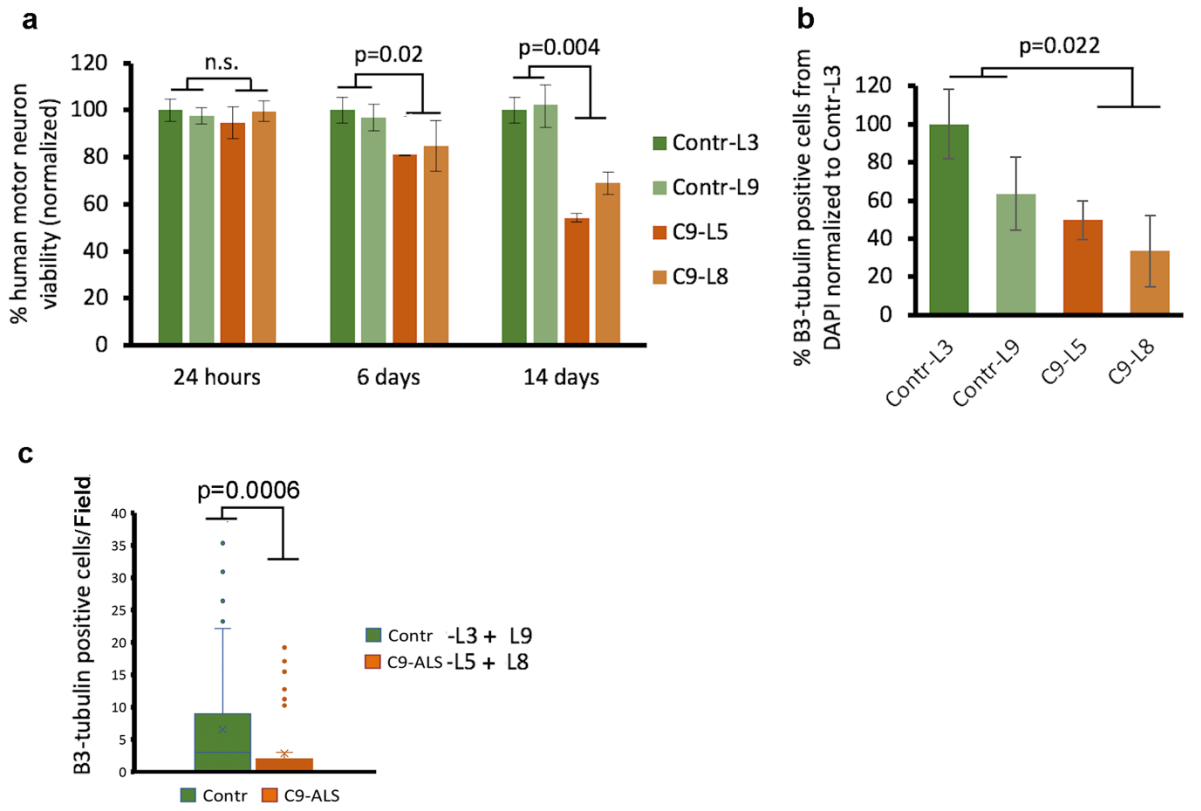
Supplemental Fig3.



a. The toxic effect of 30, 80 and 120 dFD C9-mutated astrocyte conditioned media (orange bars) compared with control (green bars) on mouse cortex neurons was evaluated by Alamar blue cell viability assay after 3 weeks of treatment. Data is represented as mean ± SEM of 3 independent experiments for each time point for each astrocyte line normalized to Contr-L3 (independent derivations of mouse cortical neurons for each experiment). b. The toxic effect of 50 dFD (light green) control astrocyte conditioned media compared with control astrocyte conditioned media at dFD 120 (dark green) on mouse cortex neurons was evaluated by Alamar blue cell viability assay after 3 weeks of treatment. Data is represented as mean ± SEM of 3 independent experiments for each time point for each astrocyte line normalized to Contr-L3 at 120 dFD. Although there is a trend of reduced ability of older control astrocytes to support

neuronal survival, this difference is not statistically significant. c. Time dependent toxic effect of 60 dFD C9-mutated astrocyte conditioned media (orange bars) compared with control (green bars) on mouse cortex neurons was evaluated by Alamar blue cell viability assay weekly for 3 weeks of treatment. Data is represented as mean ± SEM of 3 independent experiments for each time point normalized to Contr-L3 (independent derivations of mouse cortical neurons for each experiment). d. NeuN positive mouse cortical neurons were quantified after 3 weeks of treatment by C9-mutated (orange bars) and control (green bars) astrocyte conditioned media. 16 fields per experiment were imaged from each condition and the number of NeuN+/DAPI positive cells is presented. Data is represented as mean ± SEM of 3 experiments for each line normalized to Contr-L3 (independent derivations of mouse cortical neurons for each experiment). e. data represented in (d), this time represented as the number of NeuN positive neurons/field (without the normalization to the number of DAPI positive/field).

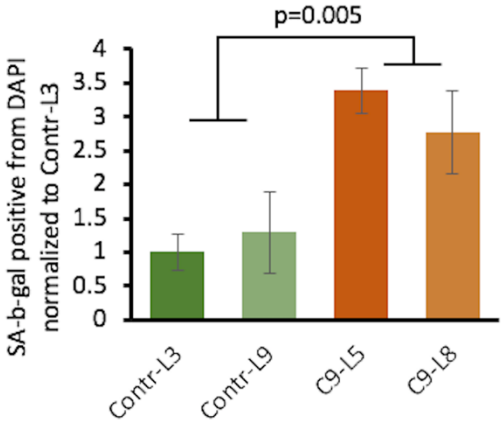
Supplemental Fig4.



a. Time-dependent toxic effect of C9-mutated astrocyte conditioned media (orange bars) compared to control (green bars) conditioned media on human MNs was measured by Alamar blue cell viability assay after 6 days and 2 weeks of treatment. The treatment was started after

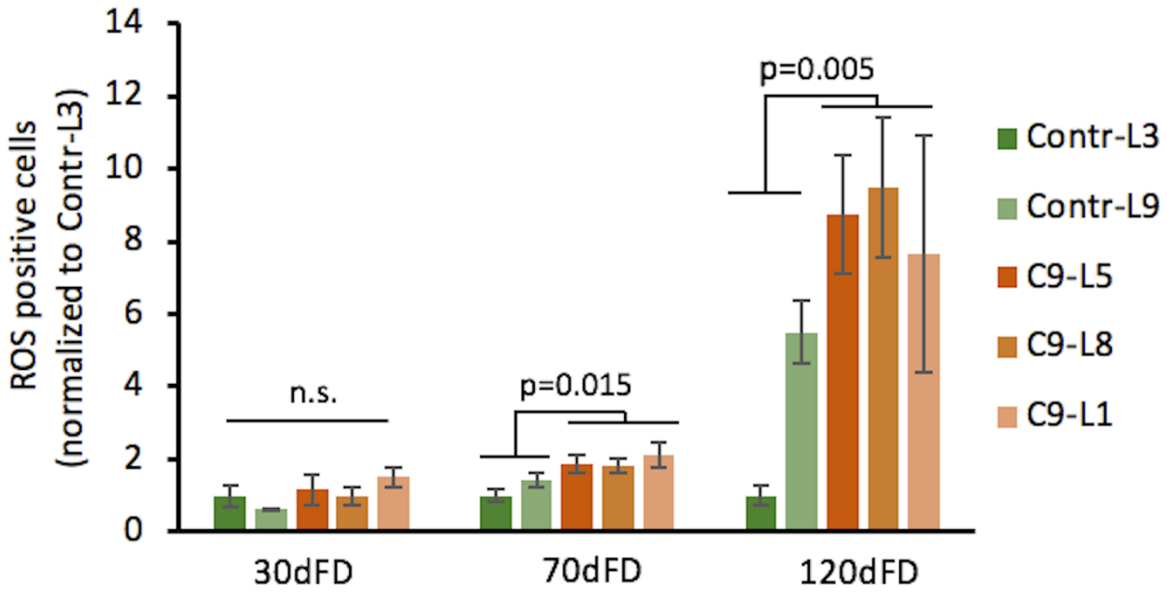
the first evaluation of MN viability 24 hours after the sorting. Data is represented as mean ± SEM of 3 independent experiments normalized to Contr-L3. b. MNs were immunostained for β3-tubulin after 2 weeks of treatment by C9-mutated (orange bars) and control (green bars) astrocyte conditioned media. 10 fields were imaged from each condition and β3-tubulin positive MNs and the total out of the total cells (DAPI positive) were quantified. Data is represented as mean ± SEM of 3 independent experiments for each line normalized to Contr-L3. c) Number of MN that stained positive for β3-tubulin (from b) presented per field.

Supplemental Fig5.



Sa-β-gal positive astrocytes from C9-mutated (C9-L5, C9-L8, orange box) and control (Contr-L3, Contr-L9, green box) cultures were quantified. 10 fields were imaged from each condition. Results are expressed as the percentage of Sa-β-gal positive cells from DAPI normalized to Contr-L3. Data is represented as mean ± SEM of 4 independent experiments normalized to Contr-L3 (independent differentiations of astrocytes for each experiment).

Supplemental Fig6.



a. The relative number of cells positive for ROS were determined by FACS analysis of C9-mutated (C9-L5, C9-L8, C9-L1) and control (Contr-L3, Contr-L9) astrocytes at different time points of FD (30, 80 and 120 dFD). Cells with increased ROS levels were detected by using the DCFDA Cellular Reactive Oxygen Species Detection Assay Kit. Only live cells were counted by gating for PI. Contr-L3 at dFD30 defined as 1. All other points are presented relative to it. Data is presented as mean ± SEM of 3 independent experiments for each time point.