# Supporting Information

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### SI Materials and Methods

**iPSC Lines.** For this study, four iPSC lines were used: two independent clonal lines derived from a 56-y-old male ALS patient with the M337V TDP-43 mutation (M337V-1 and -2), one age-matched male CTRL iPSC line (CTRL-1), and one age-matched female CTRL line (CTRL-2). The derivation, characterization, and validation of these iPSC lines were as described (1).

**Generation of Astrocytes from iPSC Lines.** Generation of neurospheres from iPSC lines and their patterning to acquire MN progenitor identity were as described (1). The neurospheres were mechanically chopped at the beginning of the enrichment phase and cultured in NSCR EL20 medium for 2–4 wk (depending on the size of the chopped spheres). After enrichment, the spheres were propagated in NSCR medium and passaged mechanically by chopping them every 2 wk. Monolayer cultures were generated by dissociating the spheres with the Papain Dissociation System (Worthington Biochemical) and plating them on plates coated with Matrigel (BD Biosciences; 1:80 dilution). The monolayer APC cultures were propagated in NSCR EF20 medium and passaged when confluent by using Accutase (Sigma) (split ratio 1:2–1:3). Astrocyte populations were obtained by differentiating the APCs for 14 d with AstroMED CNTF on Matrigel-coated plates or tissue culture flasks.

**Cell Culture Media.** NSCR EF20 medium contained AdDMEM/F12 with 1% N2 supplement and 0.1% B27 supplement, 1% nonessential amino acids, 1% penicillin/streptomycin, 1% GlutaMAX solution (all from Invitrogen), 20 ng/mL FGF-2 (PeproTech), and 20 ng/mL EGF (R&D Systems). NSCR EL20 medium contained AdDMEM/F12 with 1% N2 supplement and 0.1% B27 supplement, 1% nonessential amino acids, 1% penicillin/streptomycin, 1% GlutaMAX solution (all from Invitrogen), 20 ng/mL LIF (Sigma), and 20 ng/mL EGF (R&D Systems). AstroMED-CNTF was neurobasal with 0.2% B27 supplement, 1% nonessential amino acids, 1% penicillin/streptomycin, 1% GlutaMAX solution (all from Invitrogen), and 10 ng/mL CNTF (R&D Systems). NSCR neuron medium contained AdDMEM/F12 with 1% N2 supplement and 0.5% B27 supplement, 1% nonessential amino acids, 1% penicillin/streptomycin, 0.5% GlutaMAX solution (all from Invitrogen), and 0.1 μM retinoic acid (R&D Systems); 20 ng/mL BDNF (R&D Systems) and 20 ng/mL GDNF (R&D Systems) were added after the first week of differentiation. For survival analysis experiments AstroMED-CNTF has been used, whereas for survival analysis in absence of caspases activation, the same medium has been supplemented with 10 μM QVD-oph (Merck). In both cases, medium change was performed every 2 d.

**Cocultures.** Differentiated astrocytes were plated on Matrigel-coated 96-well plates at a confluence of $3 \times 10^4$ cells per well. MNs for cocultures were generated and transfected by using the methodology described (1) and then plated directly onto the astrocytes in each well. Murine primary astrocytes for xeno-cultures were prepared as described (2) and plated on 96-well plated at a density of $5 \times 10^4$ cells per well. The primary cultures were maintained for 3 d in 1:1 serum containing primary astrocyte medium and MNs maturation medium (1) before beginning the coculture.

**PCR and Quantitative PCR.** Differentiated astrocytes were detached with Accutase, and RNA was isolated by using the RNeasy kit (Qiagen), following the manufacturer's instructions. DNA contaminants were eliminated by using the Turbo DNA-Free kit (Invitrogen). cDNA synthesis was performed by using 0.5 μg of total RNA with the DyNAmo cDNA Synthesis Kit (Thermo Scientific) in a 20-μL volume. Real-time quantitative PCR (qPCR) reactions were performed in triplicate by using the DyNAmo Flash SYBR Green qPCR kit (Thermo Scientific) and examined on a CFX96 System (Bio-Rad). Relative expression levels were calculated by the 2DDCt method with b-actin as the reference gene. Primer sequences as follows:

- b-actin: Forward (5′-3′) GTTACAGGAAGTCCCTTGCCA TCC; reverse (5′-3′) CACCTCCCCTGTGTGGACTTGGG
- TDP-43: Forward(5′-3′) CGGCCTAGCGGGAAAAGTA-AAAGA; reverse(5′-3′) AGCACCGTCCCATCGTCTT
- HDAC6: Forward(5′-3′) TCGCTGCGTGTCCTTTCAG; reverse(5′-3′) CCCTCATTCATGTACTGGGTTG
- S100B: Forward(5′-3′) TGGCCCTCATCGACGTTTTC; reverse(5′-3′) CAGTGTTTCCATGACTTTGTCCA
- GFAP: Forward(5′-3′) ATCGAGAAGGTTCGCTTCCTG; reverse(5′-3′) TGTTGGCGGTGAGTTGATCG

**Glutamate Uptake Assay.** Differentiated astrocytes were plated on Matrigel-coated 96-well plates at a density of $2 \times 10^4$ cells per well and cultured in AstroMED-CNTF for an additional 7 d. For the glutamate uptake assay, astrocytes were cultured in HBSS (Invitrogen) with 100 μM L-Glu for 30, 60, or 120 min, and the concentrations of L-Glu in the medium were determined by using the Amplite Fluorimetric Glutamic Acid Assay Kit (ATT Bioquest) for each time on a FluoSTAR Optima plate Reader (BMG Labtech). L-Glu HBSS supplemented with 2 mM L-transpyrrolidine-2,4-dicarboxylic acid (Sigma) or 100 μM L-Glu HBSS without sodium (Biosera) were used as negative controls, and both were assayed at 120 min. We used CyQuant Cell Proliferation Assay (Invitrogen) to obtain an indirect estimate of number of cells per well: the kit was used as indicated by the manufacturer, and the readings for this assay were performed on FluoSTAR Optima plate Reader (BMG Labtech). The raw CyQuant data were processed by subtracting the background calculated on an empty plate with the same coating as the plates used in the glutamate uptake assay. The data were then normalized by the CyQuant value of CTRL-1 iPSC-derived astrocytes. The processed CyQuant data were used to normalize the glutamate uptake assay data. Data in Fig. 2*E* were analyzed with the one-way ANOVA and Bonferroni posttest ($^{***}P < 0.01$); data in Fig. 2*F* were analyzed with two-way ANOVA (no significant difference was found between times of the different groups). Results from the mutant lines (M337V-1 and -2) and from the CTRL lines (CTRL-1 and -2) were pooled together to be presented in Fig. 2 *A*–*C*.

**Synaptogenesis Assay.** For quantification of synapse formation, differentiating neurons derived from CTRL-2 iPSCs were cultured either in isolation or on a layer of iPSC-derived astrocytes. Neurons were generated from MN-patterned neurospheres treated as described (1). The cocultures were analyzed by immunostaining for PSY and PSD-95 after 3 wk. The average number of PSY+/PSD-95+ positive puncta per 30-μm axonal segment was calculated on five different fields for every condition, analyzing three biological replicates. The average number of puncta in isolated cultures of neurons was used as a normalizer. Results from the mutant lines (M337V-1 and -2) and CTRL lines (CTRL-1 and -2) were pooled to be presented in Fig. 2 *E* and *F* and analyzed by using one-way ANOVA with Bonferroni posttest ($^{***}P < 0.01$).

**Calcium Waves.** At 3 wk, CNTF-differentiated cultures of astrocytes were incubated in HBSS containing 5 μM Fluo-4AM dye (Invitrogen) for 1 h at 37 °C according to manufacturer instructions. Cultures were then washed three times in HBSS solution and left for an additional 30 min at 37 °C to allow complete de-esterification of the dye. At this point, 50 μM 2-aminoethoxydiphenyl borate (2-APB) (Calbiochem) was added to the negative control. For mechanical stimulation, a suspension of 200-μm-diameter glass beads in HBSS was applied to the cells during imaging. ATP-evoked calcium responses were obtained by local application of a 5 mM ATP solution (Sigma). Imaging was performed at 1 fps on a Zeiss Axiovision microscope with incubation chamber at 37 °C.

**Survival Analysis.** For survival analysis, differentiated astrocytes were plated on 96-well Matrigel-coated plates at a density of $2 \times 10^4$ cells per well and transfected with pGW1-mApple (Fig. 2*G*) or cotransfected with pGW1-EGFP and pGW1-TDP43:mApple (Fig. 2 *H* and *I*) with Lipofectamine 2000 (Invitrogen). For analysis of cocultures, dissociated MNs from patterned neurospheres were plated on 96-well Matrigel-coated astrocytes plates after transfection, as described (1). A robotic microscope system was used to perform the imaging (3, 4). After transfection, cells were imaged at 24-h intervals for 10 d, and survival was determined by using algorithms developed in MatLab and ImageJ. Cell death was determined by cell membrane rupture or loss of fluorescence. Kaplan–Meier and cumulative risk-of-death curves were plotted by using R and used to analyze the difference in survival between the various lines, and the statistical significance of differences between groups was determined with the log-rank test. Cox proportional hazards analysis, calculated in R, was used to determine the influence of the M337V iPSC background on the risk of death in astrocytes. The survival plots are presented with time in hours at the horizontal axis and the cumulative risk expressed as a logarithm at the vertical axis. The cumulative HR values are presented in nonlogarithmic form. For every experiment the baselines of comparison have a cumulative HR of 1.

**Immunofluorescence and Densitometry Analysis.** Cells were fixed with 2% (vol/vol) paraformaldehyde, permeabilized with 0.2% Triton X-100 (Sigma) at room temperature, and blocked in 3% (vol/vol) goat serum (DAKO), followed by incubation with primary and secondary (Alexa Fluor dyes; Invitrogen) antibodies. The nuclei were counterstained with DAPI (Sigma) after the secondary antibody incubation. Images were acquired by using an Observer (Zeiss) microscope and converted to a tiff format with Axiovision (Version 4.8.1; Zeiss). Densitometric analysis on TDP-43 immunostaining was performed by using ImageJ64 (Version 1.4.4.0): the DAPI channel was converted into a thresholded binary mask from which regions of interests (ROIs) were defined, and nuclear TDP-43 signals were measured in these ROIs. Cytoplasmic TDP-43 signals were measured by subtracting the binary mask from the TDP-43 channel, and the average signal intensity was normalized by the number of astrocytes on the field. For confocal image acquisition, we used a LSM 780 microscope (Zeiss) running ZEN (Version 1.4; Zeiss).

**Analysis of Relative Expression Levels of Transfected Astrocytes.** Images of astrocytes cotransfected with pGW1-EGFP and pGW1-TDP43:mApple were analyzed with ImageJ64 and original ImageJ plug-ins written in Jython. The EGFP images were used to determine a ROI around the astrocytes to be analyzed; the same ROIs were then used to calculate area, raw integrated density (RawInt) and background levels on both EGFP and TDP43:mApple images. Relative TDP43:mApple expression levels were calculated by normalizing background-subtracted RawInt of the red channel to that of the green channel. Arbitrary expression units (x) were derived by normalizing the relative expression level of each cell to the average value for each group (WT and M337V). These arbitrary units were used to stratify the transfected astrocytes into six groups with increasing relative expression (A, below 0.65×; B, between 0.66× and 0.81×; C, between 0.82× and 0.97×; D, between 0.98× and 1.14×; E, between 1.15× and 1.30×; F, above 1.30×). The astrocytes were scored by using the following criteria: cells with TDP43:mApple localized in the nucleus were assigned to the nuclear group; cells that presented TDP43:mApple in both nucleus and cytoplasm or cytoplasm alone were assigned to the cytoplasmatic group. Examples of the two phenotypes are presented in Fig. 2*H*.

**Biochemical Analysis and Semiquantitative Western Blotting.** Differentiated astrocytes were harvested by using Accutase and lysed in cold RIPA buffer (50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA). The detergent-soluble fraction was isolated by centrifuging lysates at 15,700 × *g* for 30 min at 4 °C and collecting the supernatant. The detergent-insoluble fraction was extracted by using an equal volume of urea buffer {7 M urea, 2 M thiourea, 4% (wt/vol) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 30 mM Tris (pH 8.5)}, after an additional RIPA buffer wash to minimize the carryover contamination from the soluble fraction. Samples were then sonicated three times for 10 s in a water bath, and centrifuged again at 100,000 × *g* for 30 min at 22 °C. Fresh protease inhibitors [complete Mini (Roche) and 1 mM PMSF] were added to all buffers. Soluble protein concentrations were determined by a BCA protein assay (Pierce). We resolved 15 μg of protein for each sample on a 12% SDS/PAGE and transferred them on PVDF membranes. For the insoluble fraction, we analyzed an equivalent sample volume. All PVDF membranes were blocked in Western Blocker Solution (Sigma) and incubated in primary antibody overnight at 4 °C. Proteins were detected with horseradish peroxidase-conjugated secondary antibodies, and blots were developed with Amersham ECL Plus and Hyperfilm (GE Healthcare). Semiquantitative densitometric analysis of Western blots was performed with ImageJ, by using β-actin as a loading control for the soluble fraction and Ponceau S staining for the detergent-resistant fraction.

**Antibodies.** Vimentin (Millipore), NFIA (Abcam), GFAP (DAKO), GFAP-cy3 (Mouse IgG conjugated with Cy3; Sigma), S100B (DAKO), EAAT1 (Novus Biologicals), Synapsin 1 (Millipore), TDP-43 (Proteintech), and PSD-95 (Cell Signaling) were obtained from the companies indicated.


1. Bilican B, et al. (2012) Mutant induced pluripotent stem cell lines recapitulate aspects of TDP-43 proteinopathies and reveal cell-specific vulnerability. *Proc Natl Acad Sci USA* 109(15):5803–5808.
2. Di Giorgio FP, Carrasco MA, Siao MC, Maniatis T, Eggan K (2007) Non-cell autonomous effect of glia on motor neurons in an embryonic stem cell-based ALS model. *Nat Neurosci* 10(5):608–614.
3. Barmada SJ, et al. (2010) Cytoplasmic mislocalization of TDP-43 is toxic to neurons and enhanced by a mutation associated with familial amyotrophic lateral sclerosis. *J Neurosci* 30(2):639–649.
4. Arrasate M, Finkbeiner S (2005) Automated microscope system for determining factors that predict neuronal fate. *Proc Natl Acad Sci USA* 102(10):3840–3845.

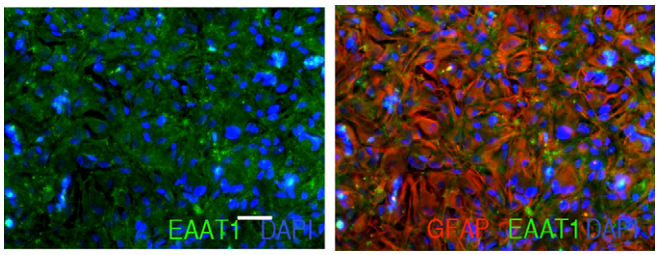



**Fig. S1.** Representative field of immunolabeling for glutamate transporter EAAT-1 and GFAP in differentiated iPSC-derived cultures of astrocytes (images represent the same field). (Scale bar: 100 μm.)

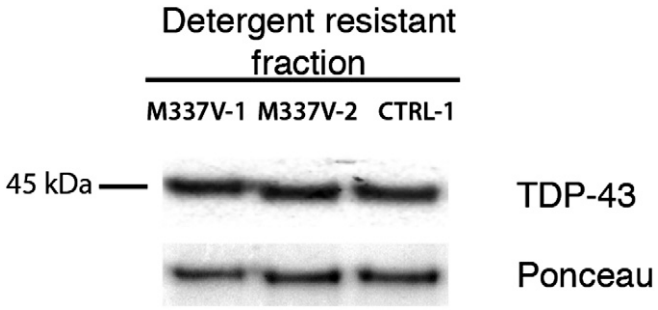



**Fig. S2.** Representative Western blot of detergent-resistant TDP-43 from M337V-1, M337V-2, and CTRL-1 astrocytes.

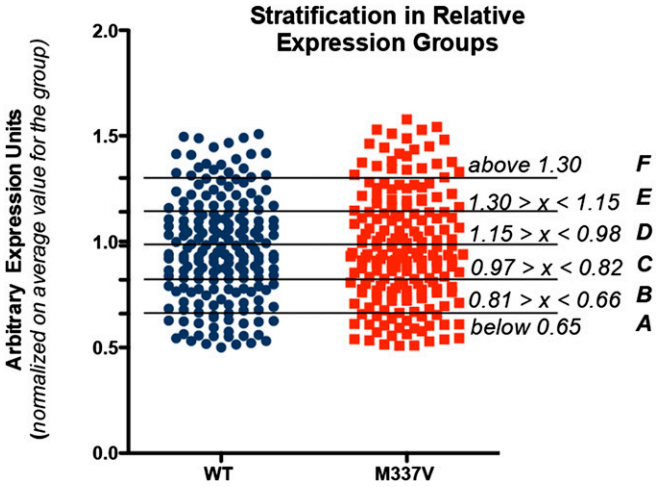



**Fig. S3.** Stratification of TDP-43:mApple-expressing astrocytes into six groups based on their relative expression levels; groups A–F contain astrocytes with progressively increasing exogenous TDP-43 expression levels.

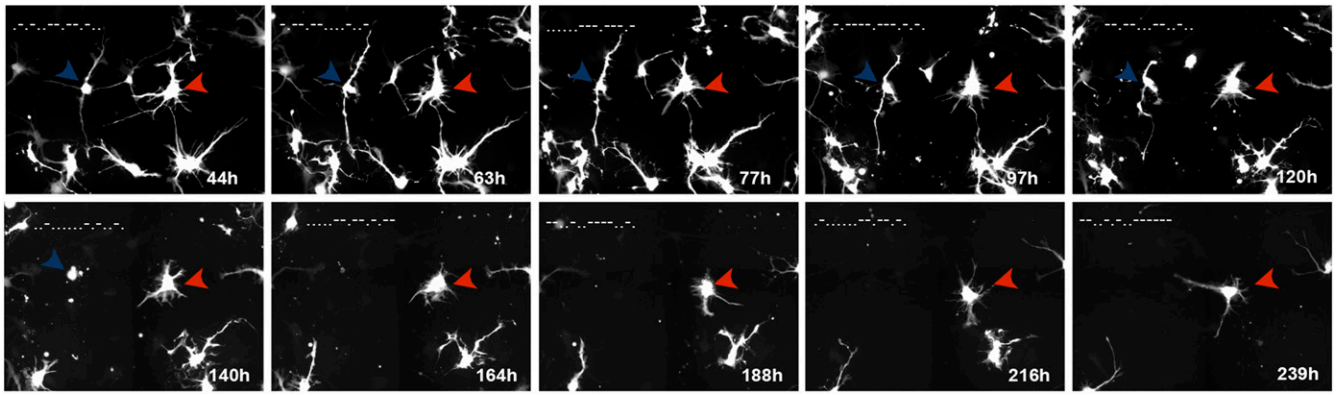



**Fig. S4.** Representative images from a typical survival analysis of EGFP-expressing astrocytes; cell fate was followed over 10 d, and cell death was determined by loss of fluorescence or dissolution of the cell itself [note as one of the astrocytes (red arrowhead) survives for the whole experiment, and in the same field, another cell (blue arrowhead) displays morphologic changes at 97 h and disappears by 140 h].

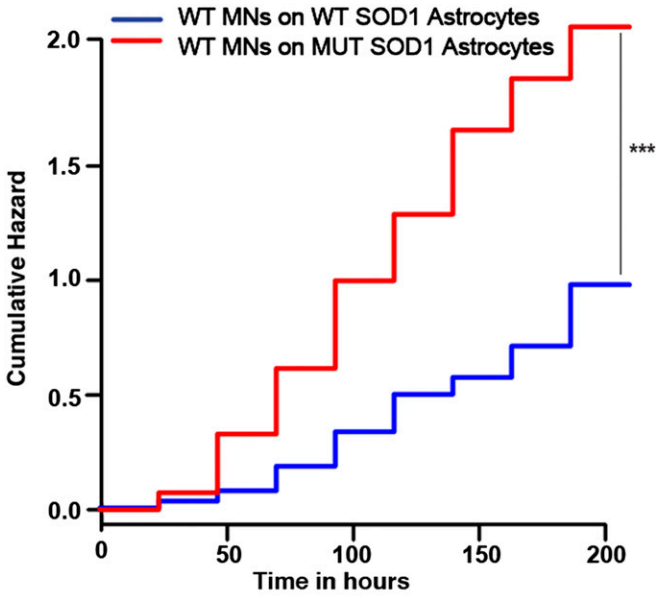
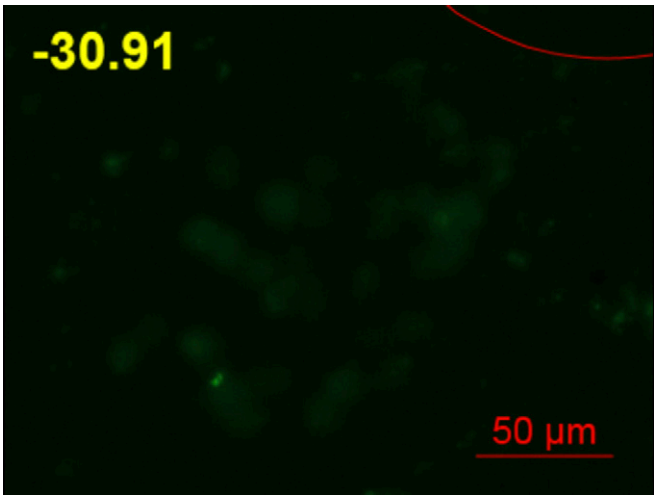
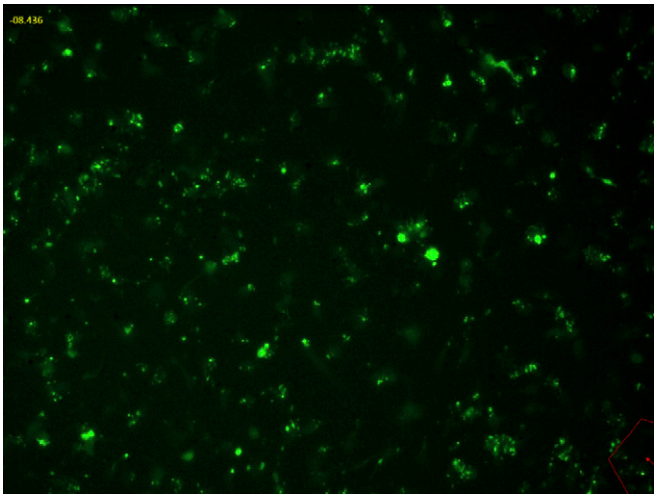



**Fig. S5.** Real-time survival analysis of WT MNs cultured on a layer of murine primary astrocytes overexpressing either WT human SOD1 or mutant human SOD1 ($n = 3$), demonstrating non-cell-autonomous toxicity of mutant SOD1 astrocytes (with WT SOD1 astrocytes coculture as a reference, in MUT SOD1 coculture, HR = 2.46; $P = 5.05 \times 10^{-10}$; $^{***}P < 0.001$). For WT SOD1 coculture, 140 cells were analyzed from three independent replicates; for MUT SOD1 coculture, 129 cells were analyzed from three independent replicates.



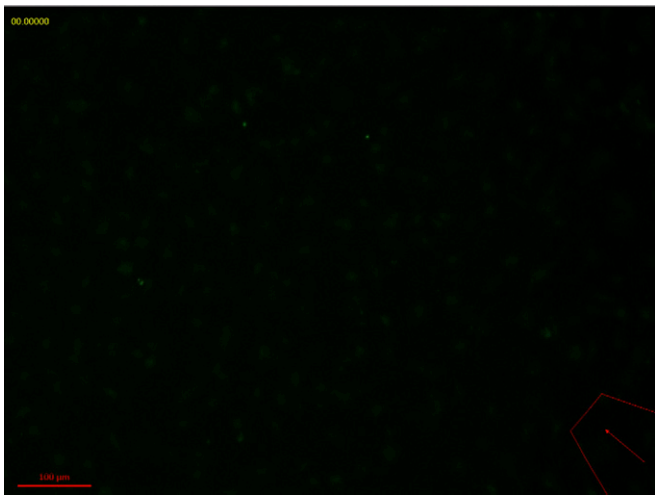

**Movie S1.** iPSC-derived astrocytes respond to local mechanical stimulation as measured by Fluo-4AM fluorescent signal intensity. Mechanical stimulation was accomplished by dropping 200-µm glass beads onto the monolayer of astrocytes. Red outlines depict a schematic of the glass bead dropped at the edge of the illuminated field of analysis. Annotation represents time in seconds to/from stimulation.

Movie S1



**Movie S2.** iPSC-derived astrocytes respond to local application of ATP by increasing intracellular calcium, as measured by Fluo-4AM fluorescent signal intensity. Red outlines depict a schematic representation of the pipette used for local application of ATP at the edge of the illuminated field of analysis. Annotation represents time in seconds to/from stimulation.

Movie S2




**Movie S3.** iPSC-derived astrocytes in the presence of 2-APB do not respond to local application of ATP, as measured by Fluo-AM fluorescent signal intensity. Red outlines depict a schematic representation of the pipette used for local application of ATP solution at the edge of the illuminated field of analysis. Annotation represents time in seconds to/from stimulation.

Movie S3