Markus Brüll et al.:

Incorporation of Stem Cell-Derived Astrocytes into Neuronal Organoids to Allow Neuro-Glial Interactions in Toxicological Studies

Supplementary Data

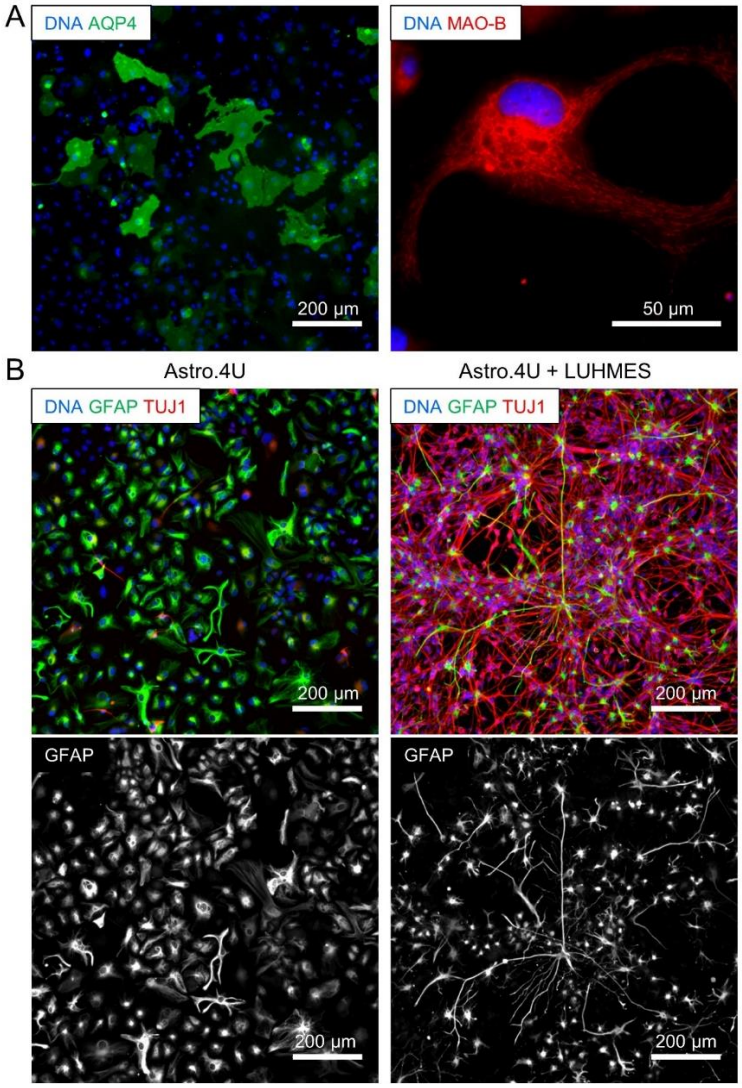

Fig. S1: Characterization of Astro.4U
(A) Cells were thawed and seeded at a density of 25,000 cells/cm^2 and cultivated in Astro.4U medium. After 7 days, cells were fixed and stained with antibodies against the astrocyte markers aquaporin-4 (AQP4) and the monoamine oxidase-B (MAO-B). (B) Astro.4U were cultivated either in mono-culture or in co-culture with 145,000 LUHMES/cm^2 and immunostaining was performed with antibodies against the astrocyte marker glial fibrillary acidic protein (GFAP) and the neuronal marker TUJ1. The lower images show the GFAP channel of the upper images.

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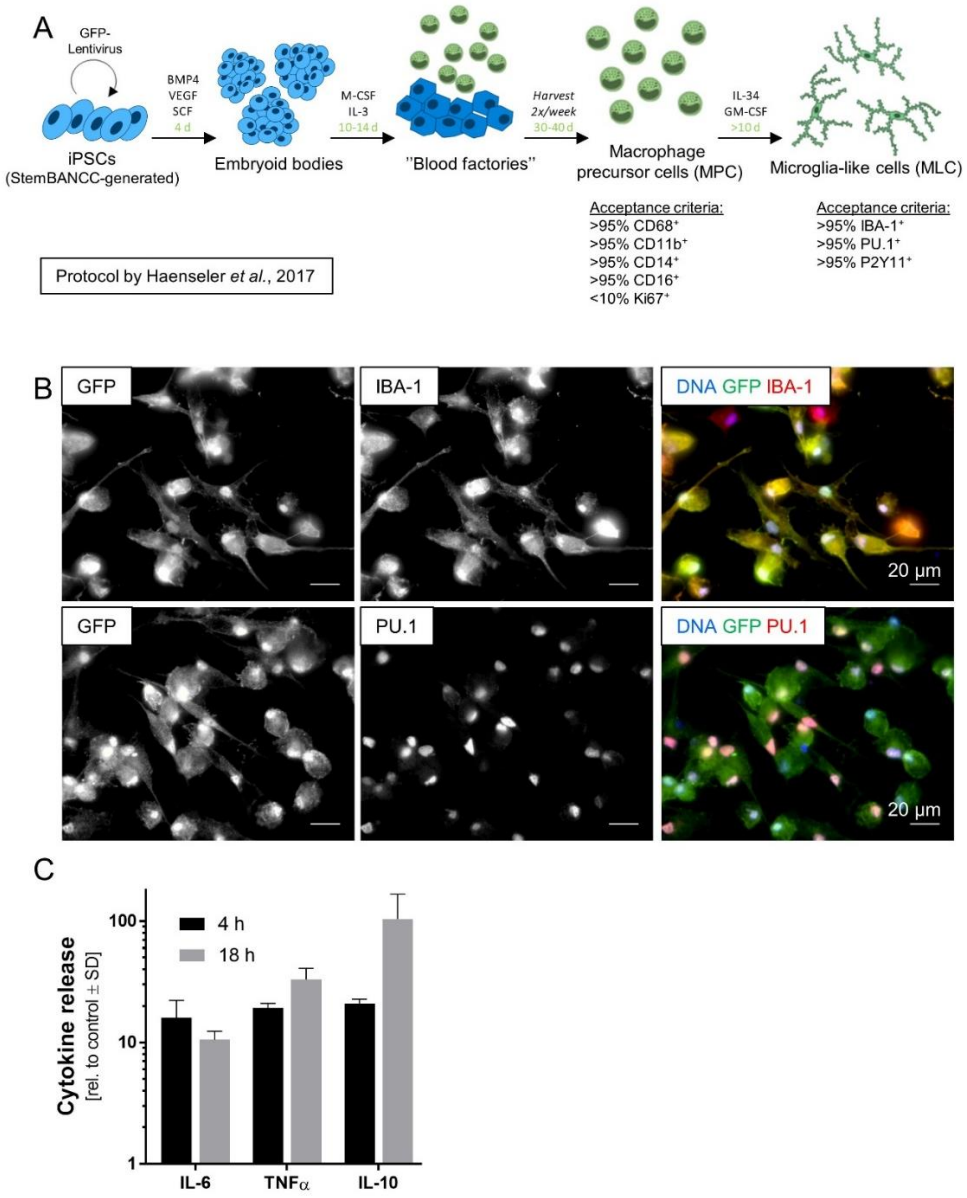

Fig. S2: Characterization of microglia-like cells

(A) Schematic differentiation of microglia-like cells from iPSC (line STBCi026-A distributed by EBiSC and ECACC). BMP4: bone morphogenic protein, VEGF: vascular endothelial growth factor, SCF: stem cell factor, M-CSF: macrophage colony-stimulating factor, IL-3: interleukin-3, GM-CSF: granulocyte-macrophage colony-stimulating factor (B) Immunocytochemistry staining of microglia-like cells after 12 days of differentiation from MPC. Cells were seeded at a density of 120,000 cells/cm^2 on PLO/laminin/fibronectin coated cover glasses (Thermo Scientific) and cultivated for three days prior to fixation with 4% para-formaldehyde (Leica Microsystems, Germany) for 30 min at room temperature (RT). After one wash with phosphate-buffered saline (PBS), cells were permeabilized with 0.6% Triton-X 100 in PBS for 10 min and blocked with blocking buffer (5% FBS (Gibco, USA), 0.1% Triton X-100 in PBS) for 30 min. Blocking solution was removed and cells were incubated with the respective antibodies diluted 1:200 in blocking solution at 4°C overnight. After washing three times with PBS, cells were incubated with the corresponding secondary antibodies (1:1000 dilution) and the DNA intercalating dye H-33342 (ThermoFisher, USA) (6.15 µM) in blocking buffer for 1 h at RT. Samples were washed three times with PBS and mounted onto SUPERFROST® microscope slides (Thermo Scientific) with Aqua-Poly/Mount (Polysciences). (C) Release of cytokines by microglia-like cells after stimulation with LPS. MLC were differentiated from MPC for 14 days at a seeding density of 150,000 cells/cm^2 and incubated with LPS (100 ng/ml). Cytokine release was measured in the supernatants after 4 and 18 h with the FirePlex assay using the human cytokines “FirePlex-HT panel 1” kit according to the manufacturer’s instructions. Plates were imaged using an Operetta CLS high-content screening system. Quantification was performed with the assay software (FireplexHT) by analyzing the barcode fluorescence and calculating the total concentration of each cytokine. LPS-dependent release was obtained by normalization to non-treated controls. Data are means ±SD, n = 3.

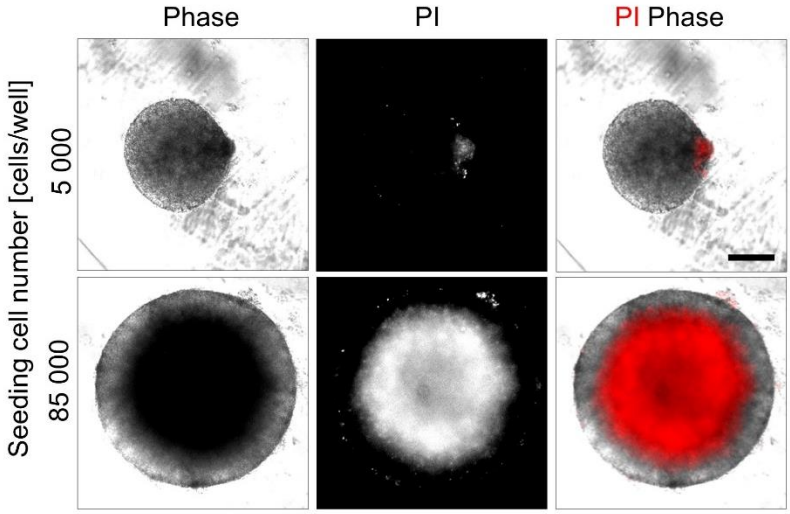

Fig. S3: Necrotic core assessment of LUHMES organoids
LUHMES cells were seeded into ULA round bottom plates on day 2 in different cell densities for the generation of organoids. On day 8, organoids were stained with propidium iodide (PI) for 1 h to visualize dead cells. Organoids were then imaged by fluorescence and phase contrast (phase) microscopy. Scale bar = 200 µm. The upper row shows the type and size of organoids normally used in this study. Upon evaluation (n =10), they never showed a necrotic core. Occasionally, peripheral structures on one side showed PI staining as handling artifact (exemplified here as control that PI was able to detect dead cells). The lower row shows large 3D structures used as positive controls for necrotic core detection. 10 of 10 such large organoids (ca. 0.8 mm diameter) showed necrotic cores.

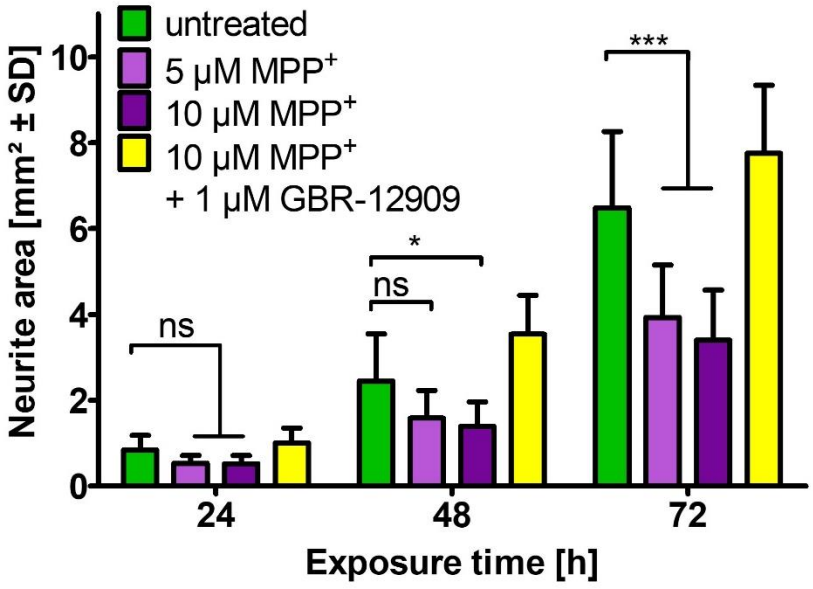

Fig. S4: Time course of neurite outgrowth of LUHMES organoids exposed to MPP^+
LUHMES organoids were generated from RFP-expressing LUHMES to allow measurement of the neurite area of the same organoids. Organoids were exposed to MPP^+ for 72 h during neurite outgrowth with or without the DAT-inhibitor GBR-12909. Neurite area was evaluated every 24 h by fluorescence microscopy and subsequent image analysis. Statistical significance was evaluated by a two-way ANOVA with Bonferroni's post hoc test. n = 10. ns = not significant, * $p < 0.05$, *** $p < 0.001$.

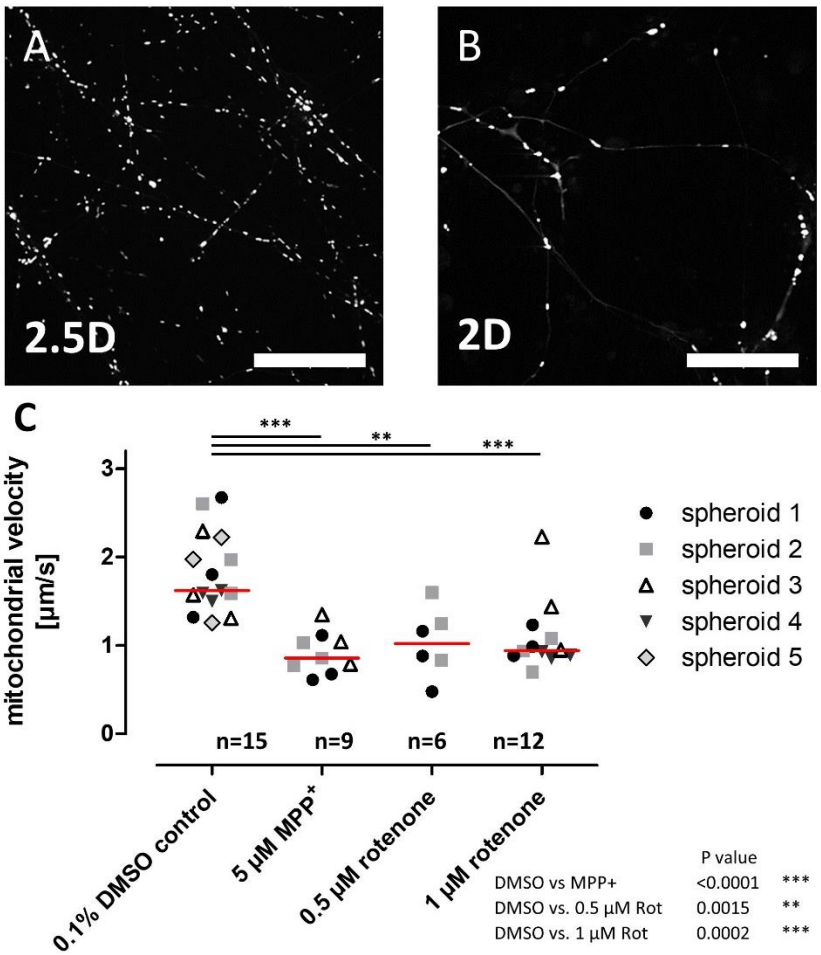

Fig S5: 3D spheres from a mixed LUHMES cell culture to visualize mitochondrial mobility
LUHMES cells were mixed with a LUHMES subclone (expressing mitochondrially targeted EGFP (Schildknecht et al., 2013) at a 10:1 ratio on d0 of differentiation. To induce sphere formation, 5000 cells were seeded on d2 to ultra-low attachment plates (ULA) and cultured until d8 of differentiation. Then, spheres were replated to Matrigel-coated LUMOX® plates, where they extended their neurites. Compound treatment was for 24 h from d10-d11, then mitochondrial mobility was assessed by confocal time-lapse microscopy. (A) The imaging of neurites grown from 3D spheres facilitated assessment of a substantially higher number of mitochondria per image than using 2D cell culture of the same composition (B). While cell bodies are automatically omitted when imaging neurites grown from spheroids, their presence on images needs to be actively avoided in 2D imaging by selection of suitable imaging areas. This is necessary, as presence of somatic mitochondria interferes with the image quantification algorithm. Scale bars indicate 50 µm. (C) MPP^+ and rotenone decrease mitochondrial mobility. The compounds were non-cytotoxic under the experimental concentration chosen, as assessed in an image-based viability assay before (UKN3b; 24 h compound exposure on mature LUHMES cells d5-d6; protocol No. 196 at the DB-ALM database accessible under https://jeodpp.jrc.ec.europa.eu/ftp/jrc-opendata/EURL-ECVAM/datasets/DBALM/LATEST/online/dbalm.html). For quantification, three time-lapse image sequences spanning 90 sec were taken per spheroid. Up to 5 spheroids per treatment condition were assessed. Mitochondrial movement was quantified using the Feature Calculator, Global Thresholder, Connected Component Analysis and Trackmate Tracker nodes of the freely available KNIME software (www.knime.com). The mean velocity of all mitochondria in an image was plotted. Data was analyzed by unpaired, two-tailed t-test using GraphPad Prism software.

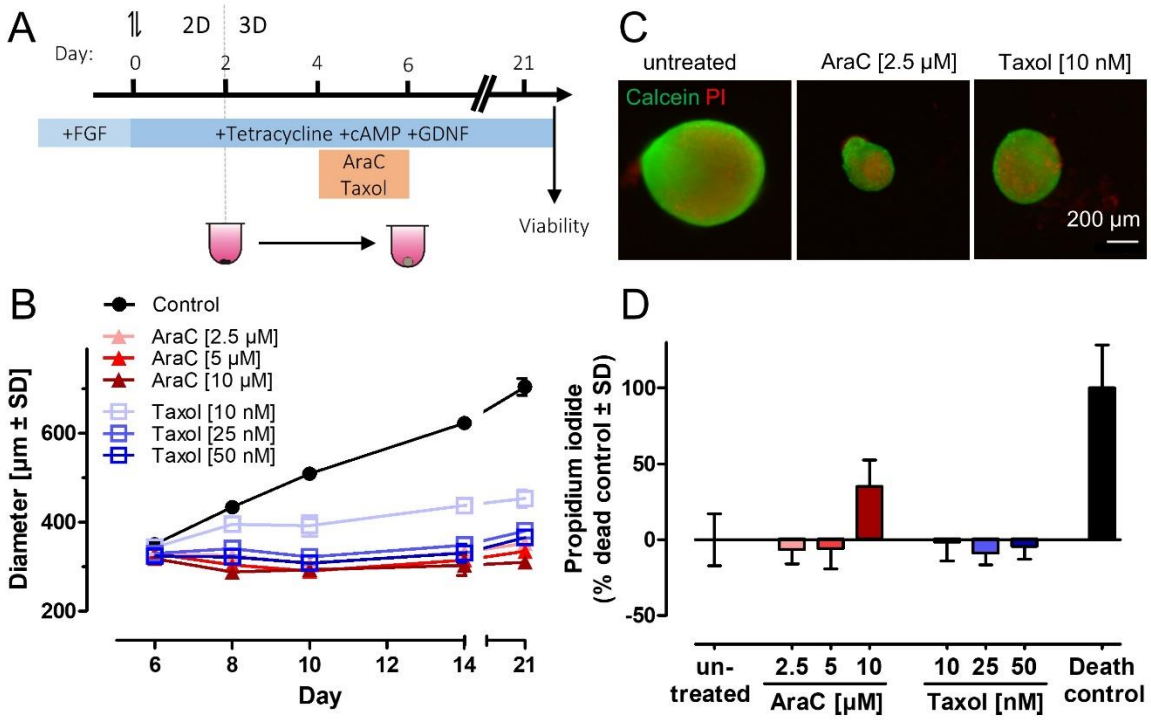

Fig. S6: Prevention of LUHMES mono-culture organoid proliferation by 48 h (day 4 - day 6) application of cytarabine (AraC) or taxol

(A) 5000 LUHMES cells were seeded per well on day 2 into ULA round bottom plates. Organoids were treated for 48 h (day 4 - day 6) with proliferation inhibitors. Antimitotics were washed out and size was measured on day 6, 8, 10, 14, and 21. (B) Size of the organoids was measured by bright field microscopy in ULA plates and subsequent image analysis. n = 6. (C) Representative fluorescence images of calcein/PI live-stained spheres on day 21. Scale bar = 200 µm. (D) Viability of pulsed organoids on day 21 was measured by fluorescence microscopy. For this, the mean grey value of PI was evaluated in ImageJ relative to a death control. n = 6.

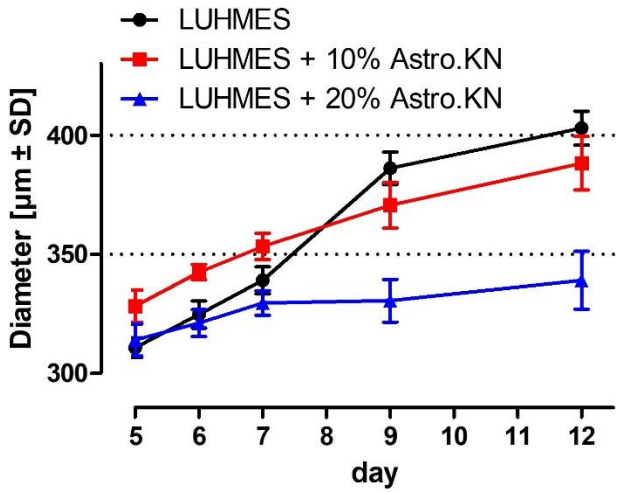

Fig S7: Prevention of LUHMES organoid growth by co-culture with Astro.KN

LUHMES mono-culture organoids and co-culture organoids with 10% or 20% Astro.KN were generated on d2 (5000 cells in total) in ULA round bottom plates. Size was measured by bright field microscopy and subsequent image analysis. n = 6.

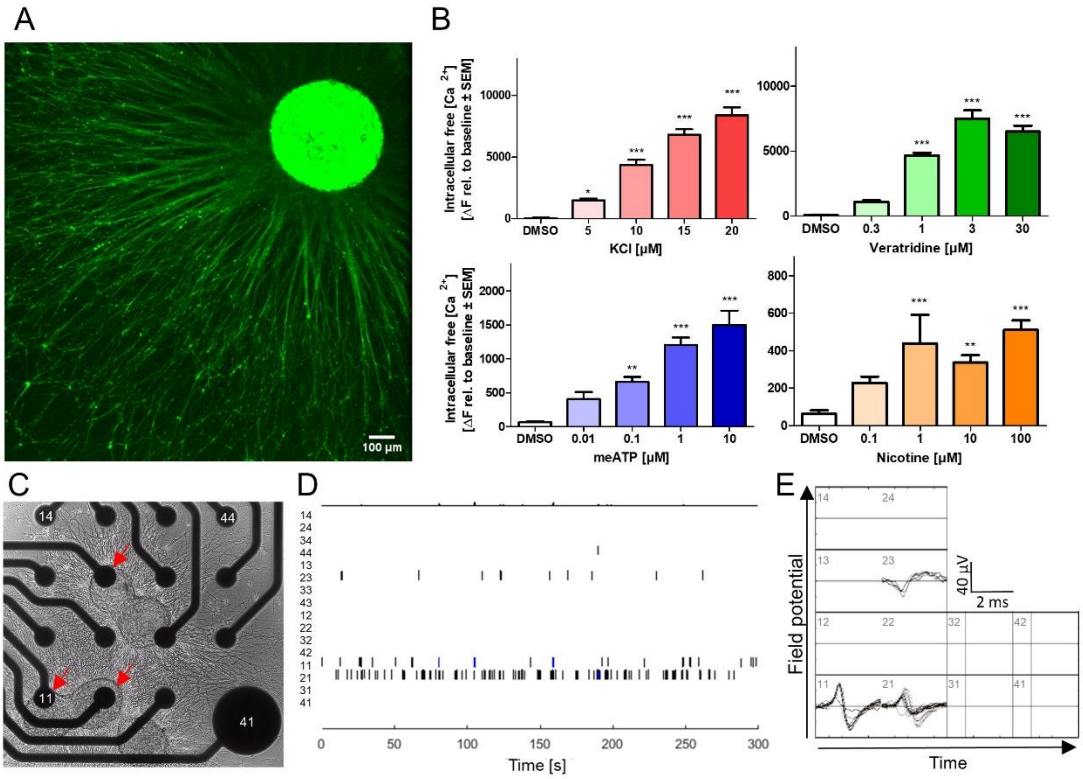

Fig S8: Electrophysiological properties of LUHMES organoids
(A) LUHMES organoids were shipped in sealed ultra-low attachment round-bottom 96-well plates (Corning 7007, USA) by overnight mail. Upon arrival, half of the medium was exchanged and the plates were kept in the incubator at 37°C with 5% CO_2 overnight. 384-well plates (Greiner Bio-One, Germany) were coated with 25 µl/well ice-cold 1% Matrigel in LUHMES differentiation medium and incubated at 37°C for 30 min. Single organoids were plated in 25 µl/well onto the Matrigel coating solution and stored in an incubator at 37°C with 5% CO_2. Fluorescence microscopy image of a Cal-520 (fluorescent calcium indicator) stained LUHMES organoid plated for three days. The staining showed that viability and capacity of organoids to grow a large neurite network was not affected by shipping. (B) Calcium response of plated LUHMES organoids after treatment with KCl, veratridine, α,β-meATP and nicotine. The Ca^{2+}-imaging was performed 72 h post-plating with the HT Functional Drug Screening System FDSS/µCELL (Hamamatsu Photonics, Japan) using the Ca^{2+}-sensitive dye Cal-520 AM (AAT Bioquest, USA) at 37°C. Recordings were performed with a sampling rate of 24 Hz. The compounds were prepared fresh daily in medium and applied as a 1:10 concentration in the wells of the 384-well plates. Compound application was executed after a 1.5 min baseline recording. Data was analyzed using FDSS software (Version 3.2, Hamamatsu Photonics, JP) and scripts written in R (Version 3.6.1, R Core Team (2019)). Calcium response is displayed as the maximum increase in fluorescence relative to the baseline recording (ΔF). Statistical significance was determined by one-way ANOVA with Dunnett's post hoc test vs DMSO control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data are means from 5 organoids. (C) Co-culture organoids (d8) were plated on Matrigel-coated 24-well micro-electrode array (MEA) plates (Axion BioSystems, USA, 16 electrodes/well). Active electrodes were identified as those generating spikes consistently (> 5 spikes/min). Phase contrast observations showed that only electrodes that were covered by the soma aggregation of an organoid were active. Active electrodes are marked with red arrows. (D) Spike raster plot of a 5 min MEA recording (Maestro Edge, Axion BioSystems, USA). Spontaneous activity was measured 24 h after plating with a sampling rate of 12.5 kHz. Spikes were defined as changes in voltage > 5.5 times the SD of the baseline. Bursts were defined as a group of > 5 spikes with an inter-spike interval > 100 ms. Bursts are shown in blue; spikes are shown in black. (E) Waveforms of the active electrodes during the recording generated by the Axis Navigator software (Axion BioSystems, USA). These preliminary data show spontaneous activity of organoids. One experiment representative of three is shown.

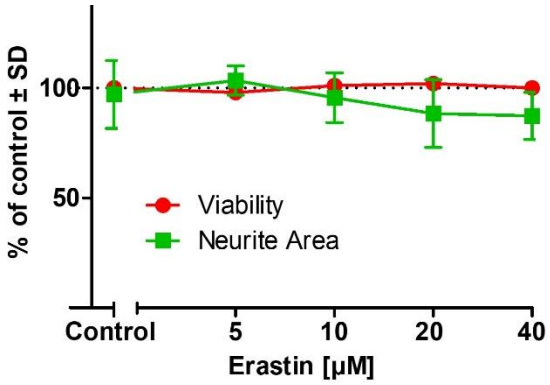

Fig. S9: Protection of astrocytes against erastin mediated neurodegeneration up to 40 µM
Co-culture organoids with 20% Astro.KN were generated on d2 in ULA round bottom plates. On day 8, they were transferred to Matrigel. After 48 h of neurite outgrowth, they were treated with erastin for 24 h. Neurite area was then evaluated by calcein staining; viability was evaluated by PI staining. N = 2, n = 3.

Tab S1: List of all antibodies used in this study

Antibody	Isotype	Dilution	Supplier	Reference Nr.
anti-DAT	rat	1:1000	millipore	MAB369
anti-NF200	mouse IgG1	1:1000	Sigma	N0142
anti-Synaptophysin	mouse IgG1	1:1000	Synaptic Systems	101011
anti-PSD95	rabbit	1:1000	Invitrogen	51-6900
anti-TUJ1	mouse IgG2a	1:1000	BioLegend	801202
anti-GFAP	chicken	1:2000	millipore	AB5541
anti-GFP	rabbit	1:500	Invitrogen	A6455
anti-Mao-B	rabbit	1:200	Sigma	M1946
anti-AQP4	rabbit	1:200	Santa Cruz	sc-20812
anti-IBA1	goat	1:200	abcam	AB5076
anti-PU.1	rabbit	1:200	Thermo Fisher	A13971
anti-chicken Alexa488	goat	1:1000	Invitrogen	A11039
anti-mouse IgG1 Alexa488	goat	1:1000	Invitrogen	A11021
anti-mouse IgG1 Alexa555	goat	1:1000	Invitrogen	A21121
anti-mouse IgG2a Alexa488	goat	1:1000	Invitrogen	A21131
anti-rat Alexa555	goat	1:1000	Invitrogen	A21434
anti-rabbit Alexa488	chicken	1:1000	Invitrogen	A21441

References

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