Supplemental Materials and Methods

Cell culture protocols

Induced pluripotent stem cells were reprogrammed from fibroblasts derived from skin biopsies under Johns Hopkins IRB protocols NA_00021979 and NA_00033726. Consents included the authorization to use DNA, fibroblasts, or cell lines derived from fibroblasts for research purposes only. Fibroblasts were cultured and induced pluripotent stem cell lines were created and initially characterized by one of the authors (G. Lee) and with an NIH-sponsored commercial agreement with iPierian (USA), who used a 4-vector method as we have previously described[1]**.**

Induced pluripotent stem cells were maintained in E8™ medium with Essential 8™ supplement (Gibco-Thermo Fisher Scientific) and passaged once every 5-6 days using Dispase (StemCell Technology). To maintain pluripotency and limit spontaneous differentiation, the stem cell colonies were manually cleaned before passaging.

Induced pluripotent stem cells were differentiated into spinal cord neural progenitor cells (NPCs) following modifications of a 25-30 day protocol described previously[2,3] that relies on extrinsic morphogenetic instructions to pattern NPCs along the rostro-caudal and dorso-ventral axis, and includes: neuralization, through dual SMAD signaling inhibition, using LDN193189 (Stemgent) and SB431542 (Millipore Sigma), followed by caudalization and ventralization, through retinoic acid (RA) and purmorphamine (PMN) (Millipore Sigma) **(Figure 1A).**

For motor neuron differentiation, NPCs were cultured with "neuronal differentiating medium", comprised of Neurobasal (Gibco-Thermo Fisher Scientific), enriched with N2 and B27 supplement (Gibco-Thermo Fisher Scientific), L-glutamine, non-essential amino acids (Gibco-Thermo Fisher Scientific), penicillin/streptomycin and supplemented with growth factors

(RA, PMN, ascorbic acid, recombinant human-brain-derived neurotrophic factor, glial cell line-derived neurotrophic, insulin-like growth factor 1, ciliary neurotrophic factor). The addition of Compound E (Santa Cruz Biotechnology), a γ-secretase inhibitor, enhanced neuronal differentiation into motor neurons. To prevent astrocyte over-proliferation, neuronal cultures were treated once with 0.02μM cytosine arabinoside (ara-C) (Millipore Sigma) for 48 h. The medium was then changed every other day. At 60 days in vitro (DIV), this protocol has been shown[3] to generate a population of spinal cord neurons, with a majority of neurons expressing MN markers, including choline acetyltransferase (ChAT) **(Figure 1A")** and ISL1/2.

For the generation of spinal cord hiPSC-A **(Figure 1A),** NPCs were cultured with "astrocyte differentiating medium", containing DMEM:F12 (Gibco-Thermo Fisher Scientific), enriched with B27 supplement (Gibco-Thermo Fisher Scientific), L-glutamine, non-essential amino acids, penicillin/streptomycin, heparin (Millipore sigma), and supplemented with 1% fetal bovine serum (FBS) (Gibco-Thermo Fisher Scientific). The medium was changed every other day, and cultures were passaged when confluent. After 90 DIV, this protocol generates a population of mature astrocytes[2,4] which express HOXB4, a marker of spinal cord regional identity[2] (**Figure 1A'**).

For MEA culture conditions (either mono- or co-cultures), the culture medium was changed at day 1 of MEA plating to "neuronal" medium enriched with 5% FBS, 0.5 μg/ml laminin and 2.0 μg/ml Amphotericin B (Gibco). Cells were fed with a half medium exchange every 3 days.

The isolation and culture of spinal cord astrocytes from postnatal mouse spinal cord was performed as previously published[5]. Briefly, the spinal cord was dissected from post-natal day 2 (P2) mouse pups, and the meninges removed to avoid the subsequent contamination of the

astrocyte culture with fibroblasts. The tissue was then enzymatically dissociated with papaine (Worthington Biochemical) and deoxyribonuclease I (Sigma), and mechanically triturated to generate a single cell suspension. Cells were plated on poly-L-lysine-coated T25 flasks, in a medium containing DMEM with 10% FBS and 1% penicillin–streptomycin. Cells were allowed to recover and proliferate for 2 weeks prior to plating.

Multi-electrode array protocols

The glass surfaces of the MEA plates represent a major obstacle to cell adhesion. In order to increase their hydrophilicity, MEA plates were first treated with 1% Terg-a-zyme (Sigma Aldrich) overnight at room temperature, and then with O_2 plasma (Harrick Plasma) for 1 minute. The plates were then coated with poly-ornithine (Millipore Sigma; concentration: 100µg/mL) and laminin (Thermo Fisher Scientific; concentration: 5µg/ml).

Voltage measurements were made at a sampling rate of 25 kHz/channel using MC_RACK software (MCS) and filtered using a second order butterworth filter with a 200Hz cutoff frequency. Spikes were identified as instantaneous time points of voltages that exceed a threshold of at least five standard deviations below baseline. In addition to filtering and to setting a threshold for spike detection, all traces were visually inspected for quality control, as recently described by one of the authors of the present study [6]. We did not include in our analysis electrodes recording artifactual activity. Bursts were defined as activity with >4 spikes/0.1 sec. Hypersynchronous (or "network") bursts were defined when over 60% of active electrodes fired within one 20-ms bin, as previously described [7]. Spike trains were exported into an HDF5 format and further analyzed using MEAnalyzer[6,8].

Immunocytochemistry

In parallel experiments with MEA and using the same cell densities, neurons and astrocytes were plated in 24 well plates on glass cover-slips for immunocytochemistry. Two time points were considered: 1 week and 4 weeks of mono-culture or simultaneous co-culture.

Cells were fixed with 4% paraformaldehyde for 10 minutes and then washed with phosphate-buffered saline (PBS) three times. The cells were then permeabilized with 0.1% Triton™ X-100 (Millipore Sigma) in PBS for 10 minutes and washed with PBS three times. A blocking solution with 3% bovine serum albumin (BSA) in PBS was then applied for 1 hour. Coverslips were stained with primary antibodies in a blocking solution containing 3% BSA in PBS and 3% species specific serum and incubated overnight at 4ºC. The next day, cells were washed with 3% BSA in PBS three times and incubated with appropriate secondary antibodies (Thermo Fisher Scientific, Alexa Fluor Dyes; concentration: 1:1000) and Hoechst® (Thermo Fisher Scientific; concentration: 2 μg/ml) in a blocking solution with 3% BSA in PBS and 3% species-specific serum, for 1 h at room temperature. Finally, the coverslips were washed with 3% BSA in PBS three times and mounted with Prolong gold with DAPI® (Thermo Fisher Scientific) and stored until ready to image. The primary antibodies used for this study are listed in **Supplemental Table 1**.

Images were acquired on a Zeiss fluorescence microscope (Zeiss ApoTome.2), using 20x and 63x oil magnification and analyzed using Image J software (NIH) and Fiji package of Image J. Five images were obtained for each coverslip, and 3 coverslips were utilized for each condition. Cell counts were performed by a person who was blinded to the experimental conditions.

Quantitative analysis of neurites and synapses.

For the morphological analysis of neurons, we used TUJ1 immunostaining and 63x oil magnification images. We manually traced the diameter of individual neurites (i.e. projections from the cell body), and for each neuron, we defined the primary neurite as the largest neurite. The area of each neuronal soma was traced manually. For these analyses, 50 randomly selected neurons were considered for each condition.

To define the complexity of neuronal connections, we first tracked individual neurites on 63x TUJ1 images using the simple neurite tracer software plugin on the Fiji package of image J[9] and then performed a Sholl analysis[10,11] on the neurite mask, with soma-centered concentric circles of increasing radius (10 μm increment); for analysis purposes, we considered the mean number of intersection for each individual neuron.

Finally, we quantified synapses as the number of co-localized punctae of synaptophysin and PSD-95 antibody staining on 63x oil images, as previously described[12]. This immunocytochemistry-based protocol determines the mean number of co-localized punctae within a defined region of interest (ROI) surrounding neuronal soma. We used circular regions, one-cell diameter radially around the soma of interest, which was identified with TUJ1 staining. For Sholl analysis and for the quantification of synapses, we considered a minimum of n=10 neurons/per coverslip, randomly selected, and 3 coverslips for each condition.

qPCR of neuronal and astrocyte RNA transcripts

Human-specific primers for neuronal and glial target genes (**Supplemental Table 2**) were designed, and the primer sequences were confirmed by BLAST analysis and tested for

specificity. Universal eukaryotic 18S rRNA primers were used as endogenous control, allowing for comparisons between human-human and human-mouse co-cultures, as previously published [13,14].

Human iPSC-A, immature hiPSC-A, mouse primary spinal cord astrocytes and hiPSC-MN were grown as monolayers in 6 well plates (3 wells per condition), in the same conditions, densities and time points used for immunostaining and in parallel experiments. After 1 and 4 weeks *in vitro*, cell cultures were lysed and homogenized using TRIzol™ reagent (Invitrogen). Human iPSC-A and hiPSC-MN from mono-cultures were collected together and in the same volume of TRIzol (i.e. 0.3 ml/well) used for simultaneous co-cultures. With this strategy, and since the number of $TUJ1^+/DAPI^+$ and $TUJ1^-/DAPI^+$ cells was not significantly different between monocultures and co-cultures (as showed in the results section), we anticipated comparable loading controls from the initial samples.

Total RNA was then extracted using RNeasy Mini Kit (Qiagen, 74104). The quality and quantity of purified RNA was examined using a NanoDrop spectrophotometer (Thermo Fisher Scientific). The RNA was converted into cDNA using the iScriptTM cDNA synthesis kit (Bio-Rad) following manufacturer's instructions. The cDNA was then amplified using the Fast SYBR green PCR master mix (Applied Biosystems) in technical duplicates for each individual sample. The expression of target genes was normalized to 18s levels using the comparative CT method [15].

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