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Supplemental Information

Neuron-Glia Interactions Increase Neuronal Phenotypes in Tuberous Sclerosis Complex Patient iPSC-Derived Models

Aishwarya G. Nadadhur, Mouhamed Alsaqati, Lisa Gasparotto, Paulien Cornelissen-Steijger, Eline van Hugte, Stephanie Dooves, Adrian J. Harwood, and Vivi M. Heine

Supplemental figures

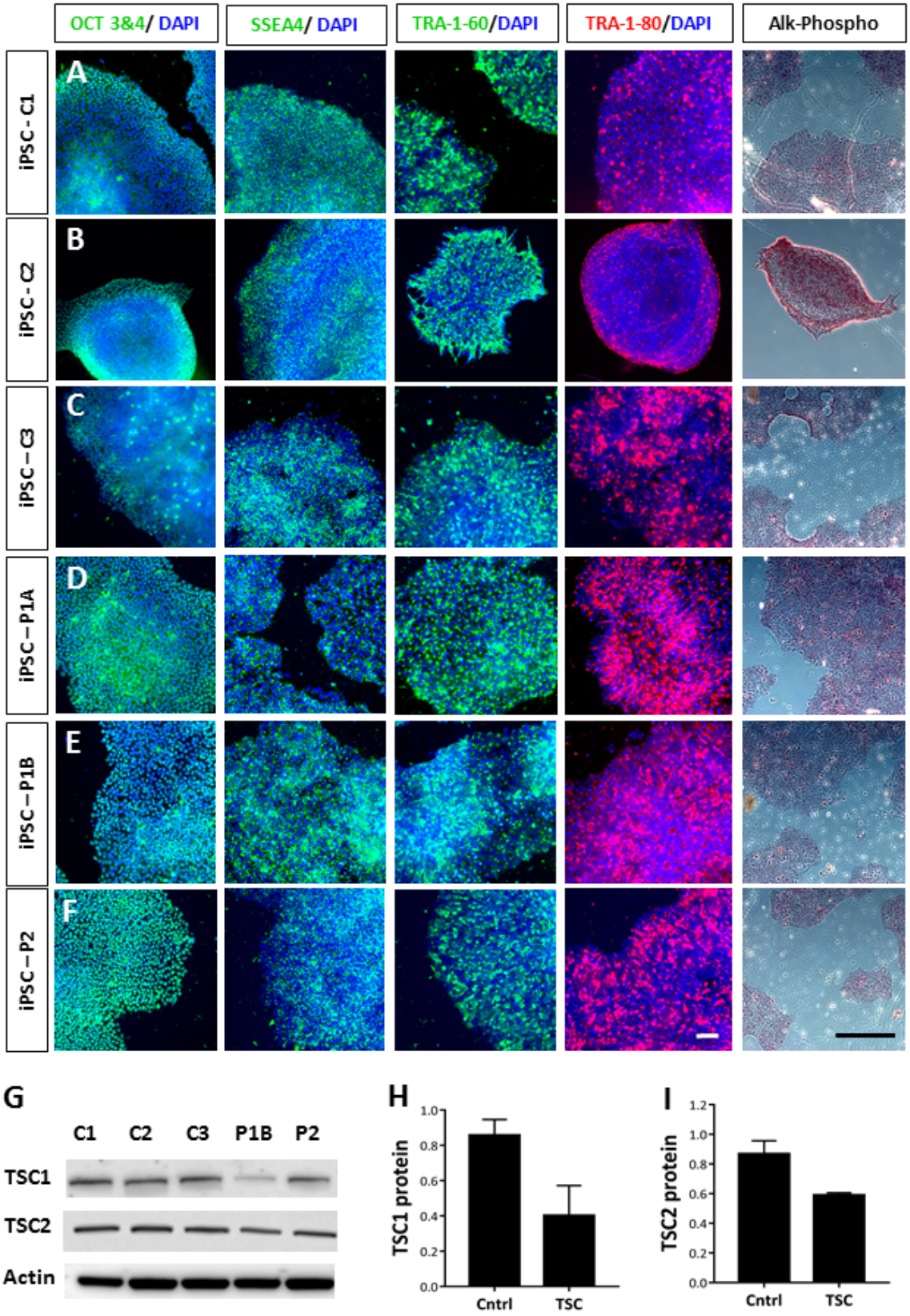

Figure S1 related to Figure 1 – TSC patient iPSC characterization and TSC protein expression at neural precursor stage. Characterization of hiPSCs derived

from controls (A) C1 (hVS-88), (B) C2 (hVS-228), (C) C3 (hVS-421) and TSC patient hiPSC lines (D) P1A (hVS-233; Clone A of P1), (E) P1B (hVS-248; Clone B of P1) and (F) P2 (hVS-417) using pluripotency markers OCT 3&4, SSEA4, TRA-1-60, TRA-1-80 and alkaline phosphatase (G) Western blot quantification of TSC1, TSC2 and actin, (H) TSC1 and (I) TSC2 protein quantification in neuroepithelial stem cells. Individual points indicate the average values of the different patients (P1B, P2) and controls (C1, C2, and C3). Scale bars of immunocytochemistry images and bright field alkaline phosphatase images (last column) are all 100 μm.

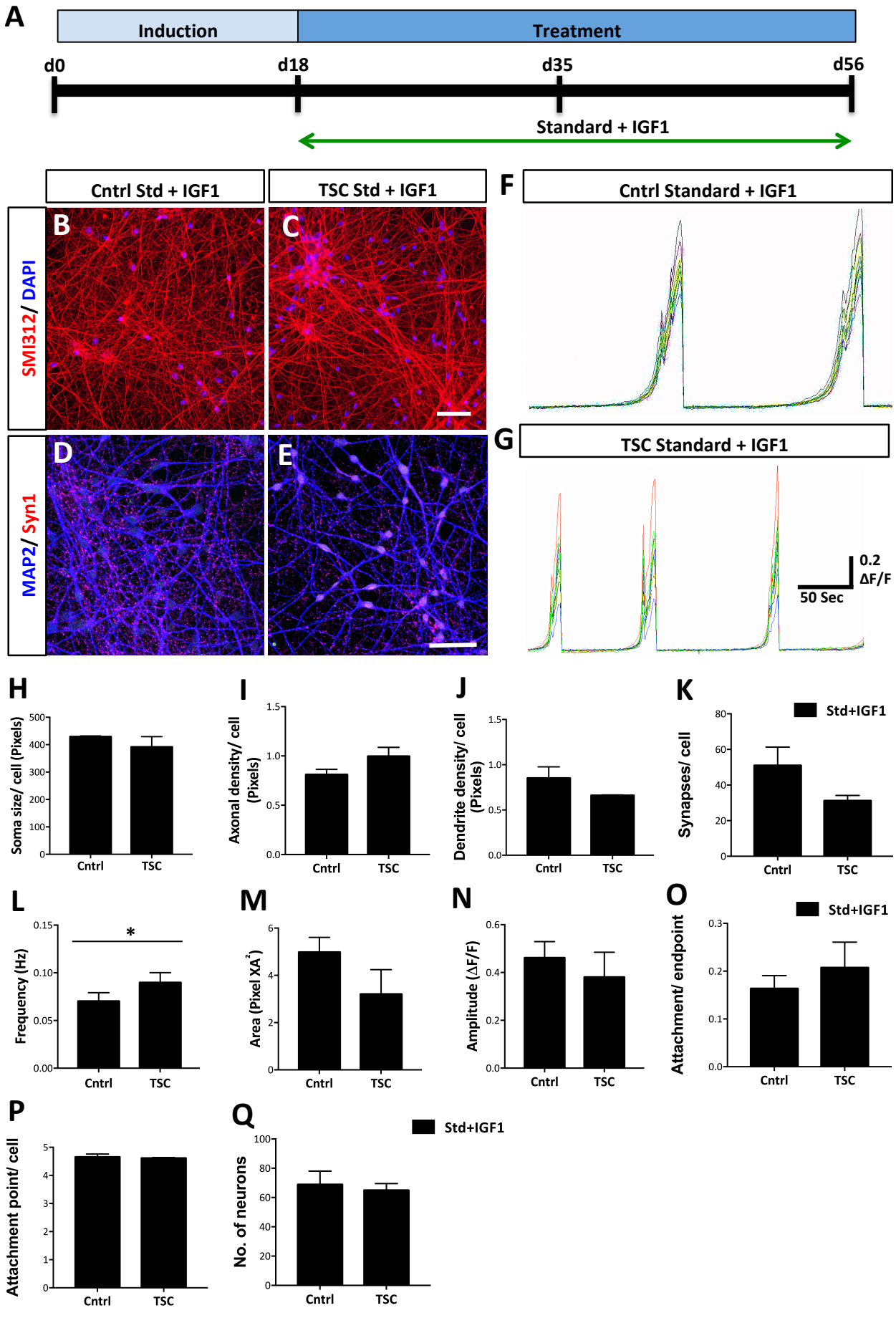
A
Induction
Treatment
d0
d18
d35
d56
Standard + IGF1
Cntrl Std + IGF1
TSC Std + IGF1
SMI312/ DAPI
MAP2/ Syn1
B
C
D
E
F
Cntrl Standard + IGF1
G
TSC Standard + IGF1
0.2
ΔF/F
50 Sec
H
Soma size/ cell (Pixels)
I
Axonal density/ cell (Pixels)
J
Dendrite density/ cell (Pixels)
K
Synapses/ cell
Std+IGF1
L
Frequency (Hz)
*
M
Area (Pixel XA²)
N
Amplitude (ΔF/F)
O
Attachment/ endpoint
Std+IGF1
P
Attachment point/ cell
Q
No. of neurons
Std+IGF1
Cntrl
TSC

Figure S2 related to Figure 2 and 3 – Neuronal properties of Std+IGF1 condition. (A) Schematic of neuronal differentiation protocol explaining time line and growth factors added at specific time point for Std + IGF1 (10ng/ml) condition. (B-E) Immunochemistry for axonal marker SMI312, nuclear marker DAPI in control Std+IGF1 (B), TSC Std+IGF1 (C), and dendritic marker MAP2 and pre-synaptic marker Synaptophysin1 in control Std+IGF1 (D), TSC Std+IGF1 conditions at day 56 for C1, C2, C3, P1 and P2 (n=6 to 8 independent differentiations; mean ±SEM). Representative traces of calcium influx measured using Fluoro-5 dye in one field of view (FOV) of C1 (F) and P1A (G) under culture condition Std + IGF1. (H) Soma size, (I) Total axonal density, (J) total dendritic density per soma and (K) number of synapses per soma measured in Std+IGF1 conditions. (L-N) Average calcium event frequency (L), event area (M) and event amplitude (N) for C1, C2, C3, P1 and P2 (n=6 to 8 independent differentiations; mean ±SEM) day 56 neurons in Std+IGF1 condition. (O) Total number of neurons present in control and patient lines. Statistical differences (*$p<0.01$)* in graph L including Std and Std+Rapa conditions were obtained using one-way ANOVA. All scale bars are 50 µm.

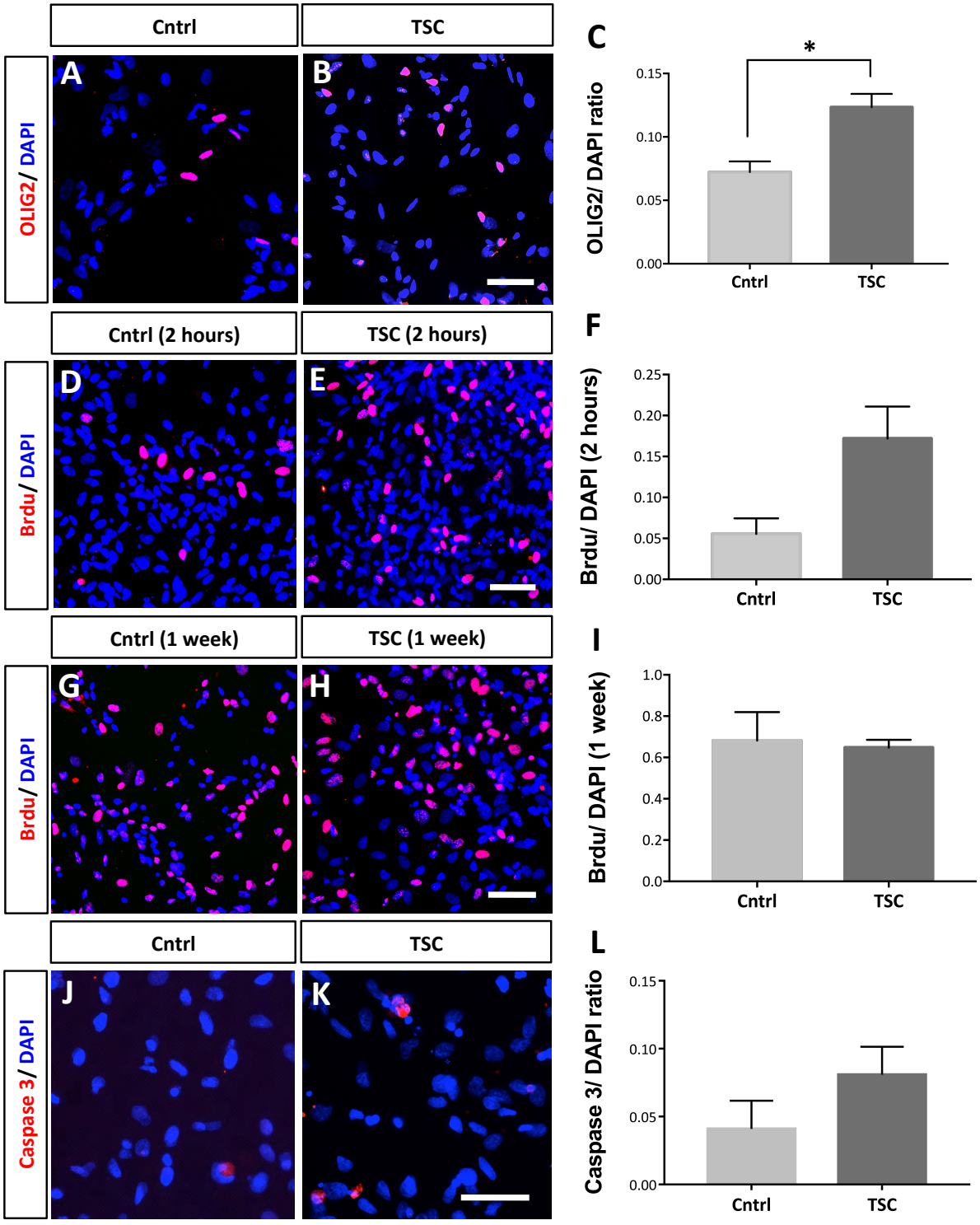

Figure S3 related to Figure 5 – Proliferation and cell survival in OL mono-cultures. We performed immunocytochemistry for OLIG2 in control (A) and TSC OL cultures (B) and analyzed the ratio of OLIG2/ DAPI cells (C). Additionally, we performed immunocytochemistry for BrdU after 2 hours in control (D), TSC OLs (E) and after 1 week in control (G), TSC OLs (H) and cleaved Caspase 3 in control (J) and TSC OLs (K). The ratio of Brdu/ DAPI cells 2 hours (F) and 1 week after Brdu treatment (I), and the ratio of cleaved Caspase 3/ DAPI cells (L). All experiments were n=3. All

scalebars are 50 µm. Statistical differences in C represents $p<0.05$ using unpaired T-test.

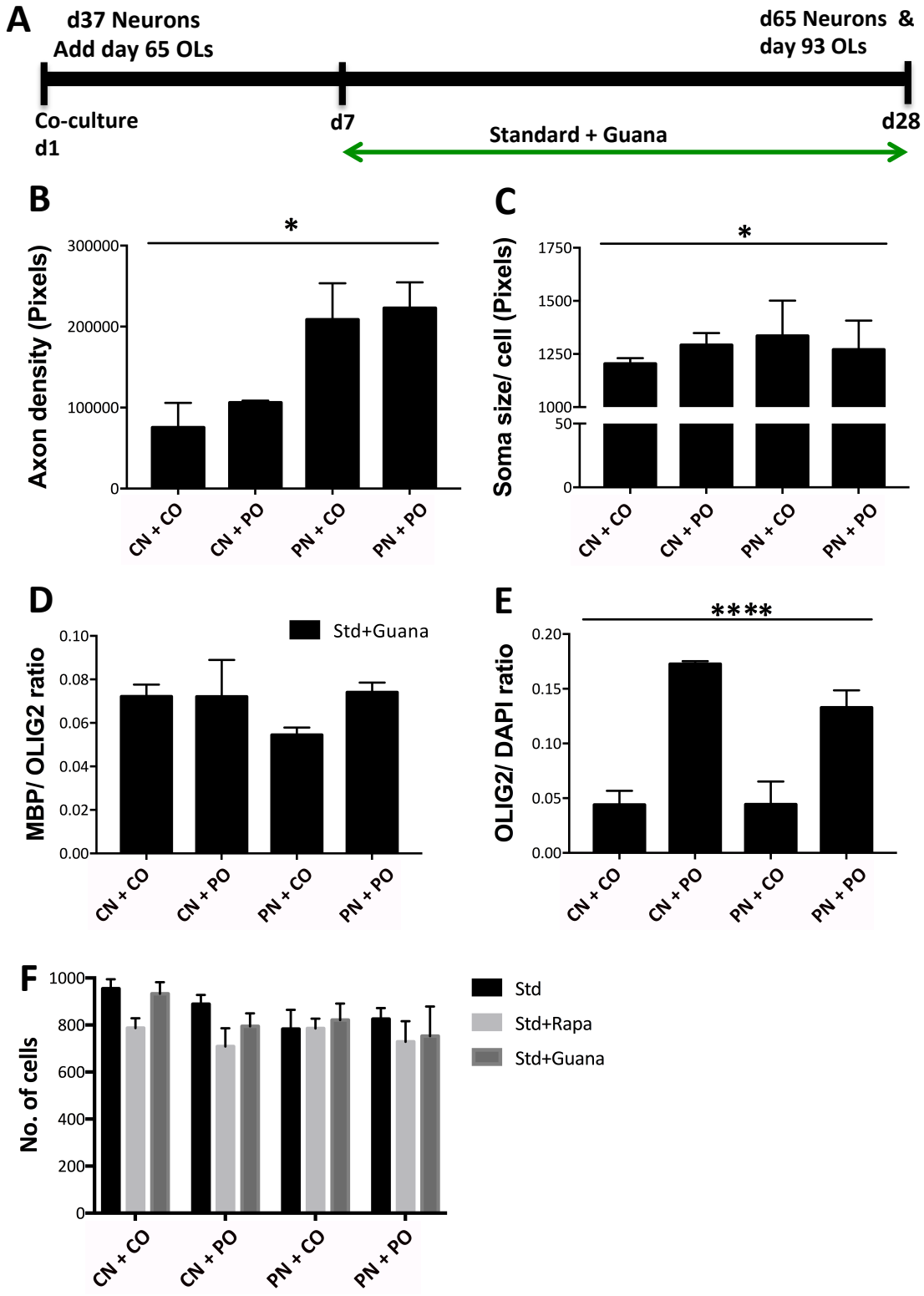

Figure S4 related to Figure 4 and 5 – Effects of Guanabenz on axonal density, hypertrophy, OL proliferation and maturation in neuron-OL co-cultures. (A) Schematic of neuron-glial co-culturing and different growth factors involved in the

Std+Guana conditions. (B) Axonal density, (C) soma size (D) MBP/ OLIG2 ratio and (E) OLI2/ DAPI ratio in co-cultures under Std+Guana condition. Statistical differences of all groups including Std, Std+Rapa in Fig 4, 5 were obtained for graphs B, D and E using one-way ANOVA where **** is $p<0.0001$ and * $p<0.05$. (F) Total number of cells in all co-cultures including Std, Std+Rapa groups shown in Fig 4, 5 as measured by DAPI staining. Mean per group was obtained from total n=4-9 experiments using C1, C3, P1B and P2 derived neurons and OLs.

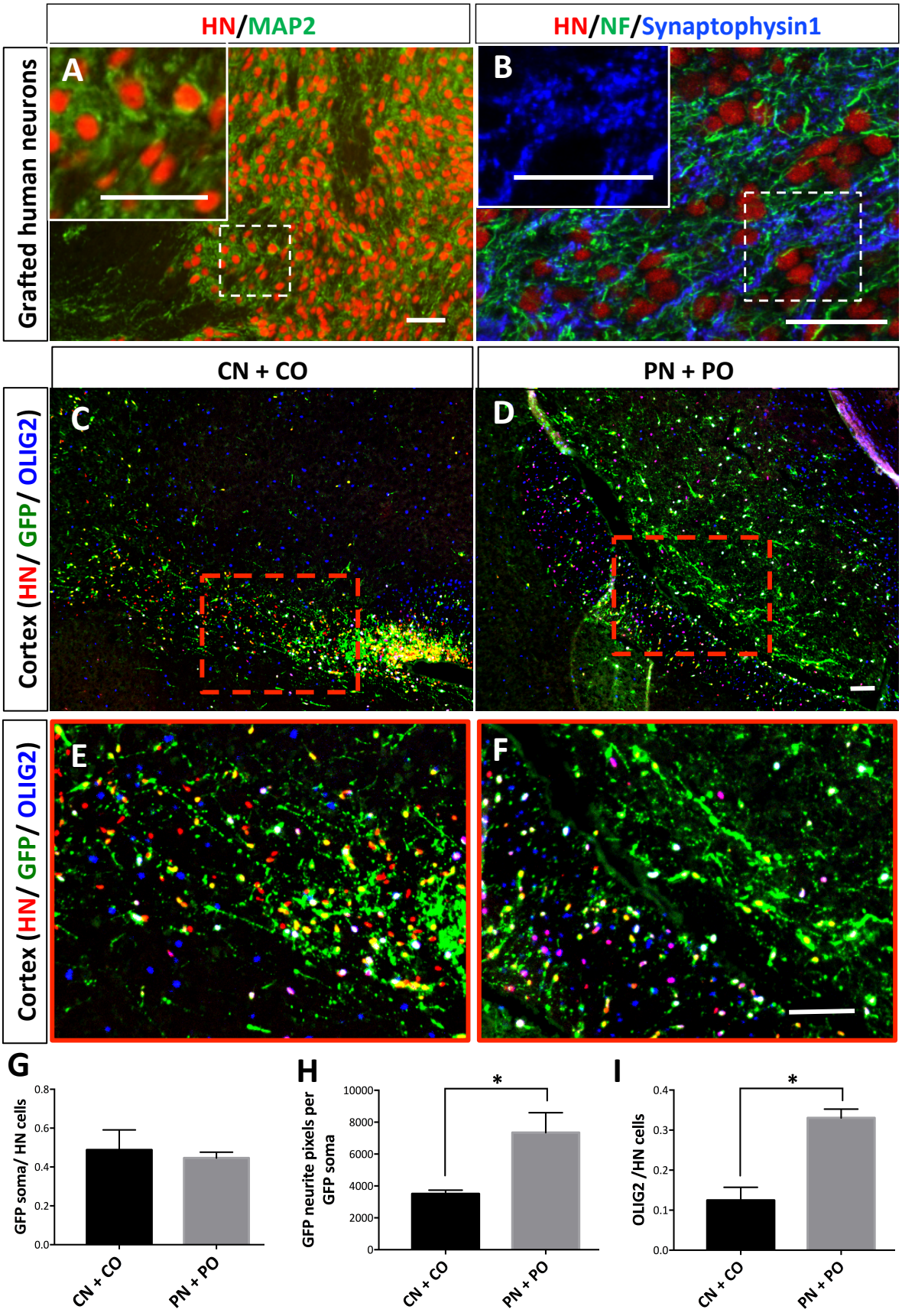

Figure S5 related to Figure 4 and 5 – TSC patient iPSC-derived co-transplants show increased neurite length and OL precursor proliferation. (A) Overview of HN-

positive cells showing MAP2 expression and (B) NF expression with NF filaments co-localizing with Synaptophysin1 puncta at 2.5 months post-transplantation. At 2.5 months post-transplantation, incorporation of HN-positive and HN-/OLIG2-double positive cells and GFP neurites in both (C) control neuron + control OL (CN + CO) and (D) patient neuron + patient OL (PN + PO) groups were quantified. (E, F) Insets show zoom images of regions in A and C. (G) Quantification of number of GFP-positive neuronal soma between CN + CO and PN + PO, (H) GFP neurite length/ GFP neuronal soma and (I) OLIG2/HN positive cells measured/ quantified regions of cortex and olfactory bulb. Neurons and OLs derived from one control (C1) and one patient (P1B) iPSC line were used from one differentiation. For groups CN + CO and PN + PO 2 and 3 animals were used, respectively with n=5 slides and 3 sections per slide. Significant differences were obtained using unpaired two-tailed t test with $p<0.02$. Scale bars in A, B are 50 µm and C-F are 100 µm.

Supplemental methods

Neuronal mono-cultures

All the neurons under Std, Std+IGF1 and Std+Rapa conditions were treated once with 2 µM cytosine β-D-arabinofuranoside (AraC) at day 26. All neuronal conditions were refreshed twice every week with half medium change, and from day 49 to 56 the Std+IGF1 (10 ng/ml) and Std+Rapa (10 nM) conditions were treated every day. At day 56 of differentiation the neuronal coverslips were processed for immunocytochemistry or calcium imaging analysis.

OL mono-cultures

OL cultures from day 65-80 were plated at a density of 100K or 50K/ 2.0cm^2 for OLIG2 or Brdu stainings. For OLIG2 stainings cells were cultured for 5 days and then fixed with 2% PFA for stainings. For Brdu 1 week, cells were treated with Brdu (10 µM) 24 hours after plating and fixed a week later; for Brdu 2 hours, cells were grown for a week and then treated with Brdu for 2 hours before fixation.

Culturing on MEAs

For multi-electrode array (MEA) experiments, day-56-neurons from Std conditions were re-plated on the MEAs. Prior to cell plating, each array was coated with 200µl of a filter-sterilized 0.1% solution of polyethylenimine (Sigma Aldrich) in borate buffer (3.10g of boric acid (Fisher Scientific) and 4.75g of sodium tetra borate (Sigma Aldrich) in 1L of distilled water) overnight at 37^{O}C. Next day, the solution was removed and the wells

were washed 3X with sterile water and air-dried for one hour in a sterile biological safety cabinet. Then, neurons were dissociated using accutase and were re-suspended at a concentration of 5000 viable neurons/µl in BrainPhys (B27 1X (Gibco), BDNF (20 ng/ml; Peprotech), ascorbic acid (200 nM; Sigma)) media with 10µg/ml laminin (Sigma Aldrich) for Std condition. A 25 µl droplet of the cell suspension (100,000 neurons) was added to the center of each array, directly over the electrode area. MEAs were incubated for 1 hour with the seeded neurons followed by gentle addition of 700ul of the culture media. The media was replaced every three days with fresh rat astrocyte conditioned medium at 1:1 ratio. Cultures were measured 50 days post plating on MEAs (i.e. 106-day-old neuronal cultures). For Std+Rapa condition a number of arrays were treated with 10nM Rapa for 30min at 37^{O}C and then recorded for 10 minutes.

Immunoflourescence

Primary antibodies used were OCT 3&4 (#5279, Santa Cruz), SSEA4 (#MC-813-70, Hybridomabank), TRA-1-60 (#21705, Santa Cruz), TRA-1-80 (#4381C3, Millipore), SMI312 (#837901, Biolegend), MAP2 (#5392, Abcam), Synaptophysin1 (#101-004, SYSY), OLIG2 (#9610, Millipore), MBP (#7349, Abcam; #SMI-99P, Covance), Brdu (#ab6326, Abcam) and Nissl (#21480, Thermofisher), Cleaved Caspase-3 (Asp175, #9661, Cell signaling), Neurofilament (#8135, Abcam), HN (#1281, Millipore) and GFP (#1020, Aves labs).

Transplantation

Experimental procedures involving mice were in strict compliance with animal welfare policies of the Dutch government and were approved by the IACUC of the VU University, Amsterdam. Frozen day 19 neuronal progenitors and day 60-65 OL progenitors from control (C1) and TSC patients (P1B) were dissociated equally into a cell suspension of 40,000 cells in saline with DNAse1 (100 µg/ml) per injection (total volume of 0.4 µl). Injections were done in the striatum and cortex of P0 *RAG2*null mice (Taconic). For transplantation P0 mice were placed on clay mold to keep them from moving, cryo-anaesthetized for approx. 7 minutes before the injections in the striatum and cortex. The cortex injections were bilaterally at 1.6 mm posterior to lambda and -0.3mm depth. The striatum injections were bilaterally at -2.3 mm from lambda, at 1.0 to 1.1 mm from the midline and -0.7 to -0.8 mm depth. At 2.5 and 4 months post injection animals were anaesthetized and transcardially perfused with 4% PFA in PBS.

Subsequently after perfusion the collected brains were cryopreserved with 30% sucrose embedded in OCT (Tissue Tek). Brain sections were cut at a thickness of 12 µm using Leica cryostat with 3 sections per slide and used for immunofluorescence analysis.