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Supplementary Information for

# Activity in grafted human iPS cell-derived cortical neurons integrated in stroke-injured rat brain regulates motor behavior

**Sara Palma-Tortosa[1,&], Daniel Tornero[1,2,&], Marita Grønning Hansen[1], Emanuela Monni[1], Mazin Hajy[1], Sopiko Kartsivadze[1,3], Sibel Aktay[1], Oleg Tsupykov[4], Malin Parmar[5], Karl Deisseroth[6], Galyna Skibo[2], Olle Lindvall[1] and Zaal Kokaia[1]***

Corresponding author: Zaal Kokaia, Laboratory of Stem Cells and Restorative Neurology, Lund Stem Cell Center, Lund University, BMC B10, SE-22184, Lund, Sweden. Phone number: +46 2220276

Email: zaal.kokaia@med.lu.se

**This PDF file includes:**

**SI Appendix, Materials and methods**

***It-NES cell line***. Human iPS cell-derived It-NES cells were produced as described previously (1-3) with some modifications. Briefly, human dermal fibroblasts from a healthy adult donor were subjected to sendai virus transduction with the reprogramming factors Oct4, Sox2, KLF4 and c-MYC (CytoTune$^{TM}$iPS 2.0 Sendai Reprogramming kit, Invitrogen) and split into plates with mouse embryonic fibroblasts. Colonies were then picked and expanded to establish iPS cell lines in feeder-free conditions using mTeSR medium (Invitrogen). On day 0, iPS cells were split using dispase (0.5 mg/ml) in order to collect whole colonies. Colonies from 3 wells of a 6 well plate were gently resuspended in embryoid body (EB) medium (Dulbecco's modified Eagle medium/F12 (DMEM/F12), 10% KSR, 2-Mercaptoethanol (1:1000), non-essential amino acids (NMEAA) (1:100), Glutamine (1:100)) with Rock inhibitor (1:1000), 3 µM Dorsomorphin (Sigma-Aldrich) and 10 µM SB431542 (Sigma-Aldrich) and plated into ultra low-attachment 10 cm culture dishes to generate EBs. The EB medium with freshly dissolved Rock inhibitor, Dorsomorphin and SB431542 was changed daily. On day, 5 EBs were collected and plated on poly-ornithine-laminin-coated 6-well plates in EB medium with 3 µM Dorsomorphin and 10 µM SB431542. On day 6, medium was changed to N2 medium (DMEM-F12 (without Hepes + Glutamine), N2 (1:100), Glucose (1.6 g/l)) supplemented with 1 µM Dorsomorphin and 10 ng/ml bFGF. Six days later, neural rosettes appeared in culture and were carefully picked and grown in suspension in N2 medium with 20 ng/ml bFGF. On day 14, neural rosette spheroids were collected and dissociated with trypsin. The small clumps obtained were grown in adhesion on poly-ornithine-laminin-coated dishes in the presence of 10 ng/ml bFGF, 10 ng/ml EGF (both from Peprotech) and B27 (1:1000, Invitrogen). The iPS cell-derived It-NES cell line was routinely cultured and expanded on 0.1 mg/ml poly-L-ornithine and 10 mg/ml laminin (both from Sigma)-coated plates into the same media supplemented with FGF, EGF and B27 and passaged at a ratio of 1:2 to 1:3 every second to third day using trypsin (Sigma).

***Animals***. Adult athymic, nude male rats (220 g, n=32; Charles River) were used for stroke and cell transplantation and adult male Sprague-Dawley (SD) rats (225-250 g, n=6; Charles River) for virus injections. Animals were housed in individual ventilated cages under standard conditions of temperature and humidity and a 12-hour light/dark cycle with free access to food and water.

***Distal middle cerebral artery occlusion and cell transplantation***. Focal ischemic injury was induced in cerebral cortex by distal middle cerebral artery occlusion (dMCAO) as described previously (4). Briefly, animals were anaesthetized with isoflurane (3.0% induction; 1.5% maintenance) mixed with air and the temporal bone was exposed. A craniotomy of 3 mm was made, the dura matter was carefully opened, and the cortical branch of the middle cerebral artery was ligated permanently by suture. Both common carotid arteries were isolated and ligated during 30 min. After releasing common carotid arteries, surgical wounds were closed.

Intracortical transplantation of cortically fated It-NES cell-derived neurons, which had been transduced with lentivirus carrying GFP or eNpHR3.0, was performed stereotaxically 48 h after dMCAO using anaesthesia as described above. On the day of surgery, cortically primed cells in their fourth day of differentiation were resuspended to a final concentration of 100 000 cells/µl in citocon buffer. A volume of 1 µl was injected at each of two sites at the following coordinates (from bregma and brain surface): anterior/posterior: +1.5 mm; medial/lateral: 1.5 mm; dorsal/ventral: −2.0 mm; and anterior/posterior: +0.5 mm; medial/lateral: 1.5 mm; dorsal/ventral: −2.5 mm.

***Immunohistochemistry and quantifications***. Animals were perfused transcardially with 4% paraformaldehyde. Free-floating coronal sections (30 µm) were first subjected (only for antibodies labelling nuclear epitopes) to antigen retrieval including an initial incubation with sodium citrate pH

6.0 Tween 0.05%, for 30 min at 65°C. Sections were then preincubated in blocking solution (5% normal donkey serum and 0.25% Triton X-100 in 0.1 M phosphate-buffered saline (PBS)) and incubated at +4°C overnight with primary antibodies (listed in Table S1) diluted in blocking solution. Fluorophore-conjugated secondary antibodies (Molecular Probes or Jackson Laboratories) were applied for 2 h at room temperature. Nuclei were stained with Hoechst 33342 (Molecular Probes or Jackson Laboratories) for 10 min and sections were mounted with Dabco (Sigma) on gelatin-coated slides.

Infarct volume was quantified in NeuN-immunostained sections. Intact area was identified by $NeuN^+$ cells in the ipsilateral and contralateral hemisphere, delineated and then measured using C.A.S.T.-Grid software. Infarcted area was calculated by subtracting the non-lesioned (NeuN-stained) area in the damaged hemisphere from the corresponding area in the contralateral one. Lesion volume was obtained by multiplying the infarcted area by the thickness and distance between the sections (300 µm).

Quantification of NeuN+ cells at the transplantation site was performed by stereology using C.A.S.T.-Grid software (Visiopharm). Around 500 cells per animal were counted in a predefined fraction of the graft area in an epifluorescence/light microscope (BX61, Olympus, Germany). Number of cells expressing NeuN is expressed as percentage of total number of human (STEM101+) cells. Sox10 and DCX quantification was performed using 40X confocal images (LSM 780, Zeiss, Germany) and numbers of positive cells were counted using ImageJ software by sampling different areas of the core of the transplant (($Sox10^+$-STEM101+)/total STEM101+). Coexpression was assessed in the confocal microscope as overlapping of the two selected markers in the same plane and same area.

Analysis of areas reached by human lt-NES cell-derived fibers was performed using a Virtual Slide Scanning System (VS-120-S6-W, Olympus, Germany). Fiber density was assessed semiquantitatively in 10 µm thick maximum intensity projection confocal images using 63X objective. For each area, 3-5 images were analysed.

***Rabies virus monosynaptic tracing***. Construct for the tracing vector (containing TVA receptor) was purchased from AddGene (ID: 30195). High-titer preparations of lentiviral particles were produced according to protocol from Dull et al. (5) in a biosafety level 2 environment. Pseudotyped-modified rabies (ΔG-Rabies) vector was produced as previously described (6) with minor adjustments. The protocol was stopped after step 60 as the virus was concentrated via ultracentrifugation only once and no sucrose cushion was used. Titering was performed using TVA-expressing HEK 293T cells as defined in the protocol. Titers were 20-30 x $10^6$ TU/ml. A dilution of 5% was used, as determined by testing different dilutions for a concentration that gave specific infection and tracing in the absence of toxicity.

Six months after cell transplantation, intracortical lentiviral injections were performed stereotaxically on anesthetized animals in the contralateral hemisphere at the following coordinates (from bregma and brain surface): anterior/posterior: +1 mm; medial/lateral: -1.5 mm; dorsal/ventral: -2 mm.

***Immuno-electron microscopy***. Rats were deeply anesthetized with an overdose of pentobarbital and transcardially perfused with 0.1 M phosphate buffered saline (PBS) followed by ice-cold 2% formaldehyde, containing 0.2% glutaraladehyde, in 0.1 M PBS, pH 7.4. Brains were removed and then washed in 0.1 M PBS. Frontal 150 µm sections of whole brain were cut on a Vibratome VT1000A (Leica, Germany). Sections were cryoprotected, freeze-thawed in liquid nitrogen, and incubated overnight in primary goat anti-GFP antibody (1:500, Novus Biologicals) at +4ºC. Tissue was then incubated at room temperature for 2 h with biotinylated rabbit anti–goat secondary antibody (1:200, DakoCytomation), and avidin biotin peroxidase complex (ABC) (Vector

Laboratories) followed by 3,3′- diaminobenzidine tetrachloride (DAB) and 0.015% hydrogen peroxide. Following DAB reaction, sections were processed for electron microscopy. Immunostained sections were postfixed in 1% osmium tetroxide in 0.1M PBS, dehydrated in a graded series of ethanol and propylene oxide, and flat-embedded in Epon. Ultrathin sections were cut with a diamond knife and then counterstained with lead citrate and uranyl acetate. Ultrathin sections were mounted on grids, examined and photographed using a transmission electron microscope JEM-100CX (JEOL, Japan).

***Optogenetic inhibition of neuronal activity***. The lt-NES cells were stably transduced with lentivirus carrying third generation halorhodopsin (pLenti-hSyn-eNpHR3.0-EYFP) for 48 h. Efficiency of transduction was about 75% of the neurons generated. Cells were cortically fated and transplanted as described above. For eNpHR3.0-injected rats, 1 μL of AAV-CaMKII-eNpHR3.0-EYFP was injected at the same two coordinates as used for cell transplantation. At 5 months after cell transplantation or in the same session as virus injection, an optical fiber cannula (Prizmatix) with 250 μm core diameter and no protrusion was implanted in the surface of the brain at the location of the graft (coordinates: anterior/posterior: +1 mm; medial/lateral: +2 mm) or at the two locations of the virus injection (coordinates: anterior/posterior: +1 mm; medial/lateral: +2 mm and anterior/posterior: +1 mm, medial/lateral: -2 mm). For silencing neuronal activity in the grafted cells or endogenous transduced cells, the cannula was connected by an optical fiber (Optogenetics-Fiber-500um-2.5mm NA 0.63 an 0.5m length; Prizmatix) to an orange-LED optogenetic light (LED-595 nm, Prizmatix) and light was constantly applied for 5 min until the end of the behavioral session. The eNpHR3.0-injected rats were then sacrificed and brains stained with YFP antibody to verify the location of transduced endogenous cells.

***In vitro and ex vivo electrophysiology***. Slices and coverslips were constantly perfused with carbogenated artificial cerebrospinal fluid (acsf, in mM: 119 NaCl, 2.5 KCl, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 26 $NaHCO_3$, 1.25 $NaH_2PO_4$, and 11 glucose, pH 7.4) at 34°C. Recording pipettes were filled with intracellular solution (in mM: 122.5 potassium gluconate, 12.5 KCl, 10 HEPES, 2.0 MgATP, 0.3 $Na_2$-GTP, and 8.0 NaCl). Biocytin (1-3 mg/ml) was freshly dissolved in the pipette solution for *post-hoc* identification of recorded cells. The eNpHR3.0 transfected cells (YFP+) were visualized under fluorescent light and infrared differential interference contrast microscopy was used for visual approach of the recording pipette. Whole-cell patch-clamp recordings were performed with a HEKA double patch-clamp EPC10 amplifier using PatchMaster for data acquisition. In current clamp mode, cells were kept around -70 mV in most cases, but cells were also kept at more depolarised potentials to induce spontaneous generation of APs. Current was injected, when needed, to keep the membrane potential of interest. A series of current steps, going from -40 pA to 200 pA in 10 pA steps and lasting 500 ms, or a current ramp protocol going from 0-300 pA in 1 s was performed from a membrane potential of approximately -70 mV to determine the cells' ability to generate APs. In voltage clamp mode, cells were held at -70 mV. Data were analysed offline with FitMaster (v2x90.2), IgorPro (v6.37) and NeuroMatic (v2.8b).

***Behavioral test***. Cylinder test was performed as previously described (7, 8). Animal was placed in a 20 cm-diameter glass cylinder and forelimb activity was recorded with a digital video camera. Quantification of forelimb use was done by using two perpendicular mirrors placed behind the cylinder to make the complete surface of the cylinder clearly visible. Forelimb use was defined by the placement of the whole palm on the wall and contacts were counted off-line by an observer blinded to the group identity of the animals for a total of 5 min.

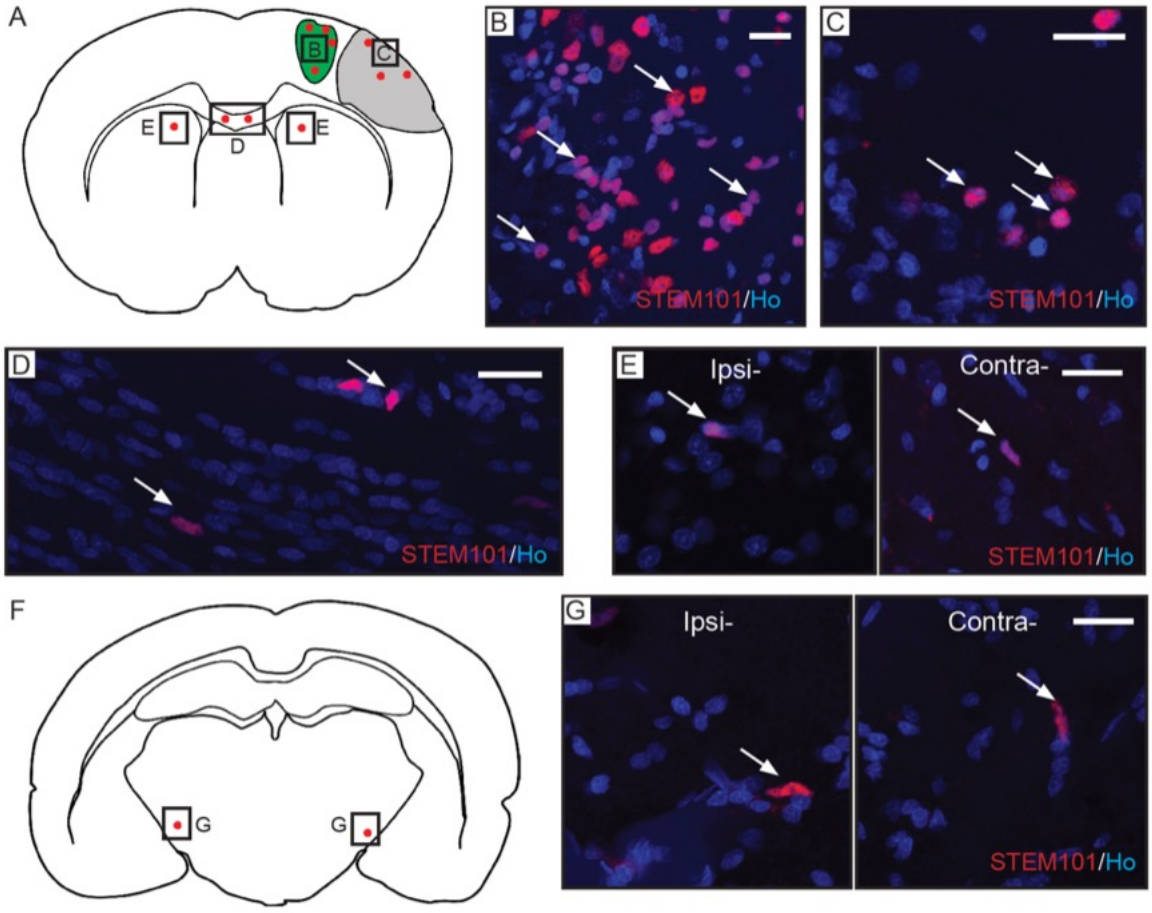


**Fig. S1.** Migration of human It-NES cells to different areas of stroke-injured rat brain 6 months after intracortical transplantation. (**A**) Location of illustrated areas **(**in **B-E)** reached by grafted cells. **(B-E)** Immunohistochemical images of grafted cells immunoreactive for human nuclear marker (STEM101) in the (**B**) transplant core, (**C**) periinfarct area, (**D**) corpus callosum and (**E**) caudate-putamen. Location **(F)** and immunohistochemical images **(G)** of grafted cells in the internal capsule. Arrows show colocalization of STEM101 (red) and Hoechst (blue). Scale bar: 20 μm.

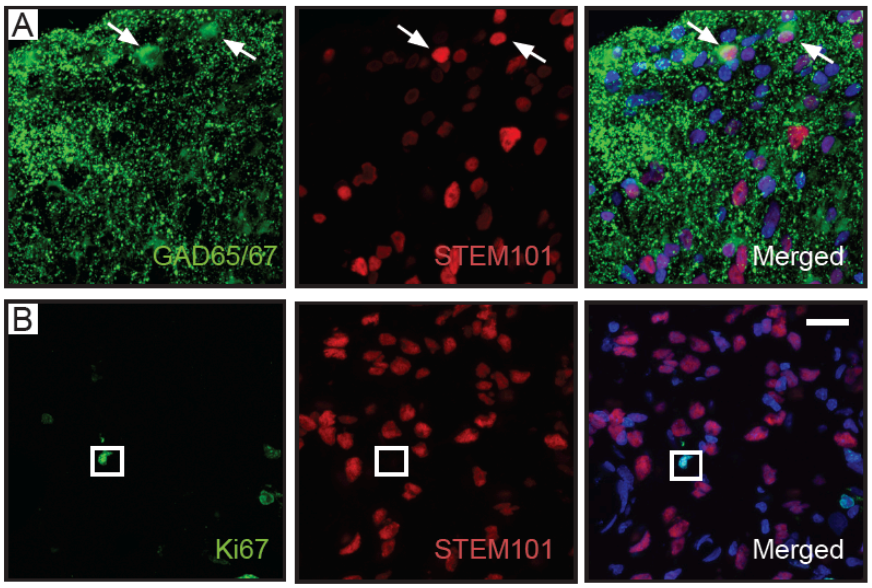


**Fig. S2.** Cellular composition of cortically fated human It-NES cell-derived grafts in stroke-injured rat somatosensory cortex. Confocal immunohistochemical images showing GABAergic neurons (**A**, GAD65/67) and proliferative cells (**B**, Ki67) in the transplant core (human cells are STEM101+). Arrows indicate colocalization. Square indicates no colocalization. Scale bar: 20 μm.

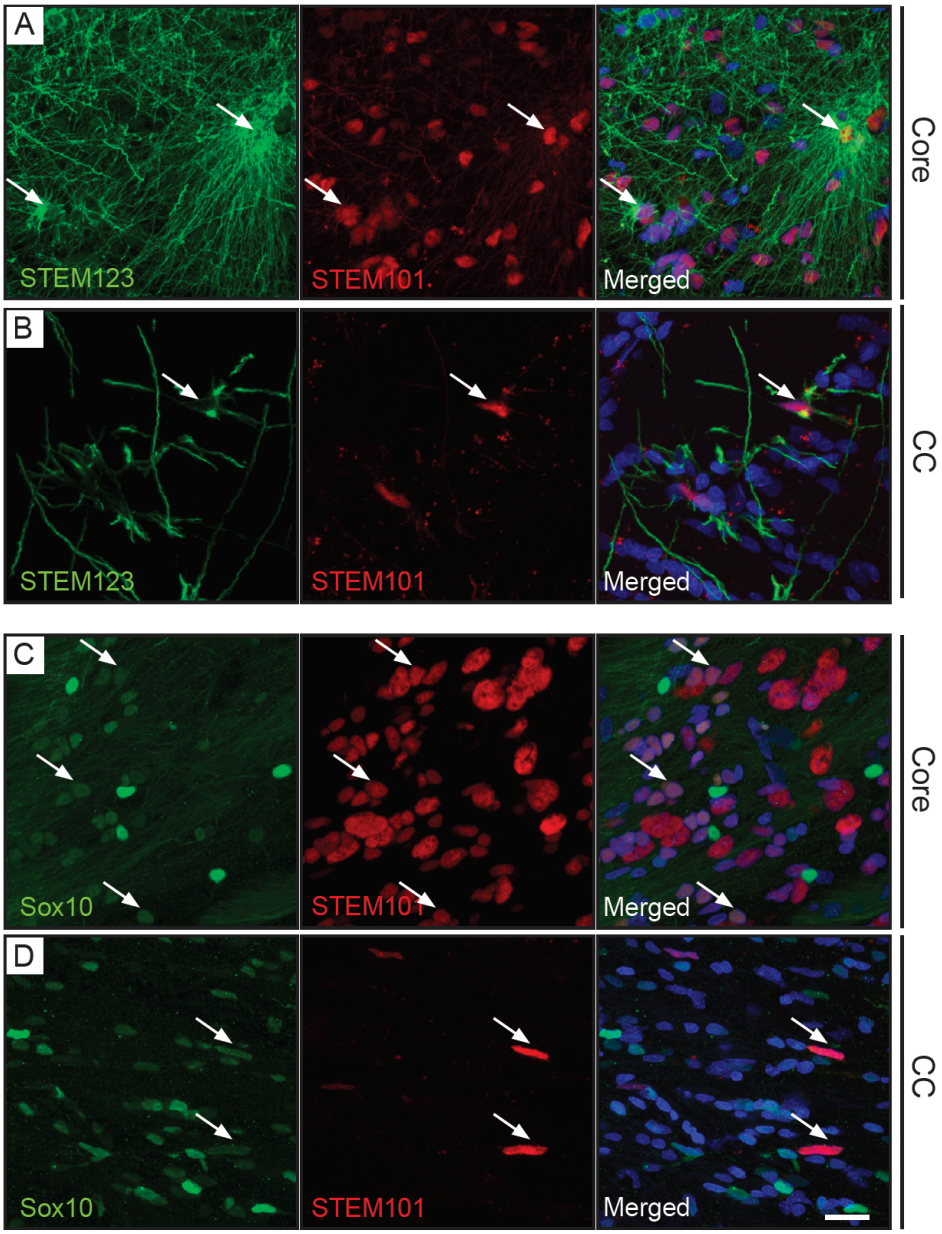


**Fig. S3.** Occurrence of human It-NES cell-derived glial cells in stroke-injured rat brain 6 months after intracortical transplantation. **(A-B)** Confocal immunohistochemical images showing colocalization of human nuclear marker (STEM101, red) with human astrocytic marker STEM123 (green), recognizing GFAP+ astrocytes, in (**A**) transplant core and (**B**) corpus callosum (CC). **(C-D)** Colocalization of human nuclear marker (STEM101, red) with nuclear oligodendrocytic marker Sox10 (green) in both locations. Arrows indicate colocalization. Scale bar: 20 μm.

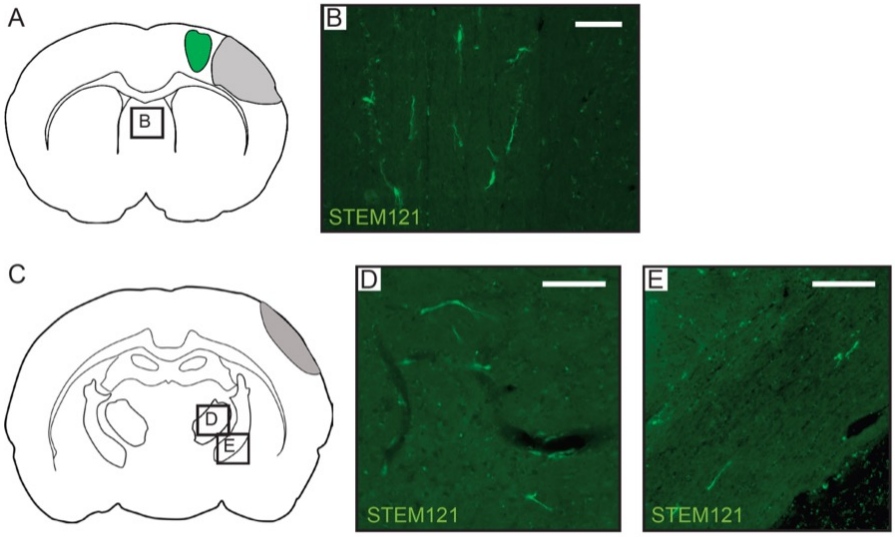


**Fig. S4.** Intracortical grafts of human lt-NES cell-derived cortical neurons project to various subcortical areas in stroke-injured rat brain 6 months after transplantation. (**A, C**) Location of illustrated areas. (**B**, **D** and **E**) Immunohistochemical images of graft-derived fibers stained with human cytoplasmic marker STEM121 in septum (**B**), ventral anterior thalamic nucleus (**D**), and internal capsule (**E**). Scale bar: 100 μm.

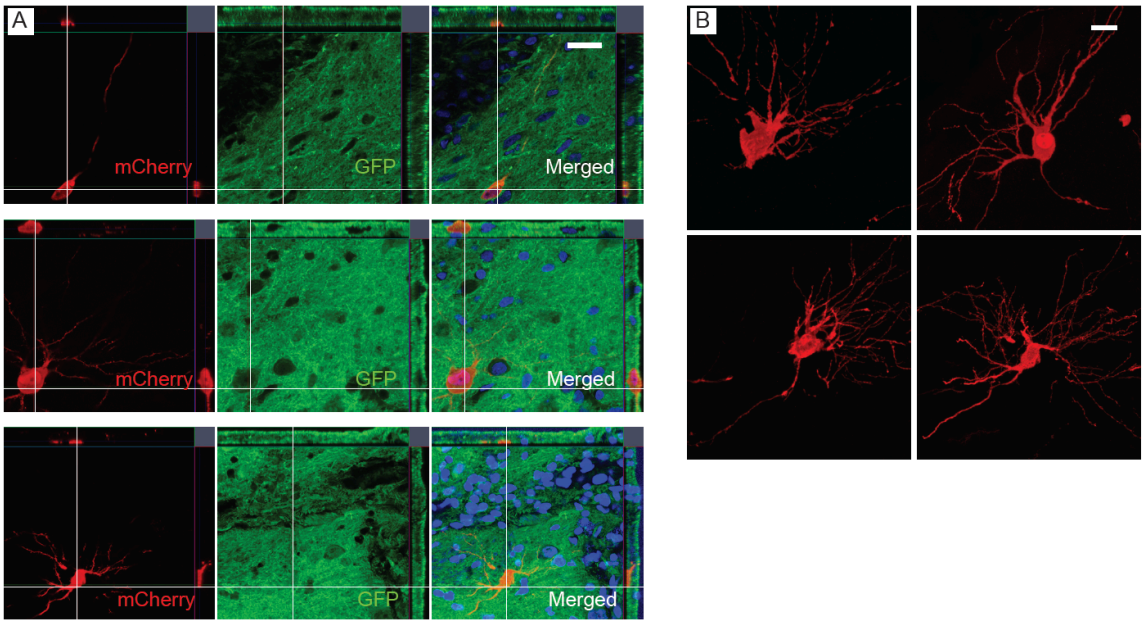


**Fig. S5.** Grafted human lt-NES cell-derived cortical neurons establish monosynaptic connections with host neurons in contralateral cortex of stroke-injured rats. (**A**) Confocal images of mCherry+/ GFP+ grafted cells located in the core of the transplant. Nuclear staining (Hoechst, blue) is included in merged panel. (**B**) Confocal images of mCherry+-grafted cells in the core of the transplant showing pyramidal morphology. Scale bar in B: 20 μm.

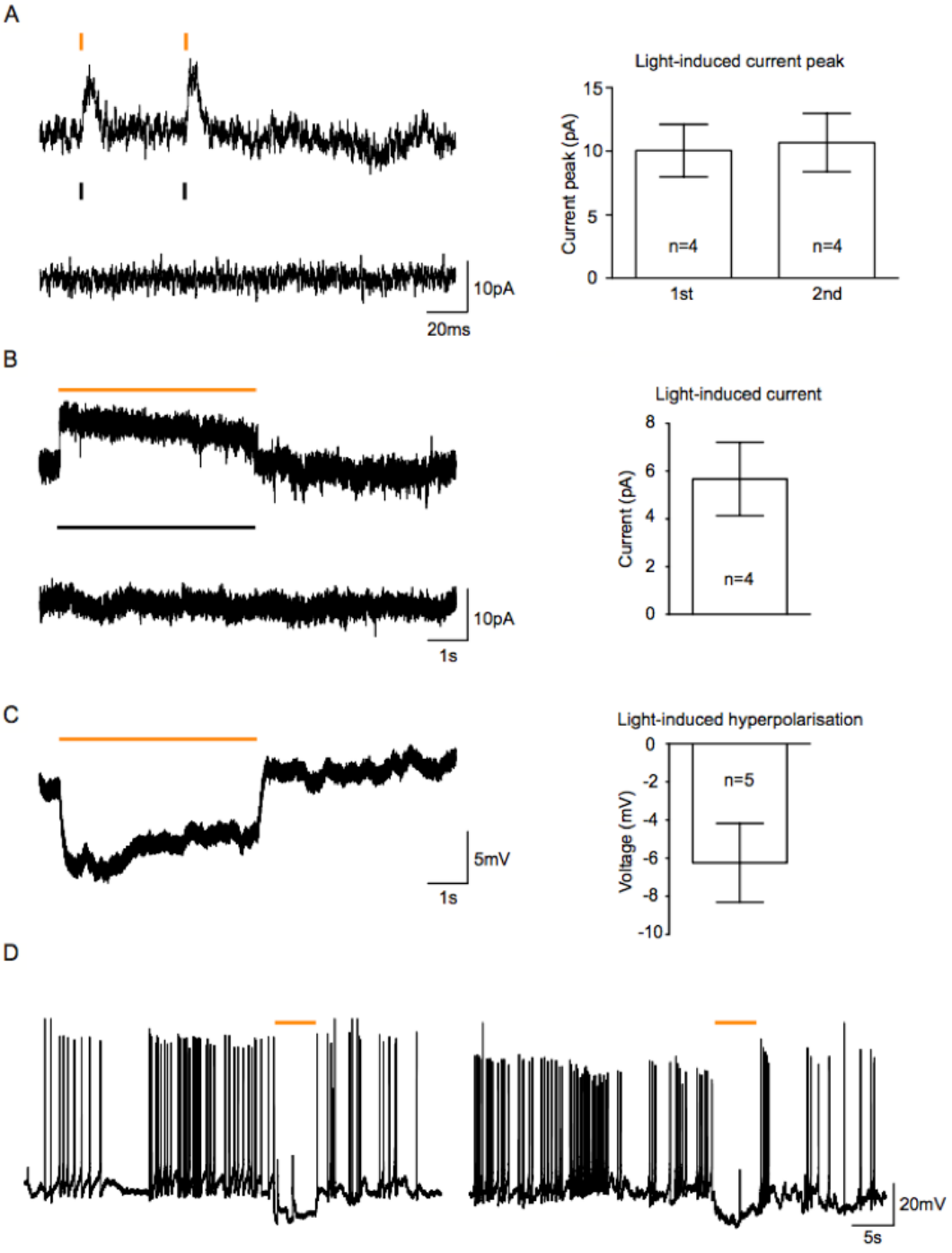


**Fig. S6.** Validation of eNpHR3.0-transduced cortically fated human It-NES cell line. Cells were transfected with pLenti-Syn1-eNpHR3.0 and differentiated *in vitro* to cortical phenotype; electrophysiological recordings were performed at day 52 of *in vitro* maturation. **(A-B)** Response of neurons to two short (**A**) or one long (**B**) stimulation with orange LED light (orange bar) in the voltage-clamp configuration. Graphs on the right show the average ± SEM of the light-induced change in current observed in both cases (n=4). Recordings below in (**A**) and (**B**) show the absence of response when repeating the same protocol with the orange LED light off. (**C**) Response of

neurons to long stimulation with orange LED light (orange bar) in current-clamp configuration. Graph on the right shows the average ± SEM of the light-induced change in voltage (n=5). (**D**) Two examples of the silencing of neuronal activity induced by orange LED light (orange bar) in lt-NES cells expressing eNpHR3.0 *in vitro*.

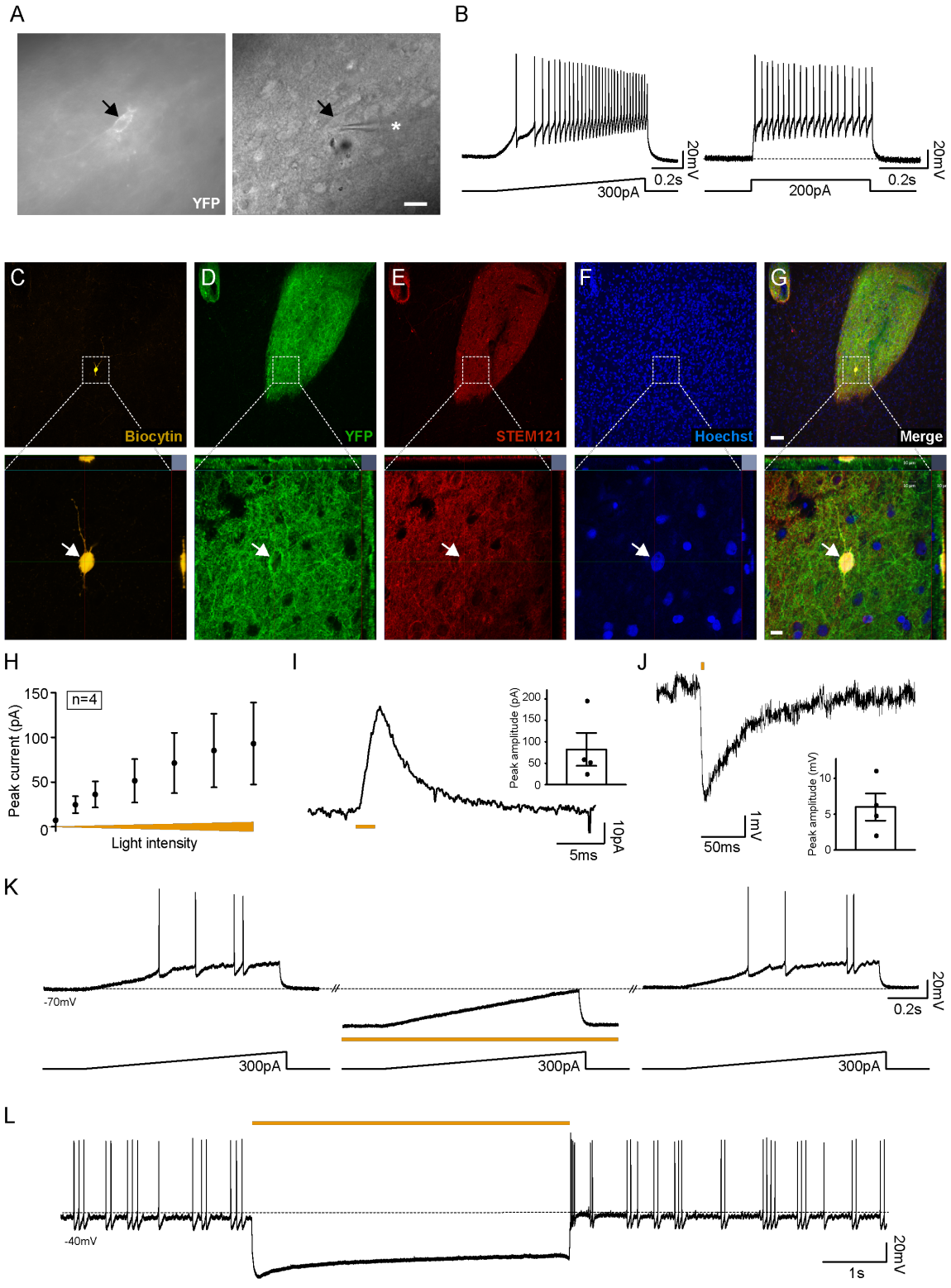


**Fig. S7.** Response of grafted eNpHR3.0-transduced human cortically fated lt-NES cells to orange light and suppression of AP generation in acute brain slices at 6 months after intracortical transplantation into stroke-injured rats. (**A**) Patched YFP+ cell which responded to orange light.

Black arrow depicts cell and white asterisk patch pipette. Scale bar: 20 μm. (**B**) Voltage traces illustrating the generation of current-ramp (*left*) and -step (*right*) induced APs. (**C-G**) Low (top) and high (below) magnification confocal images of the patched cell (arrow) shown in **A**. Biocytin-filled cell (**C**), YFP+ cells belonging to graft (**D**), human marker STEM121 (**E**), Hoechst (**F**) and merged picture (**G**). High magnification images are presented as orthogonal view**.** (**H**) Light-induced current peak plotted against light intensity, demonstrating that current is enhanced upon increasing light intensities. (**I-J**) Averaged current (**I**) and voltage (**J**) traces (average of 3 traces) illustrating the induction of an upward-deflecting current (**I**) and a downward-deflecting voltage (**J**) upon 2 ms orange light illumination. Bar diagram showing averaged current (**I**) and voltage (**J**) peak amplitude (average from 2-3 traces per cell and determined from 4 cells). (**K**) Voltage traces illustrating current ramp-induced generation of APs. Orange light illumination hyperpolarise the cell and suppress the generation of APs. (**L**) Voltage traces showing spontaneously occurring APs when the cell is held at approximately -40 mV. The cell is hyperpolarised upon orange light illumination and spontaneously occurring APs are abolished.

**Table S1.** List of primary antibodies used for immunohistochemistry

| Antibodies | Host species | Dilution | Company |
|---|---|---|---|
| STEM101 | Mouse | 1:200 | Stem Cells |
| STEM121 | Mouse | 1:400 | Stem Cells |
| STEM123 | Mouse | 1:200 | Stem Cells |
| GFP | Chicken | 1:3000 | Merk Millipore |
| NeuN | Rabbit | 1:2000 | Abcam |
| DCX | Goat | 1:1000 | Santa Cruz |
| Ki67 | Rabbit | 1:1000 | Abcam |
| Sox10 | Goat | 1:100 | Santa Cruz |
| Fog2 | Rabbit | 1:200 | Thermo Fisher |
| mCherry | Mouse | 1:300 | Abcam |
| MBP-Cy5 | ------- | 1:200 | Biolegend |
| Tbr1 | Rabbit | 1:300 | Abcam |
| Brn2 | Goat | 1:400 | Santa Cruz |
| Satb2 | Mouse | 1:50 | Abcam |
| Ctip2 | Rabbit | 1:100 | Sigma |
| hu Mit-Cy3 | ------- | 1:100 | Millipore |
| KGA | Rabbit | 1:500 | Abcam |
| GAD 65/67 | Rabbit | 1:1000 | Abcam |

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