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Supplemental Information

Electrical Guidance of Human Stem Cells in the Rat Brain

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Supplemental Figures 1-6

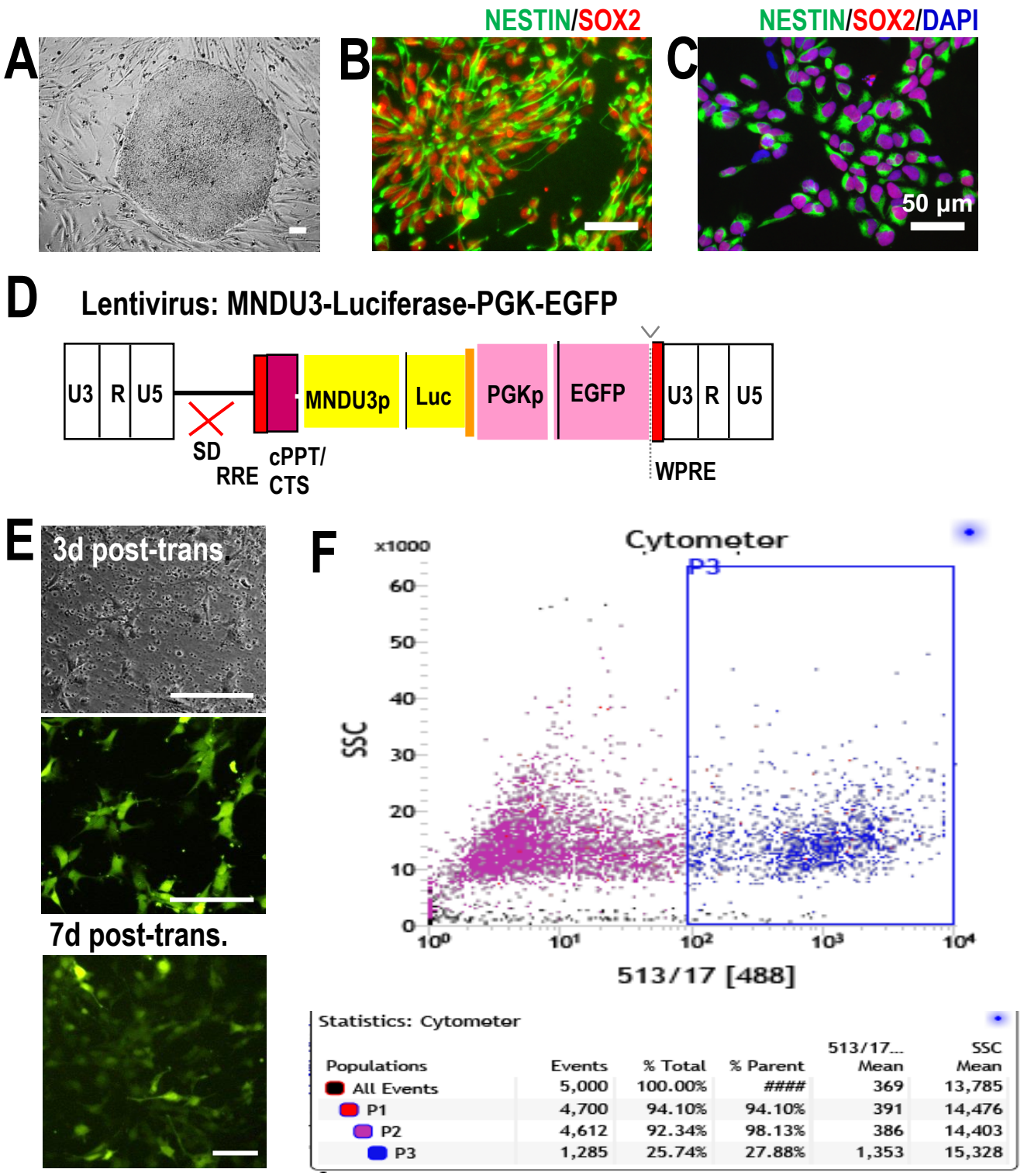
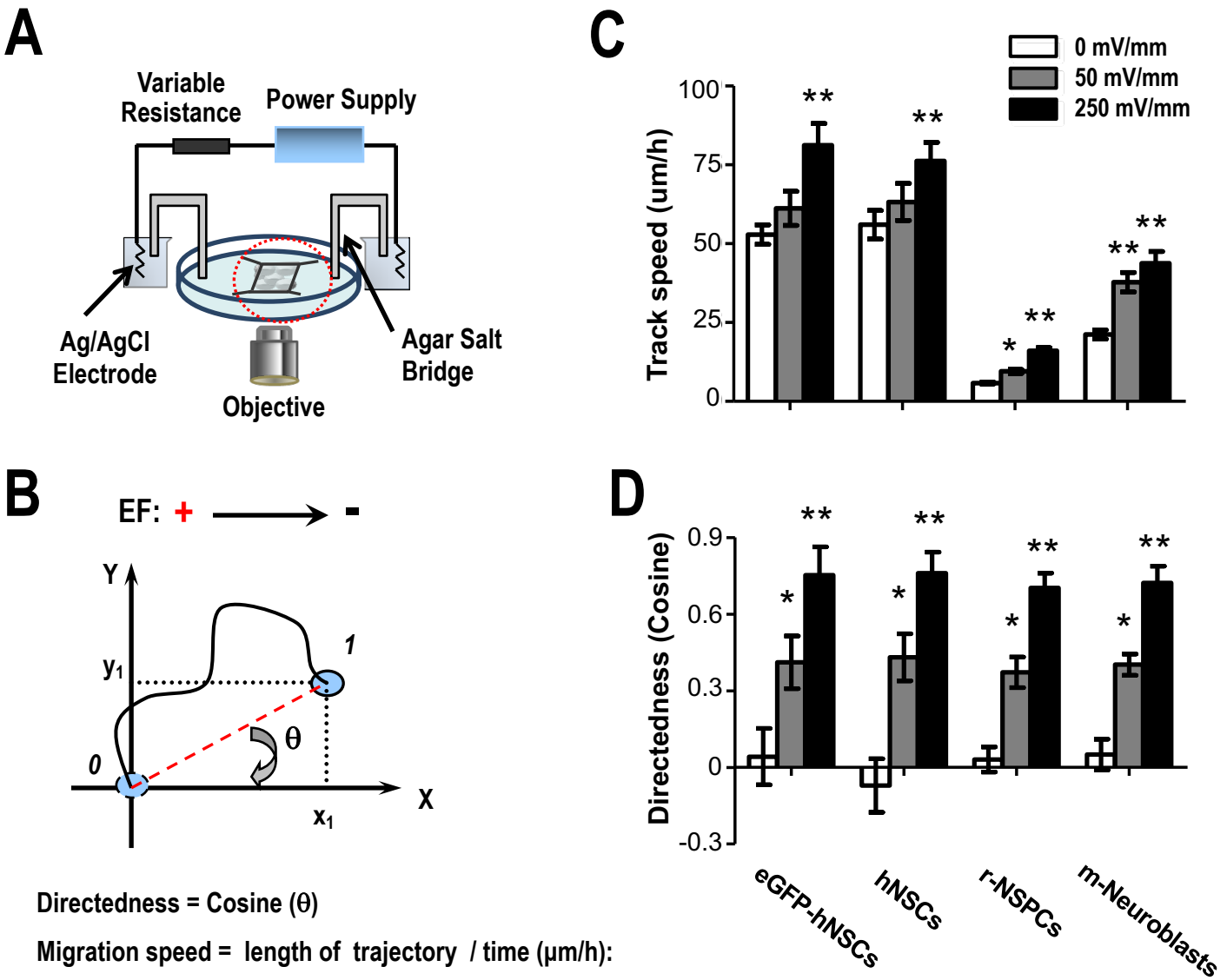


Figure S1. Derivation of human neural stem cells that express EGFP



Directedness = Cosine (θ)

Migration speed = length of trajectory / time (μm/h):

Figure S2. ***In vitro*** **electrotaxis setup and investigation for different originated neuroblasts**

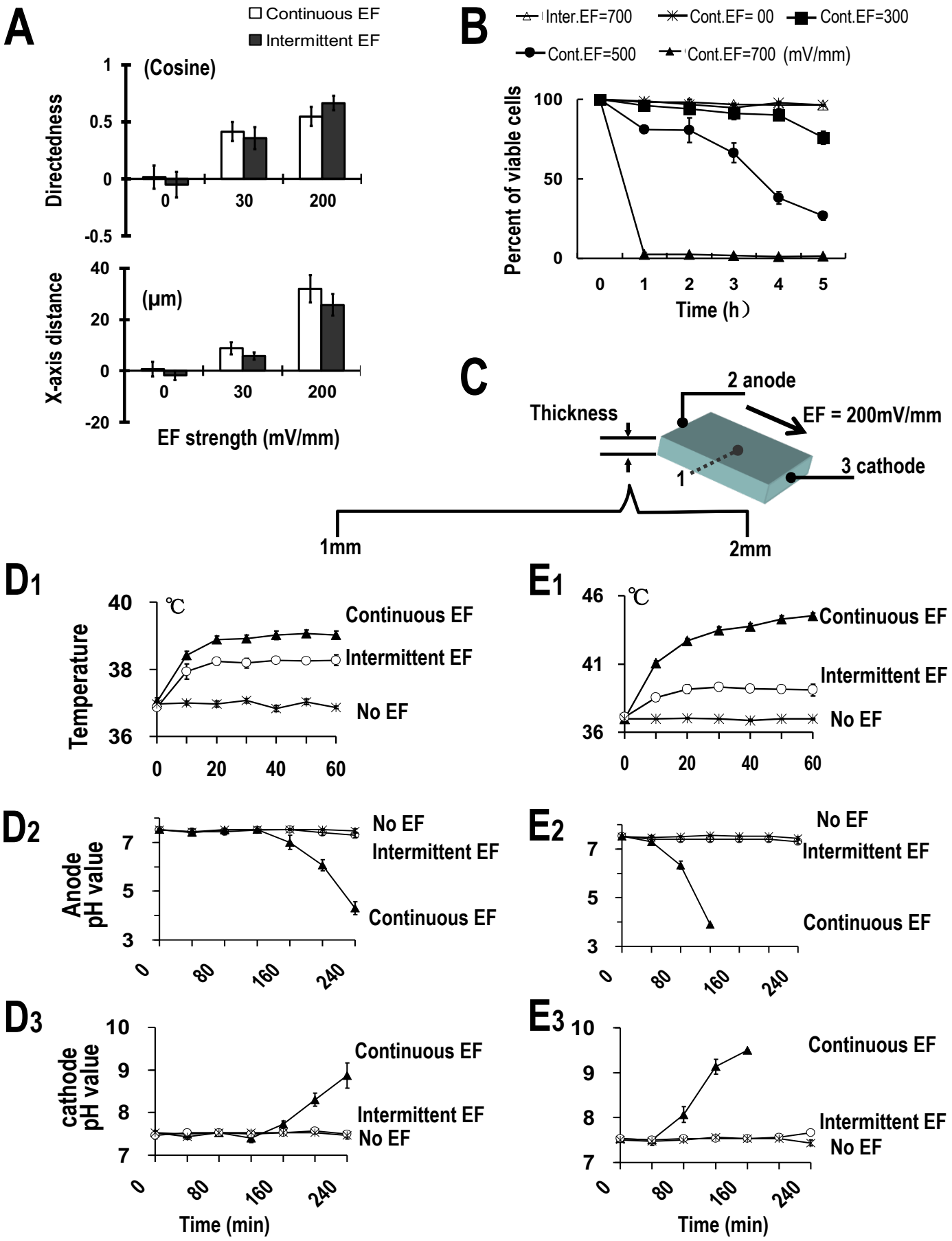


Figure S3. Intermittent EF stimulation guided migration of hNSCs with minimal cell damage and side effects

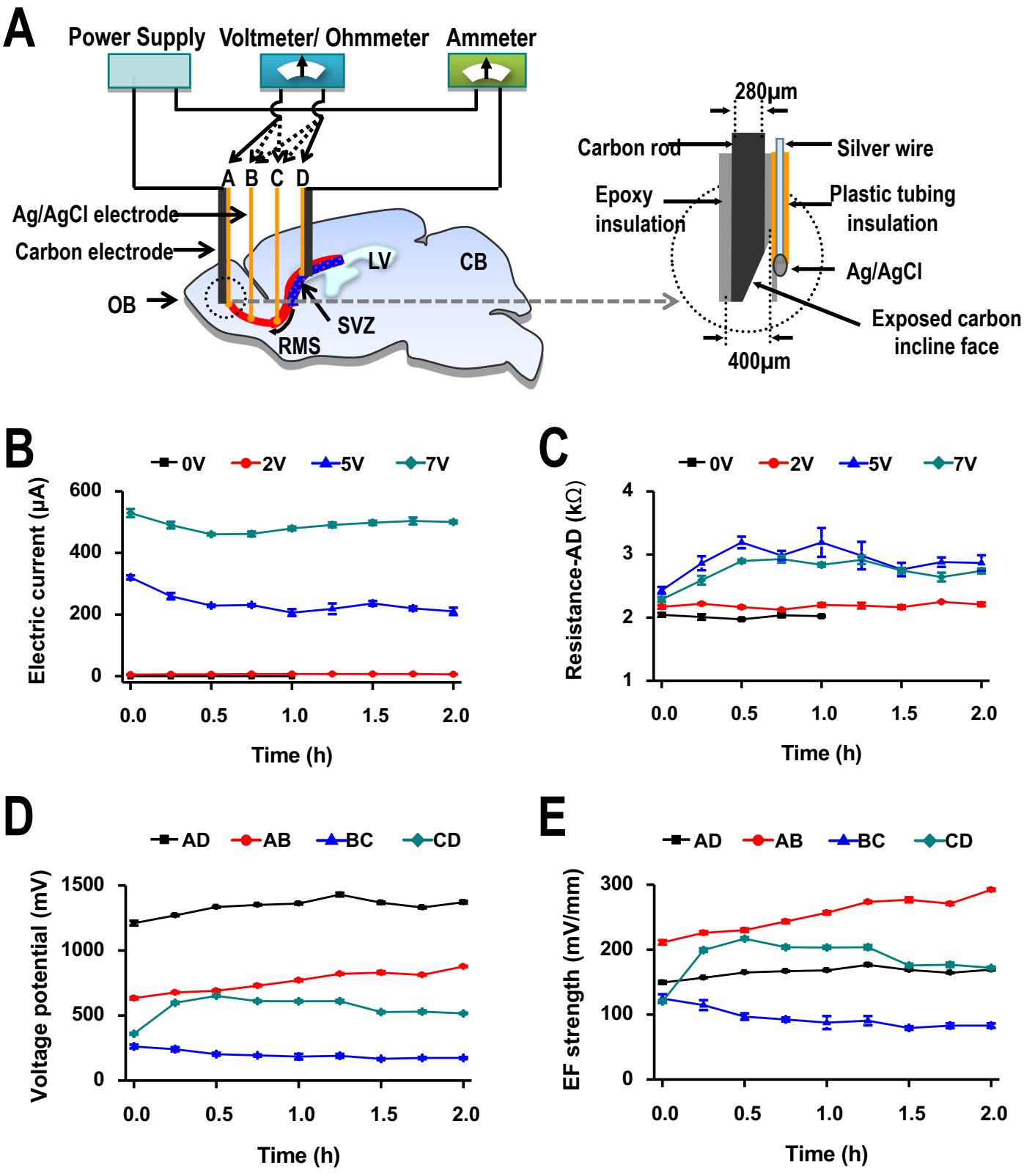


Figure S4. Electric fields and currents delivered which were stable in live animal brain

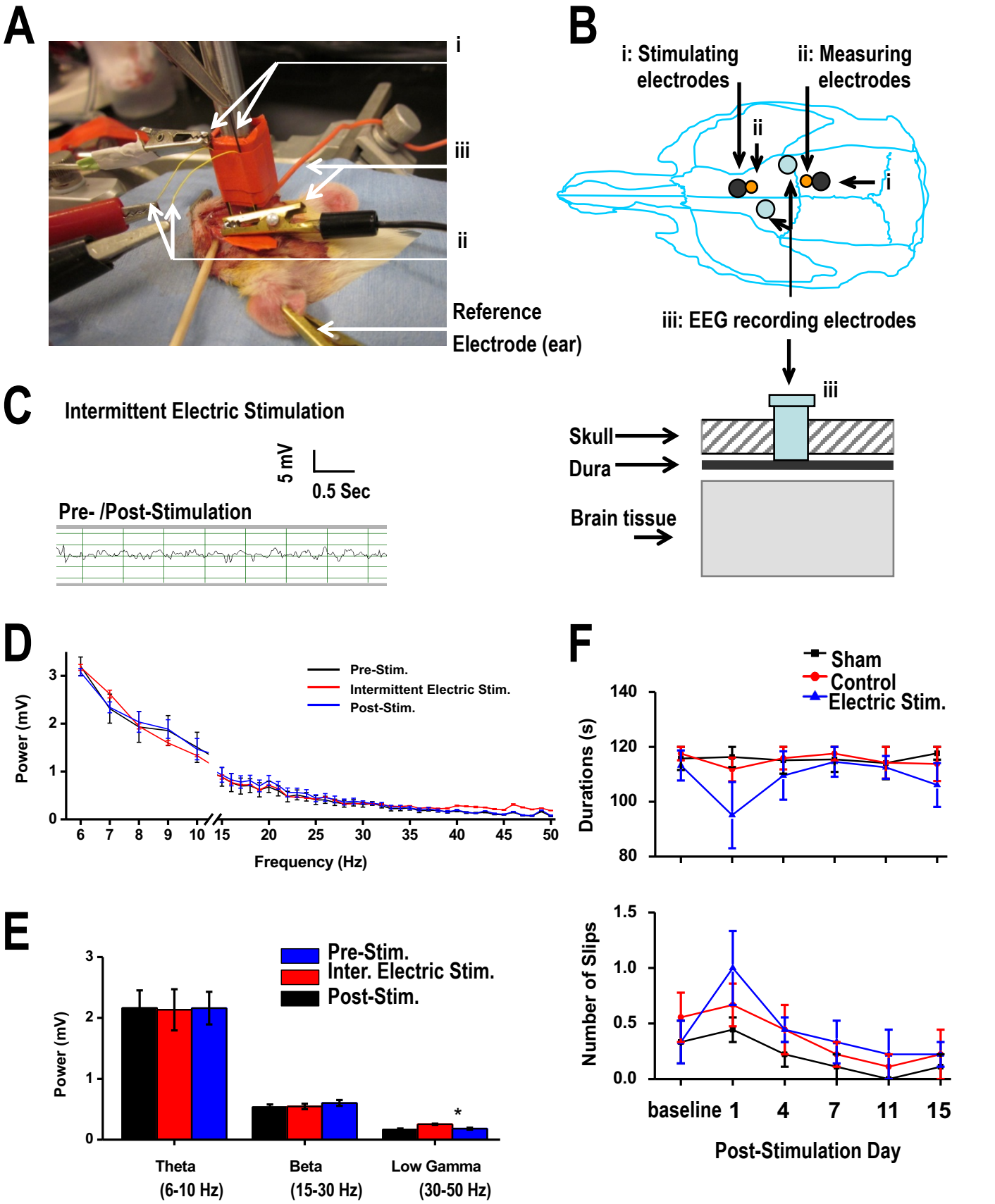


Figure S5. Electroencephalograph (EEG) monitoring during electrical stimulation and motor function evaluation after stimulation

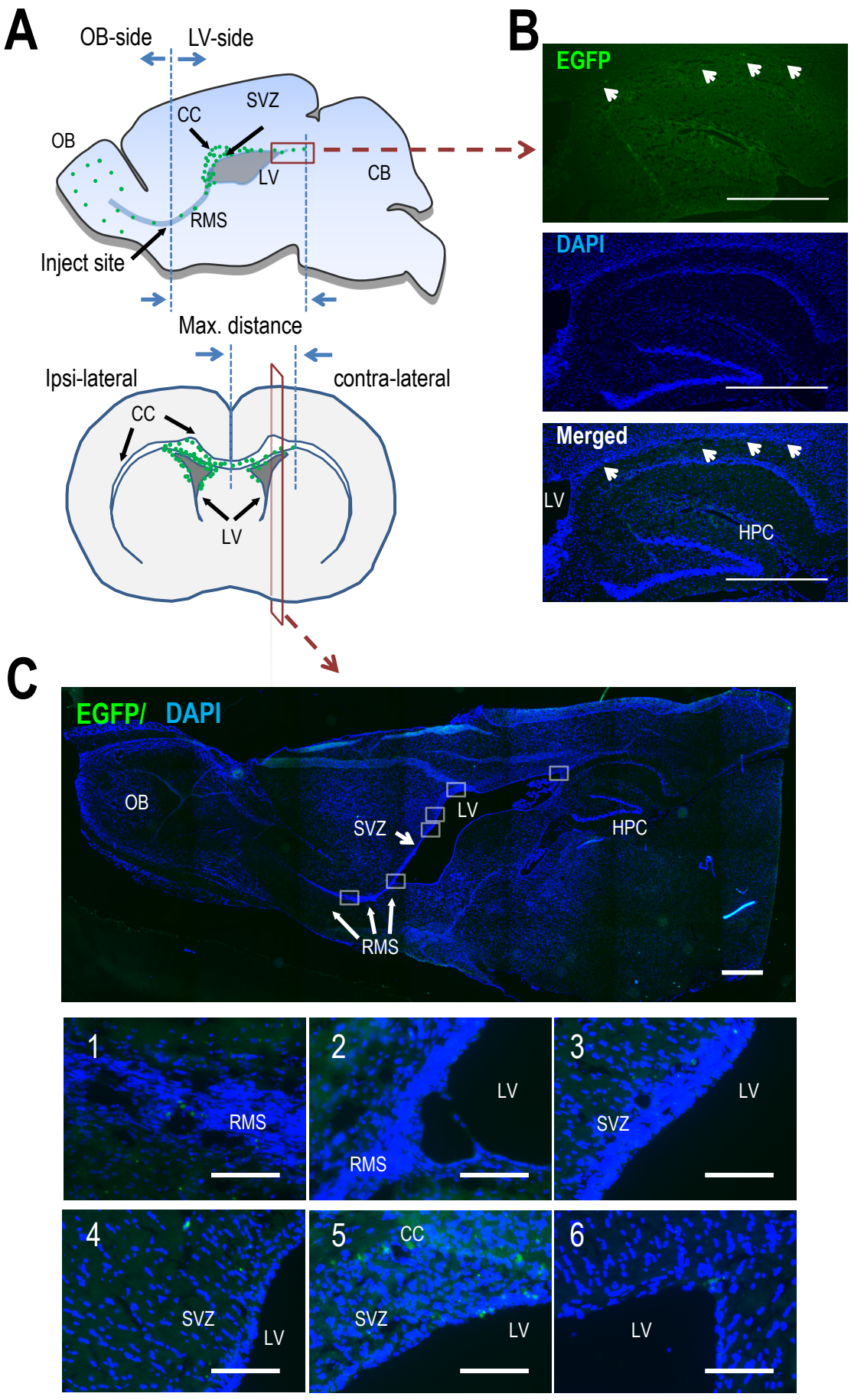


Figure S6. EGFP signals in lateral ventricle wall and the contralateral hemisphere

Legends for Supplemental Figures 1-6

Figure S1. Derivation of human neural stem cells that express EGFP

(A-C) Human embryonic stem cells cultured on feeder cells. The rosettes (B) and then the dissociated single human neural stem cells (hNSCs) (C) were positively labeled with anti-NESTIN and SOX2 antibodies.

(D) Lentivirus structure with the EGFP reporter.

(E-F) Transduction and expression of EGFP in hNSCs, and the enrichment of EGFP positive cells. EGFP: enhanced green fluorescence protein. Scale bar = 50 μm (A, B, C and E)

Figure S2. *In vitro* electrotaxis setup and investigation for different originated neuroblasts

(A) Experimental setup for *in vitro* electrotaxis assay. Cells were seeded in electrotaxis chamber, exposed to electric currents and migration behavior recorded with digital video microscopy.

(B) Cell migration is analyzed using migration speed and directedness value. An example of cell migration from the origin (0, 0) to (x_1, y_1).

(C, D) Electric fields significantly increased migration speed and guided migration of EGFP-hNSCs. (D) Galvanotaxis of EGFP-hNSCs in comparison to that of parental hNSCs, rat neural stem/progenitor cells (r-NSPCs) and mouse neuroblasts (m-neuroblasts).

Data are Means ± SEM from three or more independent experiments. $*P < 0.05$, $**P < 0.01$, when compared to cells not subjected an applied EF.

Figure S3. Intermittent EF stimulation guided migration of hNSCs with minimal cell damage and side effects

(A) Intermittent electric field (EF) stimulation achieved effective guidance the same as that induced in continuous EF stimulation on EGFP-hNSCs for 1 hr in an EF with strength indicated.

(B) Intermittent stimulation has evident advantage over continuous stimulation in that cell viability is significantly improved. Inter.: intermittent; Cont.: continuous. Data are presented as Means ± SEM from three or more independent experiments. Intermittent fields here shown were 1 sec on and 1 sec off.

(C) Measurement positions in an electrotaxis chamber with different chamber thickness to mimic volume conductive tissues. Electric field is as shown. The electrotaxis chamber used was of

dimension length (2.2 cm) and width (1 cm) with chamber thickness of 1 mm (D1-D3) or 2 mm (E1-E3). Data are presented as Means ± SEM from three or more independent experiments.
(D1, E1) Continuous stimulation induced significant changes in the temperature of the culture medium (measured at position 1). Intermittent stimulation reduced the change significantly.
(D2, E2) Continuous stimulation induced significant decrease in pH of the culture medium at the anode side, whereas intermittent stimulation completely prevented the changes.
(D3, E3) Continuous stimulation induced significant increased pH of the culture medium at the cathode side, whereas intermittent stimulation completely prevented the increase.

Figure S4. Electric fields and currents delivered which were stable in live animal brain
(A) Monitoring of the intermittent electrical stimulation in rat brain. Ag/AgCl electrodes at positions A, B, C and D to measure four positions along the RMS. Right panel is the schematic picture showing a carbon electrode and an Ag/AgCl electrode, for delivery and monitoring of the electric currents/fields. The electrodes were insulated with epoxy or plastic tubing and with the tips exposed.
(B) Stable electric currents recorded by the Ammeter.
(C) Electrical resistance between positions A and D. When 0 or 2 Volts of output was applied, the resistance value was registered directly on an ohmmeter between points A and D. When 5 or 7 Volts were applied, the resistance was deduced by R = V/I. R: resistance, V: voltage potential of AD, I: electric current value.
(D, E) The potentials at each point (D) and field strength (E) along the RMS when intermittent electric output was set at 7 Volts. Data are presented as Means ± SEM. In (B) and (C), each value at a single time-point was the mean of 3 values registered with 1-min time interval during the intermittent electrical stimulation (each from 1 rat). In (D) and (E), each value was the mean of 3 independent tests (each from 3 rats).

Figure S5. Electroencephalograph (EEG) monitoring during electrical stimulation and motor function evaluation after stimulation
(A) Various electrodes were implanted in an anesthetized rat.
(B) Schematic top view and lateral view illustrate the positions of stimulating electrodes (dark - i), measuring electrodes (yellow- ii), and EEG recording electrodes (blue - iii).

(C) Representative EEGs during intermittent electrical stimulation (1 sec on and 1 sec off as a cycle) at 4.4 V of output and that pre-/post stimulation.
(D-E) Frequency dependence of the power analysis of EEGs showed no changes in theta and beta waves. Low gamma wave increased however after stimulation. Data are presented as Means ± SEM from 3 rats. *$P < 0.05$ comparing to pre-stimulation.
(F) Motor function evaluation in rats with or without 10 hrs iEF stimulation post-transplantation. Rotarod (up) and Horizontal ladder-walk (down) tests showed no significant changes. Motor function appeared to be affected slightly in day 1 from the control group or sham group, but without significant statistic difference. From day 4 after stimulation, both tests showed the same motor functions between the experimental group, and the control and sham groups.

Figure S6. EGFP signals in lateral ventricle wall and the contralateral hemisphere
(A) Representative sagittal and re-constructed coronal brain sections. Green signals were detected in region close to hippocampus on the side with cell transplanted and the contralateral side of brain 4 months after electrical stimulation. Maximum distance is the distance from inject site to green signals farthest in ipsilateral hemisphere, and from the midline to farthest green signals on the contra lateral brain.
(B) EGFP signals are found far away from the inject site toward the hippocampus along the SVZ.
(C) EGFP signals are found on contralateral, distributed widely in CC, SVZ and RMS. Few green signals are co-labeled blue for DAPI.
Scale bar = 1000 μm (B and upper panel of C) or 100 (C1-6) μm. EGFP: enhanced green fluorescence protein, SVZ: subventricular zone, CC: corpus collosum, RMS: rostral migration stream.

Supplemental Experimental Procedures

1. Derivation of hNSCs and establishment of EGFP-hNSCs

Use of human embryonic stem cells was approved by UC Davis Stem Cell Research Oversight Committee. hNSCs were derived from H9 hESC as described in our previous publication *(Feng et al., 2012)*, then stably transduced with lentiviral vectors containing an EGFP reporter driven by the PGK promoter (EGFP-hNSCs). Cells were then enriched through cell sorting. Medium containing Glutamax, NEAA, N2 (1%), bFGF (20 ng/ml), 0.1% B27 and 10 ng/ml EGF on poly-l-ornithine/laminin coated dishes. To confirm the neural stem cell properties, cells were immunostained with NESTIN (MAB1259, R&D) and SOX-2 (#3579, Cell Signaling). EGFP- hNSCs were induced to differentiate into neurons and astrocytes as described previously and confirmed with staining of $EGFP^+/TUJ1^+$ or $EGFP^+/GFAP^+$ respectively *(Feng et al., 2012)* (Figures 2A-2D and Figure S1).

2. Electrotaxis of EGFP- hNSCs *in vitro*

EGFP-hNSCs were seeded in an electrotaxis chamber in CO_2 independent medium (Invitrogen) plus 1 mM L-Glutamine for 0.5-1 h. Cell migration was recorded using time lapse digital video-microscopy *(Song et al., 2007)* (Figure S2A). Following parameters were used to quantify cell migration: (1) Directedness was determined as the cosine (θ), where θ is the angle between the electric field vector and a straight line connecting the start and end position of a cell. A cell moving directly along the field lines toward the cathode (to the right) would have a directedness value of +1. A cell moving directly along the field lines toward the anode (to the left) would have a directedness value of -1. An average of cosine (θ) yields the directedness value for a population of cells, giving an objective quantification of the direction of cell migration. The cosine (θ) of a population of cells range from −1 to +1. A value close to 0 indicates cells migrate in random direction, whereas a value approaching +1 indicate guided migration of the cell population to the right (the cathode). (2) Migration speed (μm/h): accumulated migrated distance over time. (3) Displacement speed (μm/h): the straight line distance (in red) from the starting point to the final position of cell over time. (4) X-axis distance (μm): the distance which is projected on the X-axis (parallel to the electric field direction) from the starting point to the final position of cell's migration (Figure S2B).

3. Stimulation schemes for guidance of cell migration with tolerable detrimental effects

Continuous direct current (DC) electric fields can induce changes in temperature and pH in the culture medium, which are harmful to cells (Figure S3B). To minimize these effects, we optimized intermittent DC electric field stimulation scheme that effectively guide cells migration with minimal changes in temperature and pH. Temperature was recorded with a needle temperature probe (Physitemp unit TH-5, probe MT-29/2, Clifton, NJ) in the center of culture medium for 1 hr. pH values at both sides of the chamber were directly recorded for 4 hrs (Figures S3A, S3C-S3E3).

4. Design of electrodes for implantation into the brain *in vivo*

Carbon rod (Ø 280 μm) was used for current delivery electrode, with the tip exposed on a wedge face (30-40 degree) and other part insulated with epoxy. A silver wire insulated with plastic tubing (total Ø 200 μm) except the exposed tip of silver/ silver chlorides (Ag/AgCl) was used as the measuring electrode. The exposed tip was positioned in front of the carbon incline face (Figure 4A). The delivery and monitoring electrodes could be paired and implanted into brain tissues together to form a feedback-circuitry in brain tissue. An ammeter was used in the circuit for measuring the magnitude of electric current, a voltmeter connected to the Ag/AgCl electrodes for monitoring of voltage, and an ohmmeter for measuring the electric resistance. The output of power supply could be adjusted accordingly.

5. Design of a programmable stimulator for deliver electrical stimulation *in vivo*

We developed a programmable stimulator and fixed it on rat head for stimulation *in vivo* with rat free movement (Figure 3E). A 4 volts cell battery was assembled with a programed chip. The stimulation program was preset as 1 sec on and 1 sec off once the stimulation condition was initiated. When the stimulator was assembled to the carbon electrodes and electric circuit connected, the voltage potential tested from the carbon electrodes was 4 volts.

6. Implantation of electrodes in the brain

The Institutional Animal Care and Use Committee at the University of California at Davis approved all animal procedures in this study. Sprague-Dawley rats (Harlan, weighing 310–350 g) were used in the *in vivo* experiments. At least 7 days prior to surgery, animals were housed in individual cages in a temperature (22 °C) and humidity-controlled (50% relative) animal facility with unrestricted access to food and water and a 12 h light/dark cycle.

For surgery rats were anesthetized with 4% isoflurane in a carrier gas mixture of nitrous oxide/oxygen (2:1 ratio), intubated, and mechanically ventilated with a rodent volume ventilator (Harvard Apparatus model 683, Holliston, MA). A surgical level of anesthesia was maintained with 2% isoflurane. Rats were mounted in a stereotaxic frame, a scalp incision made along the midline, and four ~2 mm burr holes through the skull were performed on the right frontal and parietal bones (centered at X1=8.0, X2=5.0, X3=2.0, X4=-1.0 mm, anterior from bregma; and Y1=1.0, Y2=1.0, Y3=1.0, Y4=1.5 mm, lateral from mid-line, respectively). Dura was then carefully cut open under a surgical microscope. Two assembled carbon-Ag/AgCl electrodes and two single Ag/AgCl electrodes were arranged according to the four coordinates (X, Y as described at the center of four burr holes, Z1=-4.0, Z2=-6.0, Z3=-5.0, Z4=-4.0 mm, respectively) and fixed on a metal bar which clamped on the xyz-manipulator (Figure 3C). After alignment with Bregma via X, Y and Z controls, the metal bar lifted up slightly to locate the points of implant. Individual electrodes were adjusted slightly to avoid damaging cortical vessels. Electrodes were lowered and inserted into the brain tissue at a rate of 1 mm/min.

Following anesthesia, temporalis muscle temperature was monitored with a 29-gauge temperature probe (Physitemp unit TH-5, probe MT-29/2, Clifton, NJ) between the skull and temporalis muscle. Rectal temperature was monitored and maintained during surgical procedures by a feedback temperature controlled pad (CWE model TC-1000, Ardmore, PA).

7. Delivery of electric stimulation to the brain *in vivo*

The electric circuitry was completed when the implanted electrodes were connected with the power supply or battery and voltmeter/ammeter respectively. When electric current was delivered, actual in tissue currents, voltage and resistance were recorded at indicated measuring positions (Figure 1C and Figure S4A).

Ten rats were used to evaluate the electric delivery in brain *in vivo* (Figure S4). Animals were divided into three groups: three rats for continuous electric stimulation at 2, 5 or 7 volts for two hrs. Four rats for intermittent electric stimulation at strength of 0, 2, 5 or 7 volts, respectively (Figures S4B-S4C). The remaining three rats were used for measurements of electric fields at 7 volts of intermittent electric stimulation for two hrs (Figures S6D-S6E). Animals were observed for indications of seizures or abnormal activity during electric stimulation and during long-term

follow-up. Similar evaluation for the electric delivery in brain on the animals following transplantation of EGFP-hNSCs was performed (Figure 1D).

8. EEG during electric field stimulation

Six rats were used for this part of experiment. A level of anesthesia was maintained with ~1.5% isoflurane for minimizing anesthesia effect on EEG. The two assembled carbon-Ag/AgCl electrodes were used for electric stimulating and recording, positioned as previous described (X1=8.0, Y1=1.0, Z1=-4.0 mm, and X4=-1.0, Y4=1.5, Z4=-4.0 mm). Two skull screws (2.1mm diameter, 6.0 mm length) were placed into burr holes through the skull while keeping dura intact, 3 mm rostral to Bregma on left and 1 mm rostral to Bregma on right (see details in Figure S5A-S5B). In three rats, the screws were connected to EEG recording device (Grass Instruments model 7P5, Quincy, MA) for EEG recording pre-/ during/ post- intermittent DC electric field stimulation at 4.4 volts for 5 hrs using a programmable power supplier (Quadtech, 42006-300-8, CA). The other three rats experienced all the procedures except for electric stimulation. EEG data was filtered between 1 and 3kHz, digitized at 100 Hz, and stored on a computer. Frequency power analysis of EEG was measured using standard fast Fourier transform (Polyview 16 version 1.1 software; Grass Instruments, Quincy, MA).

9. Transplant of EGFP-hNSCs and animal groups

EGFP-hNSCs were prepared at 50,000 cells/μl in NSC medium. At a speed of 1ul/min, 5 ul cell suspension was injected to the middle point of RMS (X=3.7 mm, anterior from bregma; Y=1.2 mm, lateral from mid-line; Z= -5.9 mm). Burr hole was then filled with bone wax and scalp incision was sutured. Rats were placed into clean cages for recovery (1 rat/ cage post-operation).

After transplant of cells, animals were randomly divided into three groups: control group (no electrodes implant, n=7), sham group (electrodes implant with no electric stimulation, n=7) and electric stimulation group (n=10). For the rats of long-term survival, animal care was continued including the observation on vocalizations, seizures, hemiplegic paralysis and body weight. For sham-cell control, five μl neurobasal medium with no cells was injected (n=3, euthanized at 34 hrs, 3 weeks and 4 months post injection, respectively).

10. Delivery of electric currents to guide migration of transplanted EGFP-hNSCs

Twenty-four hours post cell transplant, electrode implantation was performed. The two assembled carbon-Ag/AgCl electrodes were used for electric stimulating and recording, positioned (X1=8.0, Y1=1.0, Z1=-4.0 mm, and X4=-1.0, Y4=1.5, Z4=-4.0 mm) (Figure 3D). Programmed power supply (Quadtech, 42006-300-8, CA) was connected to carbon electrodes and an ammeter for testing the electric current of the circuit. Ag/AgCl electrodes were connected to voltmeter and ohmmeter (Figure 1D). The output of power supply was adjusted for target electric field strength at 50-70 mV/mm during the 10 hrs' intermittent electric field stimulation. Parameters were read from voltmeter, ohmmeter and ammeter every 15 min.

Anesthesia was decreased to 1.5% isoflurane during intermittent electric field stimulation. Every 2 hrs, 1 ml saline was injected subcutaneously for fluid replacement. Saline was occasionally dropped on the burr holes to maintain moisture of brain tissue. In some groups with cell transplant, rats were euthanized immediately post stimulation (n=3), three weeks post stimulation (n=3) or four months post stimulation (n=1).

Another three rats in the electric stimulation group were fitted with the programmed stimulator and survived for two days before euthanization. Two carbon electrodes were implanted into rat brain (X1=8.0, Y1=1.0, Z1=-4.0 mm, and X4=-1.0, Y4=1.5, Z4=-4.0 mm) (Figure 3E). When the programmed stimulator was connected and fixed to the carbon electrodes, paraffin was used to seal the stimulator in a plastic cover which was secured over the exposed skull with cyanoacrylate glue. Animal care was continued including observation of vocalizations, seizures, hemiplegic paralysis and body weight until time of euthanasia.

11. Fixation of the brain, tissue section and immunohistochemistry

Rats were euthanized by injection of sodium pentobarbital (100 mg/kg, ip), followed by transcardial perfusion with 100 ml of 0.1 M sodium phosphate buffer (PB) (pH=7.4), then perfusion fixation with 300 ml of 4% paraformaldehyde at 4 °C (pH=7.4). Brains were removed and additionally fixed for 1 hr. in 4% paraformaldehyde at 4°C. Brains were cryoprotected in 10% sucrose solution for 1 day followed by 2 days in a 30% sucrose solution, embedded with optimal cutting temperature compound (O.C.T. Compound, Tissue-Tek), and 8 um sagittal sections were cut on a freezing cryostat. Sections were stored at -80°C before immunohistochemistry.

Brain sections were rehydrated in phosphate buffered saline (PBS) for 10min and then blocked with the blocking buffer containing 2% goat serum (Gibco), 1% BSA (Sigma), 0.1%

cold fish skin gelatin (Sigma), 0.1% Triton X-100 (Sigma) and 0.05% Tween 20 (Sigma). Primary antibodies were incubated at room temperature for one hour followed by the application of second antibodies (Alexa Fluor 594 nm, Invitrogen). After washing with PBS, the tissue sections were mounted with an anti-fade mounting media/DAPI mounting media. The primary antibodies used were rabbit anti- human SOX2 (#3579, Cell Signaling, 1:200); mouse anti-human Nuclei (MAB1281, Millipore, 1:200); rabbit anti- GFAP (AB5804, Millipore, 1:200); rabbit anti-TUJ1 (ab24629, Abcam, 1:200); mouse anti- Neu-N (MAB377, Millipore,1:500); Goat anti-IBA1 (ab107159, Abcam, 1:1000); Mouse anti-MBP (ab24567, Abcam, 1:500).

12. Motor functional evaluation

Animals were trained to criterion on the behavioral tasks two days prior to cell transplant, with the final day serving as the pre-surgery baseline. Electric stimulation or controlled or sham interventions were applied 24 hours post cell transplant. Animals were first tested on the rotarod followed by horizontal ladder on post electric stimulation days 1, 4, 7, 11, and 15.

Rotarod test

Duration that the animals could maintain ambulatory activity on the rotarod was measured to evaluate coordination, balance, and endurance by trained personnel blinded to the identity of the animal groups as previously described *(Hamm et al., 1994)*. Animals were placed on a 4-lane rotarod (Ugo Basile model 7700; Comerio, Italy) with a constant speed (12 rpm, 3.6 cm/sec). 120 sec was the maximum time allowed on the rotarod. Rats were tested three times on each testing day, with a 5 min rest period in between trials, and the mean latency of three trials was calculated for each animal.

Horizontal ladder-walk test

Fine motor coordination was assessed with the ladder-walk test. Rats were trained to walk across an elevated, horizontal, level ladder 1.1m length X 10cm width. This ladder was bounded between 20cm high transparent Plexiglas walls on both sides. The ladder steps consisted of 2.5mm diameter rungs, with 1.25cm spaces between them. The rats were trained to escape from an aversive auditory stimulus by walking across the ladder in order to enter a goal box at the opposite end. The noise was terminated when the rat entered the goal box at the opposite end of the ladder. The test consisted of 3 such trials. Rats remained in the escape box for 15 sec after

each trial. The number of "slips" in which a leg extended down through the rungs without grasping the rung was counted. The total time required for the rat to traverse the ladder was also recorded. The rats were trained on the ladder for 2 days before the electrical stimulation and then baseline was recorded prior to the surgery on the day of the electrical stimulation. The test was performed on days 1, 4, 7, 11 and 15 post electrical stimulation. Each trial was recorded using a video camera for later scoring and analysis.

13. Migration analysis for EGFP signals in rat brain

Brain sections were examined and analyzed with a fluorescent microscope system (Keyence, model BZ-9000 (BIOREVO); Itasca, Il). For each individual rat brain, the center sagittal section was defined as most clearly showing the stem cell injection track and with the highest accumulation of EGFP signals. Two additional sections which was +16um and -16um away respectively were also selected for further detailed analysis. Thus 3 sections for each brain (hemisphere) were chosen for initial detailed analysis of cell migration.

For the animals euthanized immediately post electric stimulation (n=3 for each group - control, sham and electric stimulation), semi-quantification was performed to assess the EGFP-cells distribution. In each section, all the EGFP positive cells on both OB- and LV-region were counted as 100%. The percent of EGFP positive cells on LV-region was detailed defined based on the cell number: 0% ~ no signals (< 5/section), 1% ~ find very few scattered green signals (< 10/section), 5% ~ (<100/section), 10% ~ (>200/section). Furthermore, the EGFP positive cells number in each brain section was counted under microscope individually. The maximum migration distance was obtained directly under the microscope for each section on both regions. If the maximum distance was within 200um from inject site, we considered as no migration. For the animals euthanized three weeks post stimulation (n=3 for each group) and four months post stimulation (n=1 for each group), the same analysis was performed for EGFP percent on LV-region and maximum distance on LV-region. The contra-lateral hemisphere of these rats were also cut and reserved as sagittal sections, for assessing the EGFP migration on contra-lateral (Figure S6C). Since only 1 rat/ group in the four-months survival group, fifteen sections were analyzed from each rat (3 sections for each primary cellular antibody NEUN, GFAP, IBA 1, MBP, and 3 for control) rather than 3 sections.

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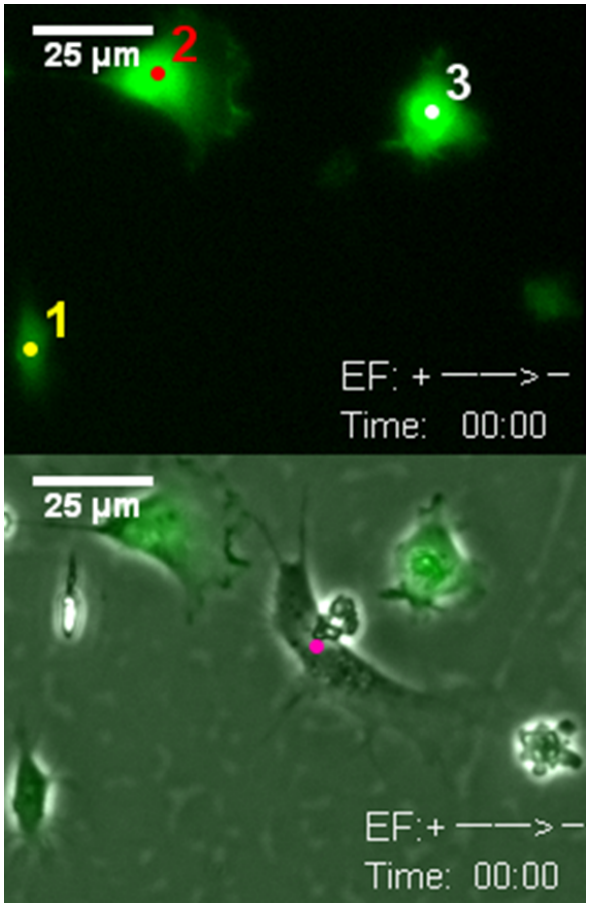
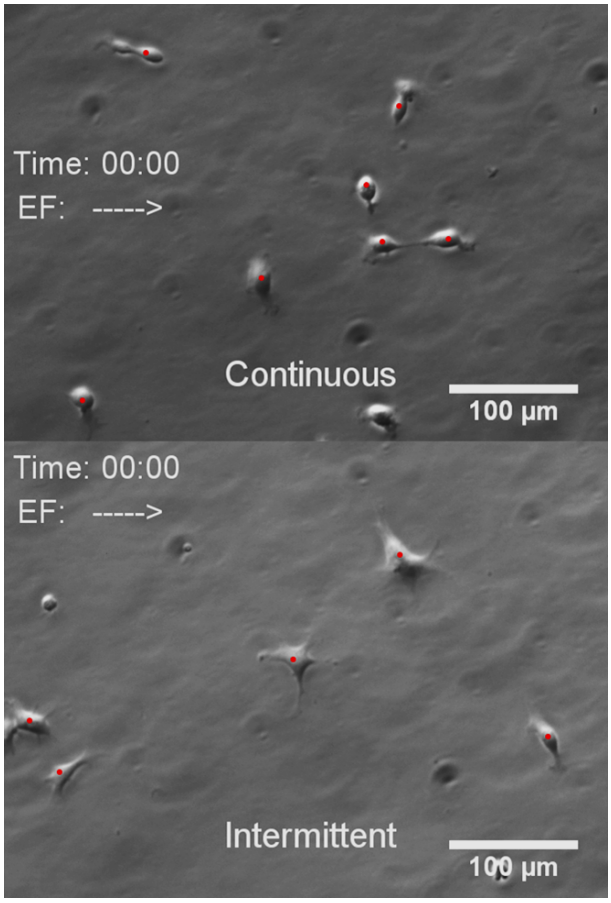
Supplementary Videos legends



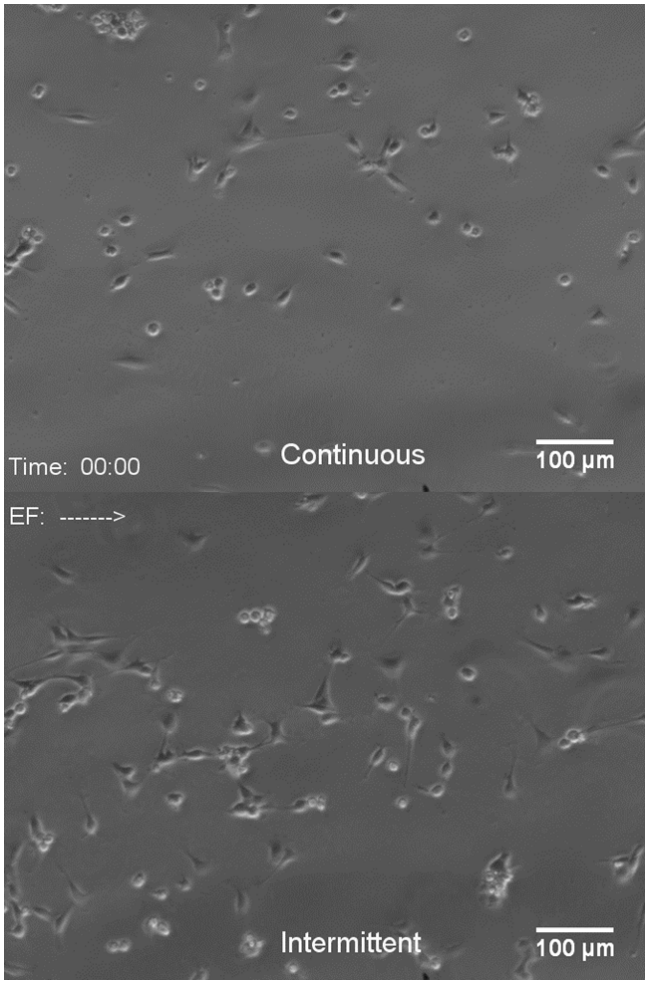
Video S1. EGFP-hNSCs showed robust cathodal migration in EF as that of non-transfected hNSCs

Upper panel: EGFP positive hNSCs shown in GFP channel. Lower panel: Merged video shows EGFP positive and negative hNSCs in the same electric field (EF). Cells with EGFP migrate as robust as the cells not transfected with EGFP. Cells migrated directionally to the cathode in the first 100 min and then migrated to the new cathode after the reversal of EF in the second 100 min. EF = 250 mV/mm. Polarity, time (hour: min) and scale are indicated. EGFP: enhanced green fluorescence protein, hNSCs: human neural stem cells.



Video S2. Electrotaxis of hNSCs in continuous and intermittent direct current EFs

Robust cathodal migration of hNSCs in both continuous (upper panel) and intermittent (lower panel) direct current EFs. EF = 200mV/mm. Polarity, time (hour: min) and scale as shown. hNSCs: human neural stem cells; EF: electric field.



Video S3. Safety advantage of intermittent EF stimulation over continuous EF stimulation

In an intermittent EF (lower panel), cells survived, whereas died rapidly in continuous EF stimulation (upper panel). EF = 700 mV/mm. Polarity, time (hour: min) and scale as shown. EF: electric field.