Optochemogenetic Stimulation of Transplanted iPS-NPCs Enhances Neuronal Repair and Functional Recovery after Ischemic Stroke

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Cell transplantation therapy provides a regenerative strategy for neural repair. We tested the hypothesis that selective excitation of transplanted induced pluripotent stem cell-derived neural progenitor cells (iPS-NPCs) could recapitulate an activity-enriched microenvironment that confers regenerative benefits for the treatment of stroke. Mouse iPS-NPCs were transduced with a novel optochemogenetics fusion protein, luminopsin 3 (LMO3), which consisted of a bioluminescent luciferase, *Gaussia* luciferase, and an opsin, *Volvox* Channelrhodopsin 1. These LMO3-iPS-NPCs can be activated by either photostimulation using light or by the luciferase substrate coelenterazine (CTZ). *In vitro* stimulations of LMO3-iPS-NPCs increased expression of synapsin-1, postsynaptic density 95, brain derived neurotrophic factor (BDNF), and stromal cell-derived factor 1 and promoted neurite outgrowth. After transplantation into the ischemic cortex of mice, LMO3-iPS-NPCs differentiated into mature neurons. Synapse formation between implanted and host neurons was identified using immunogold electron microscopy and patch-clamp recordings. Stimulation of transplanted cells with daily intranasal administration of CTZ enhanced axonal myelination, synaptic transmission, improved thalamocortical connectivity, and functional recovery. Patch-clamp and multielectrode array recordings in brain slices showed that CTZ or light stimulation facilitated synaptic transmission and induced neuroplasticity mimicking the LTP of EPSPs. Stroke mice received the combined LMO3-iPS-NPC/CTZ treatment, but not cell or CTZ alone, showed enhanced neural network connections in the peri-infarct region, promoted optimal functional recoveries after stroke in male and female, young and aged mice. Thus, excitation of transplanted cells via the noninvasive optochemogenetics treatment provides a novel integrative cell therapy with comprehensive regenerative benefits after stroke.

Key words: functional recovery; iPS cells; neuronal repair; optochemogenetics; optogenetics; stroke

Significance Statement

Neural network reconnection is critical for repairing damaged brain. Strategies that promote this repair are expected to improve functional outcomes. This study pioneers the generation and application of an optochemogenetics approach in stem cell transplantation therapy after stroke for optimal neural repair and functional recovery. Using induced pluripotent stem cell-derived neural progenitor cells (iPS-NPCs) expressing the novel optochemogenetic probe luminopsin (LMO3), and intranasally delivered luciferase substrate coelenterazine, we show enhanced regenerative properties of LMO3-iPS-NPCs *in vitro* and after transplantation into the ischemic brain of different genders and ages. The noninvasive repeated coelenterazine stimulation of transplanted cells is feasible for clinical applications. The synergetic effects of the combinatorial cell therapy may have significant impacts on regenerative approach for treatments of CNS injuries.

Introduction

Stroke is a leading cause of human death and long-term disability. While clinical trials have failed to recapitulate therapeutic

benefits of neuroprotective treatments, regenerative treatments, such as cell transplantation, have provided a promising strategy for tissue repair and functional recovery after brain injuries (L.

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Wei et al., 2005; Hicks et al., 2009; N. Wei et al., 2013; Trounson and McDonald, 2015). Induced pluripotent stem (iPS) cells are particularly amenable for treating neurological diseases given their accessibility, differentiation potency, and clinical relevance. Transplantation of iPS-derived neural progenitor cells (iPS-NPCs) after stroke in animal models shows potentials of cell replacement, increased trophic support, and functional improvements (Mohamad et al., 2013; Chau et al., 2014). Nevertheless, stem cell-based therapy is still nascent with ambiguous goals regarding their clinical efficacy and therapeutic mechanisms. Neuronal differentiation of transplanted cells and integration into the existing neural circuitry are key steps for effectual neuronal repair. Meanwhile, trophic support, such as increased BDNF, can augment functional recovery after injury (Kurozumi et al., 2005; van Velthoven et al., 2013). Strategies that enact these regenerative mechanisms should significantly improve therapeutic benefits of cell-based therapies.

Appropriate neuronal activity is essential for neuronal differentiation/maturation and cell survival; it is also critical for synaptic plasticity and specific functions (Zhang and Poo, 2001; Murphy and Corbett, 2009; Duan et al., 2017). Like immature neurons in the developing brain, the fate and functional phenotype of iPS-NPCs in the adult brain depend on the microenvironment in the transplantation site. Electrical activity and resultant cellular signaling act as critical cues for the differentiation of NPCs (Berger et al., 1981; Zhang and Poo, 2001). Excitation of cultured primary and stem cell-derived neurons promotes neuronal morphogenesis (Wan et al., 2010; Kobelt et al., 2014). Increased neuronal activity elicits activity-dependent release of regenerative neurotrophins (Kolarow et al., 2007; Matsuda et al., 2009; Park and Poo, 2013). We hypothesized that selective stimulation of transplanted cells could create an enriched microenvironment and activity-dependent neuroplasticity in the poststroke brain for optimal neural network repair and functional recovery after stroke.

Optogenetics has transformed from an interrogative tool to a therapeutic means for regulating neuronal activity and restoring neural circuits in models of neurological diseases (Paz et al., 2013; Cheng et al., 2014; Cunningham et al., 2014; Steinbeck et al., 2016). The expression of light sensitive excitatory and inhibitory channels provides cell type-specific regulations with high spatial and temporal precision. In a mouse model of stroke, repeated optogenetic stimulation of the endogenous neurons in the motor cortex increased neurotrophin levels, resulting in improved functional recovery (Cheng et al., 2014). Optogenetic stimulation of neural grafts after stroke showed downregulation of inflammatory response and improved motor activity (Daadi et al., 2016). Whether the optogenetics approach incorporated in a cell transplantation therapy could improve host-graft connectivity, neural network repair, and other benefits after stroke has not been specifically tested.

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In the contest of clinical translation, optogenetics face a noteworthy restriction: the invasive implantation of an optical fiber. Moreover, light scatters within brain tissues, such that it is reduced to nearly negligible intensity within $\sim 200 \mu\text{m}$ from the tip of the fiber (Yona et al., 2016), resulting in limited spatial control of cells in the human brain. Here, we exploited a fusion protein, luminopsin 3 (LMO3), which comprises a modified variant of the bioluminescent protein, *Gussia* luciferase (sbGLuc), tethered to an excitatory light-sensitive channel, *Volvox* Channelrhodopsin 1 (VChR1). iPS-NPCs expressing LMO3 can be excited by both physical and biological light sources (hereafter referred to as optochemogenetics), providing a novel noninvasive combinatorial stimulation method in a cell transplantation therapy. The combination therapy was tested *in vitro* and after a focal ischemic stroke in mice of different genders and ages, the optochemogenetics treatment show enhanced regenerative benefits and offer a greater potential for clinical applications.

Materials and Methods

iPS cell cultures and neuronal differentiation

Mouse WP5 iPS cells were purchased from Stemgent. Undifferentiated iPS cells were cultured in 0.1% gelatin-coated T25 flasks in stem cell culture media consisting of DMEM (Corning), 10% FBS (Invitrogen), 10% NCS (Sigma-Aldrich), 2 mM glutamine (Stem Cell Technologies), 0.1 mM nonessential amino acids (Stem Cell Technologies), 55 μM 2-mercaptoethanol (Sigma-Aldrich), 2000 U/ml LIF (Miltenyi Biotec), and 100 U/ml penicillin/streptomycin (Corning). For neuronal differentiation, iPS cells were differentiated in suspension culture with the “4–/4+” protocol (4 d without and then 4 d with 1 μM all-trans retinoic acid [RA] in LIF-free medium) under rotary condition as previously described (Bain et al., 1995). Briefly, cells were dissociated from the growth flasks by trypsinization with 0.25% trypsin-EDTA (Invitrogen) for 2 min. Then cells were seeded onto standard 10 cm bacterial Petri dishes in stem cell culture media lacking LIF and β -mercaptoethanol. Within the first day, the cells formed embryoid bodies in suspension culture. In the last 4 d, 500 nM of all-trans RA (Sigma-Aldrich) was added to the media. After 4–/4+ culture, the iPS cell-differentiated iPS-NPCs were ready to be dissociated and harvested for transplantation or *in vitro* terminal differentiation on poly-D-lysine/laminin-coated dishes in modified SATO media (Bottenstein and Sato, 1979). For electrophysiology recordings, iPS-NPCs were plated on a layer of astrocytes for longer terminal differentiation up to 12 d after the “4–/4+” neural induction.

Optogenetics gene modification of mouse iPS cells and virus infections

Luminopsins are fusion proteins of luciferase and opsin that can be activated by either extrinsic physical light (i.e., laser and LED) or by intrinsic biological light with chemical substrate. To enable iPS-NPCs to be depolarized by both blue light and intrinsic luminescence, we created the episomal backbone carrying an LMO3 construct (pLenti-CAG-sbGLuc-VChR1-EYFP-IRES-PuroR). The luciferase sbGLuc in the LMO3 construct allows for its activation by the luciferase substrate coelenterazine (CTZ), which in turn activates VChR1 channels expressed in the cell membrane, resulting in membrane depolarization.

To express with an EYFP tag using a single plasmid, pEGIP (a gift from Linzhao Cheng; Addgene, plasmid #26777) was used. The GFP-IRES-Puro sequence was modified by restriction enzyme digestion and ligation to replace the GFP with the LMO3-EYFP sequence. This optogenetics and chemogenetics channel was amplified from plasmids in our previous reports (Berglund et al., 2013, 2016b). LMO3-EYFP lentivirus was added into the iPS cells seeded at 5×10^5 cells per well *in vitro*. At 4 d after infection, cells were subjected to puromycin selection at 15 $\mu\text{g}/\mu\text{l}$. Positive clones were selected, and then the highest EYFP-expression colonies were expanded to form stable LMO3-iPS cells that coexpressed the EYFP tag with several pluripotent stem cell markers SSEA-1, Oct 3/4, or Nanog (Fig. 1A). The stable cell line (LMO3-iPS) was maintained in culture medium containing puromycin (0.5 $\mu\text{g}/\text{ml}$).

Figure 1. Expression of LMO3 in iPS-NPCs and functional activities of derived neurons. **A**, A stable iPS cell line expressing luciferase (LMO3-iPS cell line) was generated by transduction of the modified LMO3 construct (pLenti-CAG-sbGLuc-VChR1-EYFP-IRES-PuroR) and subsequent puromycin selection. Immunocytochemistry showed that LMO3-iPS cells express pluripotent stem cell markers SSEA-1, Oct 3/4, and Nanog, which colabeled with fluorescent protein EYFP tagged to the LMO3 construct. **B**, Differentiated iPS-NPCs showed neuronal morphology of neurites and axons. Green is from EYFP fluorescence in transfected cells. **C**, In whole-cell recording of current clamp on LMO3-iPS-NPC-derived neuron-like cells, current injection of 30 and 60 pA induced membrane depolarization and repetitive action potentials. **D**, Demonstration of blue light induced trains of action potential in LMO3-iPS-NPC-derived neurons. **E**, Bath application of CTZ (6 μM) significantly increased the firing frequency in LMO3-expressing cortical neurons compared with vehicle control. The firing rate in the cell increased from 2.6 ± 0.9 Hz to 4.3 ± 0.5 Hz ($p < 0.05$, t test, $n = 5$ each). **F**, In voltage-clamp recording, blue laser light (473 nm, 33.6 mW) stimuli evoked inward currents resembling EPSCs in LMO3-iPS-NPC-derived neurons. Light stimulation effect in the same cell was completely blocked by the AMPA and NMDA receptor antagonists CNQX and AP5. Different cells were recorded in **D–F**.

The point mutation, D248A, as well as a new AfeI restriction site, was introduced into the VChR1 moiety of LMO3 using a commercial mutagenesis kit following the manufacturer's instructions and their online primer design software (QuikChange XL; Agilent Technologies). First, the LMO3 cassette in the pcDNA3.1/CAG vector (Berglund et al., 2016a) was moved into the pGP/CMV vector (Addgene, plasmid #40753, a gift from Douglas Kim) using the unique NheI/BsrGI restriction sites, and then site-directed mutagenesis was performed. The correct sequence was confirmed by an AfeI restriction enzyme analysis as well as DNA sequencing. The mutated LMO3/D248A cassette was put back to the original pcDNA3.1/CAG vector using the same restriction sites and transfection-grade plasmids were prepared using a commercial kit (PureLink Midiprep Kit; Invitrogen). For electrophysiological recordings and a plate reader assay *in vitro*, human embryonic kidney 293 cells were transiently transfected via lipofection following the conventional method (Berglund et al., 2016b) and manufacturer's instructions (Lipofectamine 2000; Invitrogen). For *in vivo* experiments, a stably transfected iPS cell line was established by lipofection followed by antibiotic selection using the neomycin resistance gene in the pcDNA3.1/CAG vector. In another control experiment, iPS cells were transduced with a recombinant adeno-associated viral (AAV) vectors pseudotyped with AAV2/9 carrying the channelrhodopsin 2 (ChR2) gene from the pAAV/CaMKII α -hChR2/H134R-mCherry-WPRE plasmid in Emory Viral Core. ChR2 alone lacked luciferase, serving as a negative control for CTZ administration.

For the expression of LMO3 in naive animals, AAV vectors carrying the LMO3 gene under control of the human synapsin-1 promoter were similarly produced and injected into the sensorimotor/barrel cortex (-2.54 mm AP, 3.5 mm ML, 0.4 mm DV, 30° angle) using a Hamilton syringe on a stereotaxic platform ($1 \mu\text{l}$ or $\sim 1 \times 10^7$ viral genomes). Three to 4 weeks after infection, animals were subjected to *in vivo* tests and killed by overdose of isoflurane and decapitated for brain dissection followed by tissue and cellular examinations.

Isolation of total RNA and RT-PCR

Total RNA from LMO3-iPS-derived neurons was isolated according to the manufacturer's instructions (Invitrogen). In brief, the RNA samples ($1 \mu\text{g}$) were reverse transcribed in $20 \mu\text{l}$ of a reaction mixture containing $2\times$ RT buffer and $20\times$ RT enzyme mix at 37°C for 60 min. The samples were then incubated at 95°C for 5 min and transferred to 4°C . RT product ($1 \mu\text{l}$) was subjected to PCR amplification with 10 pmol primer, $10\times$ standard Taq reaction buffer, 10 mM dNTP, and 0.625 unit Taq polymerase in $25 \mu\text{l}$ PCR buffer (New England Biolabs). PCR primers were used as follows ($5'$ – $3'$): stromal cell-derived factor 1 α (SDF-1 α): GCATAAA-GACACTCCGCCAT (forward) and TGAGGAGAATGGGGATGAAG (reverse); synapsin-1: CAGCACAACATACCCTATGC (forward) and GGTCTTCCAGTTACCCGACA (reverse); postsynaptic density 95 (PSD95): GGCACCGACTACCCACAG (forward) and AACACCATTGACCGACAGGA (reverse); 18s: GACTCAACACGGGAAACCTC (forward) and ATGCCAGAGTCTCGTTTCGTT (reverse). PCR mixtures were heated to 95°C for 10 min and cycled 30–37 times for each primer; cycles consisted of 95°C for 15 s, 60°C for 1 min, and 72°C for 30 s. After additional incubation at 72°C for 10 min, the PCR samples were transferred to 4°C . PCR products were subjected to electrophoresis in 2% agarose gel with ethidium bromide. Relative intensity of a PCR band was analyzed using InGenius3 manual gel documentation systems (Syngene).

Immunocytochemical and immunohistochemical staining

Cultured cells were fixed with 4% PFA in PBS and postfixed with 2:1 mixture of ethanol:acetic acid for 5 min, then permeabilized with 0.2% Triton X-100 and blocked with 1% fish gelatin. Cells were incubated overnight at 4°C with primary antibodies against GFP (1:200; Novus Biologicals); used to stain for the EYFP tag in LMO3, NeuN (1:400; Millipore), β -III tubulin (Tuj-1, 1:400; Covance), synapsin-1 (1:400; Millipore), neurofilament (NF, 1:400; Millipore), CaMKII (1:400; Millipore), FoxG-1 (1:200; Abcam), SEEA-1 (1:200; Abcam), Nanog (1:100; Santa Cruz Biotechnology), Oct 3/4 (1:100; Santa Cruz Biotechnology), vesicular glutamate transporter 1 (VGLUT1, 1:200; Abcam), and vesicular glutamate transporter 2 (VGLUT2, 1:200; Abcam). For the secondary

antibodies, either AlexaFluor-488 (1:100; Invitrogen) or Cy-3 (1:400; Jackson ImmunoResearch Laboratories) conjugated antibody against the respective IgG was used. DAPI within the DAPI-Vectashield (Vector Labs) was used to stain cell nuclei.

For immunohistochemistry, animals were subjected to cardiac perfusion with PBS followed by 4% PFA in PBS. Fixed brains were sectioned at $10\text{-}\mu\text{m}$ -thick coronal sections with a cryostat (Leica Microsystems). Brain sections were postfixed in 10% buffered formalin for 10 min followed by 2:1 mixture of ethanol:acetic acid for 5 min. Standard staining protocols were then followed for GFP (1:200; Novus Biologicals), NeuN (1:400; Millipore), synapsin-1 (1:200; Millipore), glucose transporter-1 (Glut-1, 1:400; Millipore), and myelin basic protein (MBP, 1:400; Millipore). The GFP antibody was used to stain for the EYFP tag in LMO3. For BrdU (1:400; AbD Serotec) staining, slides were fixed by cold methanol followed by hydrochloric acid (HCl) treatment for 1 h. Then standard staining procedures were followed. Pictures were taken by using a fluorescence microscope (BX61; Olympus) or a laser scanning confocal microscope (Carl Zeiss) along the length of the penumbra region defined morphologically as the region just outside the stroke core. The colabeling was confirmed using the confocal microscope (FV1000, Olympus). For systematic random sampling in design-based stereological cell counting, six coronal brain sections per mouse were selected, spaced $90 \mu\text{m}$ apart across the same ROI in each animal. For multistage random sampling, six fields per brain section were randomly chosen in the peri-infarct/penumbra region of the brain. The fluorescence density was measured using National Institutes of Health ImageJ software.

Western blot analysis

Proteins were extracted from iPS-NPC-derived neurons using the lysis buffer containing the protease inhibitor mixture containing AEBSE, aprotinin, bestatin, E-64, leupeptin, and pepstatin A (1:100; Sigma-Aldrich). Protein concentrations were quantified by the BCA assay. Protein ($30 \mu\text{g}$) from each sample were loaded into a gradient gel and run at constant current until protein markers had adequately separated. They were transferred onto PVDF membranes that were then probed by using standard protocols. Primary antibodies, synapsin-1 (1:1000; Cell Signaling Technology), PSD95 (1:500; Cell Signaling Technology), BDNF (1:500; Santa Cruz Biotechnology), and mouse β -actin antibody (1:6000; Sigma-Aldrich) were applied overnight at 4°C . Alkaline phosphatase-conjugated secondary antibodies were applied for 1–2 h at room temperature. Alkaline phosphatase-conjugated antibodies were developed by using NBT-BCIP solution. The intensity of each band was measured and subtracted by the background using National Institutes of Health ImageJ software. The expression ratio of each target protein was then normalized against β -actin.

Calcium imaging in cultured iPS-NPCs and neurons

iPS-NPCs were loaded with the fluorescent Ca^{2+} dye, fura-2, in a membrane-permeable (AM) form (Invitrogen; $5 \mu\text{M}$ in $100 \mu\text{l}$ HEPES-buffered solution), inside a CO_2 incubator. fura-2 fluorescence was alternately excited at 340 and 380 nm light, and emission ratio to the 340/380 nm excitation was determined (BX51; Olympus).

Lactate dehydrogenase (LDH) release of cell death assay

The potential effect of optogenetic/chemogenetic stimulation on cell death was measured using the LDH released assay. LDH release from cells was detected using the Cytotoxicity Detection Kit (Roche Diagnostics) and a fluorometric plate reader (FL-600; Bio-Tek Instruments) following the manufacturer's instruction manual.

Immunoelectron microscopy of brain sections

One to 1.5 months after transplantation, animals were killed and perfused transcardially with 4% PFA in 0.1 M PB, pH 7.2–7.4. Immunoelectron microscopy was performed at Emory Electron Microscopy Core according to previous published method for immunogold-silver labeling at ultrastructural level (Yi et al., 2001). Perfusion fixed brains were further fixed with 4% PFA in PB overnight and then sectioned coronally at $50 \mu\text{m}$ using a cryostat vibratome (Ultrapro 5000). The sections were washed thoroughly with PB and then placed in PB containing 0.1% sodium borohydride to inactivate residual aldehyde groups in the tissue

sections. Sections were washed with PB several times until the solution was devoid of bubbles. To improve antibody penetration, the sections were treated with PB containing 0.05% Triton X-100 for 20 min before incubating in a blocking solution containing 5% BSA, and 0.1% cold water fish skin gelatin. After blocking, sections were rinsed twice with PBS containing 0.2% acetylated BSA (PBS/BSA-c; pH 7.4) and then incubated overnight at 4°C in a mixture of goat anti-GFP (5 μ g/ml) and mouse anti-tubulin (1:100), or goat anti-GFP and mouse anti-neuronal nuclei (both at 5 μ g/ml) primary antibodies diluted in PBS/BSA-c. The neuronal GFP immunolabeling helped the identification of neuronal cells derived from transplanted cells in electron microscopy (EM) images. After washes with PBS/BSA-c, sections were incubated overnight at 4°C in the secondary antibody conjugate, ultrasmall gold-conjugated donkey anti-goat IgG diluted 1:100 with PBS/BSA-c. To remove unbound secondary antibody, sections were washed thoroughly with PBS/BSA-c and then with PB.

Following PB washes, sections were washed with enhancement conditioning solution and then placed in R-Gent SE-EM silver enhancement solution for 90 min. Silver enhancement was terminated using 0.03 M sodium thiosulfate in enhancement conditioning solution. After the first silver enhancement, sections were washed with PB. Sections were incubated overnight at 4°C again with the secondary antibody conjugate, ultrasmall gold-conjugated donkey anti-mouse IgG diluted 1:100 in PBS/BSA-c. Sections were washed with PBS/BSA-c and then PB. Before the second silver enhancement of 60 min, sections were fixed with 2.5% glutaraldehyde in PB. All immunoincubations were done with gentle agitation 4°C, and all immunoEM reagents were purchased from Electron Microscopy Sciences.

Following fixation, sections were washed with PB, fixed with 0.5% osmium tetroxide for 15 min, dehydrated, and flat-embedded in Eponate 12 resin (Ted Pella) between two sheets of Aclar film. After resin polymerization, small pieces of flat-embedded sections were dissected from the cortex, mounted on plastic stubs, and sectioned *en face* at 80 nm with an ultramicrotome (Leica Microsystems, UC6rt). Sections were then stained with 5% uranyl acetate and 2% lead citrate. Imaging was done on a transmission electron microscope (JEM-1400, JEOL) that is equipped with a 4 million-pixel charge-coupled device camera (US1000, Gatan).

In vitro neurite/axon morphology and outgrowth analysis

An axon isolation device (Axon investigation system, AXIS, Millipore) was used to measure the process length and distribution of iPS-NPC-derived neurons. The AXIS device has a two-chamber system, which was separated by a set of microgrooves, allowing for the separation of cell body and axons/dendrites and allow only for axon/dendrite crossing. The measurement followed the manufacturer's instruction. Briefly, after the "4–/4+" RA neural differentiation, LMO3-expressing iPS-NPCs were dissociated and plated into one of the culture chambers in SATO medium, and the other culture chamber was filled with SATO medium without cells. The cells in the photostimulation group received blue light stimuli as described below for 5 consecutive days starting from 1 d after plating. Cells in the CTZ group received CTZ treatment (1.5 μ M bath application for 15 min, 3 times a day for 5 d). Cells in the control group received the medium change without CTZ. On day 6 after plating, the cells were fixed and stained for the neurite marker Tuj-1. The Tuj-1-positive neurites were imaged using confocal microscopy. The distance and diameter of processes extended from the microgrooves were measured using National Institutes of Health ImageJ. The diameter of each process was measured at five random areas, and the average diameter was presented in this report. The number and length of processes that crossed the microgroove barrier into the axonal chamber were counted and compared among groups.

The Sholl analysis of major process sprouting was performed using ImageJ software (National Institutes of Health). Briefly, processes >20 μ m originating from the branch point were counted. Concentric circles with 15 μ m differences in diameter were drawn in the end of microgrooves, and the number of processes crossing each circle was manually counted. The investigators were blind to the treatment groups.

Experimental design

Light and CTZ stimulation of iPS-NPCs in vitro. *In vitro* experiments were performed on LMO3-iPS-NPCs. The total number of iPS cell culture dishes tested was 51 cultures dishes/plates from different batches. Starting 1 d after the "4–/4+" differentiation and plating, cells were subjected to blue light stimulation (473 nm, 36 mW for 5 ms at 10 Hz for 12 s followed by a 48 s off period repeated 15 times; 3 times a day with a minimum of 2 h interval) for 5–6 consecutive days. The optical fiber of 200 μ m core diameter, connected to a laser source (BL473T3–100FC, SLOC), was placed 5 mm below the culture dish. The peak irradiance at the level of the specimen (i.e., at the bottom of culture dish) was determined by an optical power meter console (ThorLabs) to be $\sim$ 1 mW/mm².

The same laser light source or wide-field illumination through a green filter cube in an epi-fluorescent unit in a microscope (440–520 nm; 2.1 mW/mm²) was used for all *in vitro* light stimulation experiments. Cells in the CTZ group received CTZ exposure (1.5 μ M) for 15 min, 3 times a day with a minimum of 2 h interval for 5–6 consecutive days (n = 12 cultures). Cells in the control group received medium changes that contained vehicle for CTZ following the same schedule (n = 9 cultures).

Focal ischemic stroke model of the mouse. A total of 442 adult male C57BL/6 mice (2- to 3-month-old; body weight = 24.1 $\pm$ 0.7 g for male, 22.3 $\pm$ 0.8 g for female) and 80 aged mice (18- to 20-month-old, 30.7 $\pm$ 2.1 for male, 27.1 $\pm$ 2.0 for female) (Charles River Laboratories) were used in stroke experiments. The animal number in each group was determined based on the mortality of the distal middle cerebral artery ischemic surgery ($\sim$ 5% for young adult and $\sim$ 10% for aged animals), the variables in measurements and our previous data. Randomization was performed, and the sample size was further determined using power analysis (Power and Precision 4; Biostat).

Focal cerebral ischemia was induced and housed at Emory University Animal Facility in standard cages in 12 h light/12 h dark cycles. The ischemic stroke in the sensorimotor cortex was induced based on the barrel cortex stroke model, with modified artery occlusion procedures (L. Wei et al., 1995; Choi et al., 2012). Briefly, anesthesia was induced using 3.5% isoflurane followed by the maintenance dose of 1.5% isoflurane. Both the tail and paws of the animal were pinch-tested for anesthetic depth. The right middle cerebral artery was permanently ligated using a 10–0 suture (Surgical Specialties), accompanied by bilateral common carotid artery ligations for 7 min. This modified ischemic procedure is suitable and sufficient for the induction of focal ischemia in the mouse cortex, resulting in specific infarct formation in the right sensorimotor cortex. During surgery and recovery periods, body temperature was monitored and maintained at 37.0 $\pm$ 0.5°C using a temperature control unit and heating pads. The focal ischemic surgery caused low mortality rate ($\sim$ 5% of 3 d survival). All animal experiments and surgery procedures were approved by the Institutional Animal Care and Use Committee and met the National Institutes of Health standard.

Transplantation of iPS-NPCs into the postischemic brain. Cell transplantation was performed at 7 d after the focal ischemic stroke. Three experimental groups were stroke control, iPS-NPC transplantation with vehicle, and iPS-NPC transplantation stimulated with CTZ (at least 10 animals in each group; specific numbers are mentioned in figure legends of each experiment of different figures). Animals were randomly assigned to the groups. After the "4–/4+" neural induction, 5×10^5 LMO3-iPS derived LMO3-iPS-NPCs were resuspended in 4 μ l SATO medium and transplanted to the core and peri-infarct regions using a Hamilton syringe with injections of 1 μ l each at 4 locations (site 1: AP = 1.6, L = –4.0, V = –6.5; site 2: AP = 0.6, L = –3.0, V = –5.5; site 3: AP = –0.6, L = –4.5, V = –7.0; and site 4: AP = –2.6, L = –6.5, V = –4.5) in the peri-infarct cortex. A 2 min waiting period at the end of injection allowed the cells to settle before needle removal. Stroke control animals received vehicle injection (4 μ l of SATO medium). In some experiments, iPS-NPCs were prelabeled with Hoechst 33342 (1:10,000 v/v) for 1 h, which facilitated tracking of these cells after transplantation. To enhance cell survival and regenerative property after transplantation, all iPS-NPCs were exposed to hypoxic preconditioning (1% O₂) for 8 h before transplantation (Theus et al., 2008; Yu et al., 2013). To label proliferating cells in the brain, animals received daily administration of

BrdU (50 mg/kg; i.p.) starting the day of transplantation until the day of death.

Intranasal CTZ administration in vivo. For delivery of CTZ *in vivo*, we used the nasal route that allows reagents to bypass the blood–brain barrier and achieve brain targeted drug delivery (Hanson and Frey, 2008; Chen et al., 2015; Meredith et al., 2015). Intranasal administration of CTZ followed previous procedures at a dosage of 50 μ g per animal ($\sim$ 2 mg/kg, dissolved in sterile saline) each time (Chen et al., 2015). Animals received two CTZ treatments per day for 2 weeks (at least 10 animals in each group; specific numbers are mentioned in figure legends of each experiment).

In vivo bioluminescence imaging. We performed the *in vivo* bioluminescence imaging using a chemiluminescence reader (LAS-3000; Fujifilm), which is equipped with a charge-coupled device camera for high-sensitivity detection of bioluminescent emission. For each experiment, the animals were anesthetized with the ketamine/xylazine mixture (ketamine 80–100 mg/kg, xylazine 10–12.5 mg/kg, i.p.) and given CTZ (50 μ g) intranasally followed by imaging ($n = 3$ animals). Using the chemiluminescence mode, the images were acquired as cumulative emission intensities every 4 min over the course of 60–90 min. The background-corrected luminescence intensity in an ROI was measured.

Electrophysiological recordings in cultured cells and brain slices. Whole-cell patch-clamp recording in dissociated cells in cultures was performed using an inverted microscope (IX71; Olympus) equipped with an epifluorescent unit (Olympus), an electromechanical shutter (Uniblitz; Vincent), a mercury lamp (Olympus), and a scientific CMOS camera (OptiMOS; QImaging) on iPS cell-derived neurons 7–12 d after neural induction, which were growing on top of an astrocytes layer. Whole-cell recording was also done on acute brain slices of naive mice expressing LMO3 using an upright microscope (LABOPHOT; Nikon). The membrane currents or potentials were collected using an amplifier (EPC9; HEKA Elektronik) at room temperature ($\sim$ 22°C). The external solution contained 135 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, and 10 mM glucose, pH 7.4. The internal solution consisted of 120 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM Na_2ATP , 10 mM EGTA, and 10 mM HEPES, pH 7.2. Recording electrodes pulled from borosilicate glass pipettes (P-97; Sutter Instruments) had a tip resistance between 5 and 7 M Ω when filled with the internal solution. Series resistance was compensated by 75%–85%. Linear leak and residual capacitance currents were subtracted online using a P/6 protocol. Action potentials were recorded under current-clamp mode using patch-clamp software (PatchMaster; HEKA Elektronik). Data were filtered at 3 kHz and digitized at sampling rates of 20 kHz.

In brain slice patch-clamp recordings, the forebrain was dissected and immediately placed in sucrose-enriched cutting solution containing 220 mM sucrose, 1.9 mM KCl, 6 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1.2 mM NaH_2PO_4 , 33 mM $NaHCO_3$, and 10 mM D-glucose. The solution was ice-cold and bubbled with 95% O_2 balanced with 5% CO_2 , pH 7.40. Coronal brain sections (200 or 400 μ m thickness) containing the sensorimotor/barrel cortex were obtained using a vibratome sectioning device (1000 Plus; Vibratome) and then recovered for at least 60 min in the aCSF before recordings.

For poststroke recordings, whole-cell voltage clamp was performed on brain slices containing the ischemic core and peri-infarct regions. The pipette puller was used to pull the patch pipettes with the tip resistances of 3–5 M Ω . During recording, slices were maintained at 34°C and perfused with oxygenated aCSF containing 124 mM NaCl, 3 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 1.3 mM NaH_2PO_4 , 26 mM $NaHCO_3$, and 10 mM D-glucose at a rate of 2 ml/min. The pipette was filled with a solution containing 130 mM K-gluconate, 10 mM KCl, 10 mM HEPES, 2 mM Mg-ATP, 0.3 mM Na-GTP, and 0.4 mM EGTA, pH 7.3. Recorded signals were amplified with a patch-clamp amplifier (Axopatch 200B; Molecular Devices), digitized at 10 kHz, filtered at 1 kHz, and collected with data acquisition software (Clampex 8.2; Molecular Devices). Only the neurons with membrane potential more negative than -40 mV and action potential >65 mV were accepted for further experiments. EPSCs were recorded as inward currents at the holding potential of -70 mV. The electrophysiological data were analyzed using commercial software (Mini Analysis 6.0.7; Synaptosoft).

Microelectrode array (MEA) recordings in brain slices. A high-resolution MEA2100-system (MultiChannel Systems) was used to perform simultaneous extracellular recordings at multiple locations in brain slices. A total of 31 animals were tested in MEA brain slice recordings. The MEA chamber (60pMEA200/30iR-Ti, MultiChannel Systems) in the experiments was composed of a 6-mm-high glass ring and an 8×8 titanium nitride electrode grid, including 60 electrodes (59 electrodes and 1 internal reference electrode) that cover a recording area of 5 mm². The electrode diameter was 30 μ m, and electrodes were separated by 200 μ m distance. The brain slice was placed in the MEA chamber that was perfused with oxygenated aCSF at a rate of 7 ml/min and maintained at 34°C. A stabilization time of 5–10 min was given before formal recording. The collected data were analyzed using Multi Channel Analyzer version 2.6.0 (MultiChannel Systems).

In vivo electrophysiological recording. Acute recordings were performed with a custom-built tetrode that allowed for recordings of both single-unit and local field potentials ($n = 5$ for normal and stroke control, respectively, and $n = 8$ for experimental groups, respectively). A craniotomy was made 1.3 mm posterior and 1.7 mm lateral to the bregma, and recording was made 3.4 mm inferior to bregma to record from the ventroposteromedial (VPM) nucleus. Extracellular recordings were sampled at 25 kHz using our custom-built NeuroRighter data acquisition system (Rolston et al., 2009). Local field potentials triggered by whisker stimulation were bandpass-filtered (1–500 Hz) from the raw signal and analyzed offline using custom MATLAB scripts and the Chronux toolbox (Bokil et al., 2010). Single units were detected from the bandpass-filtered (0.5–5 kHz) signal and sorted offline using superparamagnetic clustering (Wave Clus) scripts developed by Quiroga et al. (2004). The contralateral whiskers were stimulated at $\sim$ 5 Hz for 10 s that alternated with ipsilateral whisker stimulation trials as a control for at least 5 repeated trials each during a recording session.

Behavioral tests

Adhesive removal test. To evaluate sensorimotor function, time for a mouse to remove adhesive pads from the paws was measured as previously described (Bouet et al., 2009; Z. Z. Wei et al., 2015). In brief, a small adhesive dot was placed on forepaws, and the time needed to contact and remove the sticker from each forepaw was recorded. Mice were trained three times before stroke surgery, and the average time was used in data analysis. Animals with response time of >120 s were considered insensitive to the tactile stimulus and were excluded from further examinations. Sham control, stroke control, and stroke plus CTZ groups contained 10 or 12 animals in the control groups and 12 or 16 animals in experimental groups. The same animals were also tested in the following tests.

Reach and grasp test. This reaching and grasping performance tests the coordination of forelimb motor functions in rodents. Mice were placed in a cage of $11.4 \times 6.4 \times 3.8$ cm. In the front wall of each cubicle, there is a hole of 9 mm and through which a feeding plane can be accessed. On the feeding plane, small food pellets (2–3 mm diameter) were placed 1.5 cm from the wall in such a way that the mice can withdraw the food only by reaching out one of its forepaws. Before the experiment, the animals were subjected to mild fasting with 12 h diet withdrawal. The animal's attempt to reach food pellets and successful retrieval attempts were counted during a 2 min period after placing the food pellets. The successful ratio of food grasping was then calculated and compared between groups.

Corner test. The corner test was performed 1 d before ischemia and 3 d after ischemia, as described previously (Zhang et al., 2002; Choi et al., 2012). Two cardboard plates ($30 \text{ cm} \times 20 \text{ cm} \times 0.3 \text{ cm}$) were attached at a 30° angle from each other in a home cage. Each subject mouse was placed between the two plates and allowed to freely move to the corner. The number of right and left turns was counted. Twenty trials/tests were performed for each mouse.

Cylinder test. The mice were placed in a glass cylinder (9.5 cm diameter and 11 cm height), and the number of times each forelimb or both forelimbs were used to support the body on the wall of the cylinder was counted for 5 min. The animals were evaluated at different days after stroke. Two mirrors were placed behind the cylinder to view all directions. The number of impaired and nonimpaired forelimb contacts was calculated as a percentage of total contacts.

Open field test. In the open-field test, mice were allowed to freely move (25 cm × 30 cm × 25 cm) during the dark cycle. Mild stress was induced by exposure to an open-field animal cage-like box. The container was divided into 30 equal 5 × 5 squares, and the number of line crossings and duration of stay by each animal were recorded and calculated during the first exposure measured for 2 min. The middle 12 squares were considered the inner space, whereas the other 18 squares were considered the boarder space. The calculation for the relative stress-induced anxiety was performed in double-blinded manner using the total stay duration in boarder space in each 2 min.

Whisker-touching behavior. Repetitive whisker-touching behavior is a reflection of the intact neuronal controls of the barrel cortex-thalamus-whisker pathway. In stroke animals repaired with LMO3 cell transplantation, activation of the LMO3 protein and the whisker-touching reaction can be achieved by acute application of intranasal CTZ. CTZ (2 mg/kg) was intranasally delivered, and the duration of whisker-touching behaviors was counted 5 min after CTZ application. This delay helps to avoid counting false-positive reactions due to the animal's reaction to the local sensation on the nose. The counting lasts for 5 min. The ratio of the touching activity before and after CTZ is calculated and expressed.

Statistical analysis

For comparison between two groups, we used the Student's two-tailed *t* test. Prism version 5.0 (GraphPad) was used to make graphs and to perform statistical analysis. Multiple comparisons were done using one-way or two-way ANOVA followed by Tukey's test or Bonferroni's correction for multiple pairwise comparisons. Nonparametric Mann-Whitney's *U* test was applied to electrophysiological and behavioral examinations of multiple comparisons. The *F* test was performed to verify an *F* distribution under the null hypothesis in ANOVA tests of multiple groups and/or time points. All the tests were two-tailed, and the significance level (*p*) was set at 0.05. Statistical values, such as *t*, *F*, and *q*, are reported in Results and/or figure legends. The numbers in the text are reported in the form of mean ± SEM.

Results

Neuronal differentiation of LMO3-iPS cells and responses to light and CTZ stimulation

We established a mouse iPS-cell line that stably expressed the LMO3 fusion protein (LMO3-iPSs). The expression of LMO3 and pluripotency of the cell line were confirmed by the EYFP tag in LMO3 and iPS-cell markers (SSEA-1, Oct 3/4, and Nanog), respectively (Fig. 1A). After the "4-/-/4+" RA neural induction protocol (Bain et al., 1995), these LMO3-iPS cells gained neuronal morphology in EYFP fluorescence-positive cells (Fig. 1B). We characterized electrophysiological properties of LMO3-iPS-derived neurons 7–12 d after neural induction. In patch-clamp recordings, membrane depolarization caused the firing of repetitive action potentials in a current injection-dependent manner (Fig. 1C). Similarly, stimulation by both light and the luciferase substrate, CTZ, elicited trains of spikes (Fig. 1D,E). In the voltage-clamp mode at the -60 mV holding potential, photostimulation by blue laser light increased occurrence of fast inward currents, resembling EPSCs (Fig. 1F). The activated neurons were glutamatergic as they were sensitive to block by the glutamate receptor antagonists, CNQX and D-AP5 (Fig. 1F). We also conducted Ca^{2+} imaging in LMO3-iPS-derived neurons loaded with the fluorescent Ca^{2+} indicator, fura-2, using ultraviolet excitation light (Fig. 2A–C). Stimulation with blue light or CTZ increased the intracellular Ca^{2+} concentration, reminiscent of that induced by high potassium (15 mM KCl) medium ($\Delta\text{ratio} = 0.0056 \pm 0.0026$, 0.0079 ± 0.0024 , and 0.0133 ± 0.0028 for CTZ, light, and 15 mM K^+ , respectively; $p > 0.2$ among groups; one-way ANOVA; $F_{(2,12)} = 1.53$; $n = 3, 8$, and 4 assessments, each included ≥ 30 cells) (Fig. 2D–F).

Excessive Ca^{2+} increases may cause excitotoxicity. In LDH release assays, neither of the applied light or CTZ stimuli caused a significant change in cell viability (one-way ANOVA; $F_{(2,6)} = 7.057$, $p = 0.0265$, $n = 8$ –10 assessments in each condition; $q_{(6)} = 3.654$, $p > 0.09$ for control vs laser; $q_{(6)} = 1.513$, $p > 0.5$ for control vs CTZ) (Fig. 2G). These experiments indicate that LMO3-iPS cells can be differentiated into functional neurons responsive to both light and CTZ stimulations with increased Ca^{2+} and action potentials, yet without causing excitotoxicity.

In a control study, cells were transduced with LMO3 or with a point mutation analogous of nonfunctional mutation in the pore region of a channelrhodopsin chimera (D292A in C1/C2) (Kato et al., 2012) that corresponds to D248A in the VChR1 moiety of LMO3. Judged by the YFP fluorescent intensity, the expression level of these channels in cells was similar (Fig. 2H). A large inward photocurrent was recorded in patch-clamp recordings during photostimulation from cells expressing LMO3 (Fig. 2I), whereas cells expressing LMO3/D248A exhibited negligible photocurrent to the same photostimulation (Fig. 2I–L).

Expression of neuronal markers in differentiated LMO3-iPS cells

Neuronal differentiation of LMO3-iPS-NPCs was verified with neuronal fluorescent markers 7–12 d after neural induction. Transfected cells were labeled with EYFP, and their neuronal differentiation was identified by specific markers, including NF, synapsin-1, and NeuN (Fig. 3). Cell counting assays confirmed that $>80\%$ of total cells expressed NeuN and/or NF, indicating their neuronal differentiation (Fig. 3D). The forebrain marker FoxG-1 was identified in these cells (Fig. 3E). We observed glutamatergic markers, VGLUT1, localized with the EYFP tag of LMO3 in most differentiated cells (Fig. 3H), consistent with our electrophysiological recording of glutamatergic currents (Fig. 1F). This was in line with previous reports that the 4-/-/4+ RA differentiation protocol primarily induced glutamatergic neural lineage cells (Bibel et al., 2007; Boissart et al., 2013).

Light and CTZ stimulation promoted neurite growth of LMO3-iPS-derived neurons *in vitro*

The axon/neurite outgrowth of differentiating LMO3-iPS-NPCs was examined in an axon isolation device that contained compartments of cell bodies and neurites divided by microgrooves. After neural induction, LMO3-iPS-NPCs were plated into the cell body compartment for terminal differentiation. One day after plating, cells were subjected to photostimulation or CTZ exposure for 5 consecutive days. Blue laser light (10 Hz, 15 min/session, 3 sessions/d) was applied through fiber optics, while CTZ (1.5 μM) was added into the media for 15 min (3 times/d). The control group contained cells treated with regular medium and without photostimulation. Six days later, Tuj-1 staining identified significantly more axons/neurites that extended cross the microgrooves in the group that received laser light or CTZ treatment (Fig. 4A). The number of neurites extending cross the microgrooves in the AXIS chamber was significantly increased by either light or CTZ stimuli (Fig. 4B) ($n = 4$ in each condition; one-way ANOVA followed by Tukey's pairwise comparisons; $F_{(2,3)} = 14.26$, $p = 0.029$, control vs laser and $p = 0.0319$ control vs CTZ). The light and CTZ stimuli also significantly increased the length of processes extending beyond the microgrooves (Fig. 4C) ($F_{(2,3)} = 18.15$, $p = 0.0211$; $q_{(3)} = 7.146$, $p = 0.0302$ for control vs laser; $q_{(3)} = 7.593$, $p = 0.0256$ for control vs CTZ). Moreover, cells stimulated by light exhibited significantly larger process diameters (Fig. 4D) ($q_{(27)} = 6.797$, $p = 0.0001$ for control

Figure 2. Activation of LMO3 channels, Ca²⁺ influx, and cell viability. LMO3 activation-induced cellular consequences, including intracellular Ca²⁺ increases, and the influence on cell viability were examined in cultures 10–12 d after neural induction of LMO3-iPS-NPCs. **A–C**, Intracellular free Ca²⁺ concentration was measured using the Ca²⁺ dye fura-2 AM and 340/380 ratio analysis. The expression of LMO3 was demonstrated in cultured cells with the yellow fluorescence of EYFP (**A**). **B**, fura-2 image of these cells during light stimulation. **C**, Overlay of **A** and **B**. **D–F**, The time course of intracellular Ca²⁺ increases induced by bath-applied CTZ (15 μ M, 4–5 min), light stimulation (1 Hz pulse for 4 min), and 15 mM KCl solution (4–5 min), respectively. All three manipulations caused significant Ca²⁺ influx and intracellular accumulation. **D**, The binding of CTZ to the LMO3 luciferase, as its substrate, caused the emission of bioluminescence (red) followed by activation of the LMO3 and Ca²⁺ influx event. **G**, LMO3-iPS-NPC-derived neurons were subjected to 5 d treatments of blue light stimulation (10 Hz, 15 min/session, 3 times per day) or CTZ exposure (1.5 μ M, 15 min/session, 3 times per day), and cell viability was measured using the LDH release assay 12 h after the last stimulation session. There was no significant difference among all three groups ($n = 8–10$ per group). **H**, A control experiment of expressing LMO3 or LMO-D248A proteins. The latter gene has a point mutation (D248A), which made the channel moiety nonfunctional. The EYFP fluorescence intensity measurement verified similar expression levels in transfected cells. $n = 6$ wells each. **I, J**, In whole-cell patch-clamp recording, a large inward current was observed during wide-field photostimulation with blue light. Photocurrent to the same photostimulation was negligible in the cell expressing mutant LMO3/D248A. Peak and steady-state photocurrents were virtually absent in LMO3-D248A. $N = 5$ cells each. **K, L**, Summary of the patch-clamp data, showing the CTZ-induced peak and steady-state currents in different cells. *** $p < 0.001$ versus LMO3 cells (Student's t test).

Figure 3. Neuronal differentiation of LM03-iPS cells. LM03-iPS-NPCs were subjected to the “4–/4+” RA differentiation protocol. Immunohistochemical staining was performed for neuronal markers 7–12 d later. **A**, The LM03 expression was verified by EYFP tagged to the virus construct. NF labeling (NF) (red) shows the neurite growth from differentiated cells marked with the EYFP fluorescence (green). The merged image demonstrates neuronal differentiation of transfected cells. **B**, **C**, Enlarged images from the frame in **A**. **C**, The overlay of the three images in **B**, showing neuronally differentiated cells. **D**, Quantified data demonstrate >80% of cells expression the neuronal marker NF and NeuN (see **G**). **E**, The confocal image illustrates that LM03 expressing cells express the forebrain marker FoxG-1. **F**, The three high-magnification images illustrate the presynapse protein synapsin-1 in a differentiated iPS cell. **G**, Many differentiated cells (EYFP, green) were positive to NF as well as the mature neuronal marker NeuN (blue). The merged image represents a complete overlay of the three markers. **H**, These neurons also expressed glutamatergic neuronal markers, including vesicular glutamate transporter 1 (VGLUT1) and VGLUT2 (data not shown due to space limitation).

Figure 4. Light and CTZ stimulation promoted neurite growth of LMO3-iPS-NC-derived cells. **A**, LMO3-iPS-NPCs were plated in AXIS axon isolation device after “4–/4+” differentiation. One day after plating, cells in the laser group received blue light (10 Hz, 15 min) three times a day for 5 consecutive days. Cells in the CTZ group were exposed to CTZ (1.5 μ M, 15 min) three times a day for 5 d. Cells in the control group received CTZ-free medium changes and also served as the light off control. Six days after plating, cells were stained with TUJ-1. Dashed lines in lower-magnification images outline the microgrooves in the AXIS device, which separated the cell body and processes. Higher-magnification images represent samples of processes in each group. **B–E**, Quantified neurite analysis shows the number of neurites extending across the microgrooves in the AXIS chamber (**B**), the average distance that neurites extend beyond the microgrooves (**C**), the average diameter of neurites (**D**), and the number of process sprouts (**E**). Either blue light stimulation or CTZ exposure significantly increased neurite growth and maturation compared with those in the control group. $n = 4$ independent assays. For detailed statistical results, see text. **F**, Results of the Sholl analysis using the ImageJ software. The number of neurites that extended and crossed the microgroove barrier into the axonal chamber was counted at different distances from microgrooves, and their length was measured. In the control group, fewer crossings took place compared with laser and CTZ groups. Neurites in the control group grew shorter than neurites in laser light and CTZ groups. * $p < 0.05$ versus controls (two-way ANOVA). # $p < 0.05$ versus the maximal length of control axons (two-way ANOVA). $n = 4$ per group.

Figure 5. Light and CTZ stimulation increased gene expression in LM03-iPS-derived neurons *in vitro*. Cultured LM03-iPS-derived cells received chemogenetic and optogenetic stimulation from 1 to 5 d after neural induction, RT-PCR, and Western blot were performed at 6 d after induction. **A**, Representative images of RT-PCR gels indicate the expression of synaptic marker synapsin-1 (SYN-1), PSD95, and growth factor SDF-1 in each group. The mRNA level of each marker was normalized against 18S. Cells that received photostimulation (blue light, 10 Hz, 15 min, three times a day for 5 consecutive days) in the laser group and those in the CTZ treatment group (1.5 μ M, 15 min, three times a day for 5 d) had significantly higher mRNA levels of SYN-1, PSD95, and SDF-1 (for statistical analysis, see text). **B**, Represented images of Western blot bands of SYN-1, PSD95, and BDNF. Quantified data show that blue light in the laser group or CTZ (Figure legend continues.)

vs light; $q_{(27)} = 4.855$, $p = 0.0053$ for control vs CTZ) as well as increased number of sprouts (Fig. 4E) (nonparametric Mann–Whitney test, $p = 0.0001$ laser light off control vs light; $p = 0.0072$ control vs CTZ). There were no statistical differences between light and CTZ groups. Consistent with above analyses, the Sholl analysis validated that the number of processes at 200 μm from microgroves and the maximal length of processes was significantly greater in two treatment groups compared with controls (Fig. 4F).

Light and/or CTZ stimulation upregulated synaptic proteins and BDNF in LMO3-iPS-NPCs

Twelve hours after stimulation with laser light or CTZ (6 d into the terminal differentiation), RT-PCR showed that light-stimulated LMO3-iPS-NPCs expressed more mRNA of synapsin-1 ($q_{(18)} = 4.025$, $p = 0.0276$, $n = 4$; one-way ANOVA followed by Tukey's pairwise comparisons), PSD95 ($q_{(18)} = 4.556$, $p = 0.0125$, $n = 4$), and SDF-1 ($q_{(18)} = 5.405$, $p = 0.0034$, $n = 4$) (Fig. 5A). CTZ-treated LMO3-iPS-NPCs also showed significantly higher mRNA levels of synapsin-1 ($q_{(18)} = 6.982$, $p = 0.0003$, $n = 4$), PSD95 ($q_{(18)} = 7.578$, $p = 0.0001$), and SDF-1 ($q_{(18)} = 8.72$, $p < 0.0001$) (Fig. 5A). Western blot analysis verified higher protein levels of synapsin-1 ($q_{(17)} = 7.334$, $p = 0.0003$ for control vs laser; $q_{(17)} = 5.213$, $p = 0.0059$ for control vs CTZ), PSD95 ($q_{(17)} = 6.027$, $p = 0.0014$ for control vs laser; $q_{(17)} = 3.685$, $p = 0.0462$ for control vs CTZ), and BDNF ($q_{(14)} = 9.6$, $p < 0.0001$ for control vs laser; $q_{(14)} = 8.868$, $p < 0.0001$ for control vs CTZ) in LMO3-iPS-NPCs that received either photostimulation or CTZ stimulation compared with sham controls ($n = 4$ each group) (Fig. 5B). In cells without the expression of LMO3 channels, the same CTZ applications did not show significant influence on these factors (Fig. 5C,D). Examined in additional controls *in vivo* studies, CTZ treatment in stroke animals received iPS-NPCs of no LMO3 expression failed to induce significant influence on tested factors (Fig. 5E,F).

Validation of intranasal delivery of CTZ with *in vivo* bioluminescence imaging

For the translational potential of the optochemogenetic approach, we tested noninvasive brain delivery of CTZ via the well-defined nasal-brain route. Normal mice received stereotaxic injection of AAV vector carrying the LMO3 gene into the right

Figure 6. The kinetics of LMO3 bioluminescence after nasal to brain delivery. To demonstrate the effectiveness and biokinetics of LMO3 activation induced by intranasal administration of CTZ, the LMO3 luminescence was measured in the normal brain expressing LMO3 and in the stroke brain receiving LMO3-iPS-NPC transplantation. **A**, Intranasal administration of CTZ (2 mg/kg) induced luminescence in the barrel cortex area receiving LMO3 infection. **B**, AAV carrying the LMO3 construct was injected into the barrel cortex of a normal mouse. The EYFP expression appeared in the sensorimotor cortex. **C**, The time course of CTZ-induced luminescence in the brain, which lasted approximately 1 h. **D–F**, Seven days after focal ischemic stroke in adult mice, LMO3-iPS-NPCs were transplanted to the core and peri-infarct region of the right sensorimotor cortex. The 2,3,5-triphenyltetrazolium chloride staining shows the infarct area (white). Diagram illustrates the location of the cell implantation (**D**). **E**, Intranasal administration of CTZ (2 mg/kg) induced bioluminescence in the cell-grafted area. **F**, The intensity of luminescence verified that the CTZ-induced luminescence lasted ~ 1 h in the poststroke brain.

barrel cortex. Postmortem histology confirmed proper targeting of the barrel cortex with LMO3 as shown in the EYFP expression surrounding the injection site (Fig. 6B). After intranasal administration of CTZ (50 μg , or 2.0 mg/kg), bioluminescence was observed in the area of LMO3 expression and decayed over the next hour (Fig. 6A,C).

In adult stroke mice of focal cerebral ischemia targeting the right sensorimotor cortex, LMO3-iPS-NPCs were transplanted into the core and peri-infarct regions 7 d after stroke (Fig. 6D). Fourteen days later, transplanted cells and functional expression of LMO3 in these cells were confirmed with *in vivo* bioluminescence imaging (Fig. 6E). Bioluminescence was detected in the cell graft area and remained visible for ~ 1 h after intranasal CTZ administration (Fig. 6F).

CTZ stimulation of grafted LMO3-iPS-NPCs promoted neuronal differentiation, axonal remyelination, and long-term survival in the ischemic brain

In the ischemic stroke mice receiving cell transplantation or control medium, repeated stimulation of transplanted LMO3-iPS-NPCs was achieved by intranasal CTZ treatment (2 mg/kg, 10 μl) twice a day for 2 weeks starting 1 d after transplantation. Three weeks after stroke (2 weeks after cell transplantation), grafted LMO3-iPS-NPCs were identified by EYFP or mCherry tag of LMO3 (Fig. 7A–C). Colocalization with NeuN was observed in many EYFP- or mCherry-positive cells, indicating neuronal differentiation (Figs. 7A–C, 8A). Stroke animals showed a significant reduction in the expression of MBP in the peri-infarct region ($q_{(20)} = 15.48$, $p < 0.0001$ for sham vs stroke) (Fig. 7D,E). The MBP expression was noticeably greater in mice of the Stroke+LMO3-iPS+CTZ group (one-way ANOVA, $q_{(20)} = 7.679$, $p = 0.0003$ for Stroke vs Stroke+LMO3-iPS+CTZ; $q_{(20)} = 4.521$, $p = 0.026$ for Stroke+LMO3-iPS vs Stroke+LMO3-iPS+CTZ; $n = 5$ –8 per group) (Fig. 7E). Consistent with these observations, 1 month after stroke (3 weeks after cell transplantation), EYFP-positive cells colabeled with the intact nucleus of Hoechst 33342 were identified as surviving transplanted cells. In cell counting assays, stroke animals in the Stroke+LMO3-iPS+CTZ

(Figure legend continued.) exposure increased the protein levels of these factors (for nonparametric statistical analysis, see text). $n = 4$ /group independent assays. **C, D**, Western blot analysis on the expression levels of BDNF, SDF-1, synapsin-1, and PSD-95 in the cortical cultures with no LMO-3 transduction. This test served as a negative control for possible off-target actions of CTZ. There was no significant difference among saline and CTZ groups (unpaired Student's *t* test, $p > 0.05$, $n = 4$ cultures per group). **E, F**, Western blot analysis of the expression of BDNF, synapsin-1, PSD-95, and SDF-1 in the peri-infarct region 7 d after stroke. CTZ (intranasal delivery of 2 mg/kg/d, starting from 8 d after stroke) or vehicle treatment for 14 consecutive days. There was no significant difference of the expression of BDNF, SDF-1, synapsin-1, and PSD-95 in the peri-infarct region between the two groups at the end of the treatments. $n = 5$ /group. $p > 0.05$ (unpaired Student's *t* test). * $p < 0.05$ versus controls (one-way ANOVA).

Figure 7. Neuronal differentiation of transplanted LMO3-iPS-NPCs and CTZ treatment promoted remyelination in the ischemic brain. Transplantation of LMO3-iPS-NPCs was performed 7 d after stroke. At various later time points, immunohistochemical staining examined the fate of transplanted cells in the ischemic cortex. **A**, Twenty-one days after transplantation, immunostaining of the ischemic core and peri-infarct cortical regions identified transplanted cells positive with mCherry tagged LMO3. The three images show mCherry-positive (red) and NeuN-positive (green) cells at different magnifications. **B**, Enlarged images of the frame in the last image in **A**. Right, 3D image represents the overlay of mCherry and NeuN staining. **C**, In confocal imaging, EYFP fluorescence was from the EYFP tagged to the LMO3 construct; its colocalization with NeuN in the peri-infarct region verified again the survival and neuronal differentiation of grafted LMO3-iPS-NPCs 3 weeks after transplantation. **D**, In stroke mice that received LMO3-iPS-NPC transplantation, CTZ (2 mg/kg) was intranasally administered 1 d after cell transplantation for 14 consecutive days. Three weeks later, the expression of MBP (red) in the peri-infarct region was examined for myelination. In this assay, Hoechst 33342 counterstaining (blue) was used to label all cells in the peri-infarct region. **E**, Bar graph represents the relative expression of MBP per field. Stroke alone caused a significant reduction in MBP expression compared with that in sham group. More MBP staining appears in the LMO3-iPS-NPC and LMO3-iPS-NPC plus CTZ groups. $n = 5-8/\text{group}$. $*p < 0.05$ versus sham control (one-way ANOVA). $^{\#}p < 0.05$ versus stroke (one-way ANOVA). $^{\Delta}p < 0.05$ versus stroke + LMO3 cells (one-way ANOVA). For detail statistical analysis, see text. **F**, Long-term survival of transplanted cells in the poststroke brain. Cell counting was performed 1 month after stroke (3 weeks after cell transplantation). EYFP-positive cells colabeled with the intact nucleus of Hoechst 33342 were identified as surviving transplanted cells. For each animal, 30 brain sections spanning the ipsilateral cortex were counted. Cell counting was performed in the ischemic core and peri-infarct transplanted regions. $*p = 0.0034$ (paired *t* test, $F = 2.569$, $\text{Df}_n = 23$, $\text{Dfd} = 29$). $n = 30$ animals in the LMO3-iPS-NPC group (no CTZ control) and 24 animals in the LMO3-iPS-NPC plus CTZ treatment group.

group showed increased surviving transplanted cells compared with animals received LMO3-iPSs but no CTZ treatment (unpaired Student's *t* test, $p = 0.0034$ for Stroke+LMO3-iPS vs Stroke+LMO3-iPS+CTZ; $F = 2.569$, $n = 30$ and 24 animals, respectively) (Fig. 7F).

There were increased numbers of BrdU/NeuN double-positive cells (one-way ANOVA followed by Tukey's pairwise comparisons; $q_{(25)} = 8.257$, $p < 0.0001$) and BrdU/Glut-1 double-positive cells ($q_{(20)} = 6.081$, $p = 0.0018$) after stroke compared with that in the sham group, indicating pronounced neurogenesis and angiogenesis (Fig. 8). Stroke animals receiving cell transplantation (Stroke+LMO3-iPS) or a combination of transplantation and CTZ stimulation (Stroke+LMO3-iPS+CTZ) showed even greater numbers of BrdU/NeuN and BrdU/Glut-1 double-positive cells compared with those in the Stroke-only group (NeuN: $q_{(25)} = 4.129$, $p = 0.0519$ for Stroke vs Stroke+LMO3-iPS; $q_{(25)} = 7.635$, $p = 0.0001$ for Stroke vs Stroke+LMO3-iPS+CTZ; Glut-1: $q_{(20)} = 4.079$, $p = 0.0419$ for Stroke vs Stroke+LMO3-iPS; $q_{(20)} = 4.06$, $p = 0.0431$ for Stroke vs Stroke+LMO3-iPS+CTZ) (Fig. 8C,E), indicating that poststroke regeneration could be promoted by LMO3-iPS-NPCs. CTZ treatment after LMO3-iPS-NPC transplantation showed a trend of further increasing BrdU/NeuN-positive cells (Fig. 8C).

Synapse formation of transplanted LMO3-iPS-NPCs and synaptic activity/plasticity induced by light or CTZ stimulation on brain slices

Whole-cell patch-clamp recordings in brain slices 4 weeks after stroke showed current injection evoked trains of action potentials in LMO3-expressing cells marked with EYFP (Fig. 9A). Photostimulation with blue laser light (473 nm, 33.6 mW) also induced membrane depolarization and action potentials (Fig. 9B). Bath application of CTZ (300 μM) significantly increased the firing frequency of spikes compared with vehicle (Fig. 9C,D).

Immunogold EM was performed to identify synapses. LMO3-expressing cells were visualized with electron-dense gold particles by immunostaining against the EYFP tag of LMO3. The ultrastructural imaging on the brain section 4 weeks after stroke revealed synapses between immunogold-positive transplanted cells and immunogold-negative host neurons. Transplanted cells were identified either as presynaptic (Fig. 9E) or postsynaptic with host cells (Fig. 9F) as well as between

transplanted cells (Fig. 9G). Presynaptic immunogold was primarily found in Type 1 synapses with characteristic asymmetric postsynaptic density (Palay, 1956), consistent with the identity of transplanted cells as glutamatergic neurons.

Optochemogenetic stimulation of grafted LMO3-iPS-NPCs triggered synaptic plasticity in the ischemic cortex

To validate integration of transplanted LMO3-iPS-NPCs into neuronal networks, spontaneous EPSCs (sEPSCs) were recorded from LMO3-negative resident neurons under whole-cell voltage clamp. The frequency of sEPSCs significantly increased by ~2-fold during bath application of CTZ (300 μ M) with no significant change in the amplitude, indicating that CTZ caused neurotransmitter release from surrounding LMO3-iPS-NPCs (Fig. 10A–E).

Stroke in the sensorimotor cortex damages connections of the barrel cortex–thalamus pathway and causes secondary cell death in the thalamus (Carmichael et al., 2001; L. Wei et al., 2004). Brain slices from stroke animals that received LMO3-iPS-NPCs (4 weeks after stroke) were examined using the MEA containing 59 recording electrodes. The electrode template was placed in the area covering the somatosensory cortex, including part of the ischemic core and peri-infarct regions (Fig. 10F). One of the 59 electrodes located 200–400 μ m distance from the edge of the ischemic core was the stimulation electrode. Field EPSPs (fEPSPs) were evoked by electric pulses ($\pm$ 1500 mV, 0.1 ms, once every 30 s) and were simultaneously monitored in different brain areas. Among all recording locations, fEPSPs could usually be captured in 18 areas of the peri-infarct and nonischemic regions. Certain fEPSPs significantly enhanced during bath application of CTZ (100 μ M, 5 min; 7 slices from 4 mice) (Fig. 10G). Heat maps showed that CTZ significantly increased fEPSP responses at several locations (Fig. 10H,I). In the response heat map generated from MEA recordings, neurons in the cortical layer II/III and V in the same cortical/barrel column responded strongly and showed CTZ-induced LTP (Fig. 10H). In addition, neurons at a 400–600 μ m distance in a different cortical/barrel column also displayed strong response and augmented LTP (Fig. 10H). Marked potentiation of fEPSPs persisted, even after washing out of CTZ, and progressed for at least 30 min at the end of recordings (Fig. 10J). In the same or different brain slices, photostimulation (473 nm, 10 Hz for 10 s, separated by 10 s intervals, 4 min total) triggered similar potentiation effects dur-

Figure 8. Neurogenesis and angiogenesis in the poststroke brain received different treatments. Immunohistochemical staining of neuron or vessel markers and BrdU incorporation were applied to detect newly formed neuronal and vascular cells in the cortical peri-infarct region 21 d after stroke with different treatments. BrdU (50 mg/kg, i.p.) was injected 1 d after cell transplantation for 14 d. **A**, The 3D image shows the colocalization of transplanted cell marker YFP and the neuronal marker NeuN, indicating neuronal differentiation and maturation of LMO3-iPS-NPCs. **B**, **C**, NeuN and BrdU double-positive cells illustrate new neurons. **C**, Bar graph represents that the ischemic stroke was an insult and trigger inducing neurogenesis. Both cell transplantation and the cell/CTZ treatments further increased the number of newly formed neurons. CTZ showed a trend of greater effect than cell alone, although it was not statistically significant. * $p < 0.05$ versus sham control (one-way ANOVA). # $p < 0.05$ versus stroke control (one-way ANOVA). $n = 10$ –12 per group. **D**, **E**, Angiogenesis was measured using the vessel marker Glut-1 colabeled with BrdU. **E**, Bar graph represents that angiogenesis was induced after stroke, whereas both two treatments further increased the number of new vascular structures. * $p < 0.05$ versus sham control (one-way ANOVA). # $p < 0.05$ versus stroke control (one-way ANOVA). $n = 10$ –12 per group.

ing and after light application (Fig. 10K). At the end of the 30 min recording after CTZ or light stimulation, the slope of fEPSPs was doubled compared with the baseline level (Fig. 10L). On the contrary, fEPSPs in control recordings with drug-free solution or low-frequency photostimulation were stable throughout the re-

Figure 9. Regulation of action potential firing in LMO3-iPS-NPC-derived neurons and formation of synapses. **A**, Whole-cell recording in a brain slice 3 weeks after infection with the LMO3 lentivirus. Current injection via the recording electrode evoked action potentials in EYFP-positive cells. **B**, Blue laser light stimuli (473 nm, 33.6 mW) induced membrane depolarization and action potential in EYFP-labeled neurons. **C**, **D**, Whole-cell recordings in brain slices expressing the LMO3 protein demonstrate a marked increase in firing frequency upon application of CTZ (300 μ M) compared with vehicle controls. The firing frequency increased from 2.8 ± 1.1 Hz in control cells to 19.5 ± 3.3 Hz upon the application of CTZ (t test, $p < 0.05$; $n = 5$). **E–G**, Immunoelectron microscopy micrographs of synapses in the peri-infarct region 28 d after stroke (21 d after cell transplantation). The characteristic electron-dense structure postsynaptic density (an ~ 30 -nm-thick dark band located between the presynaptic and postsynaptic neurons). Clusters of synaptic vesicles indicate presynapses (*). Large black dot (arrowhead) labeled GFP/EYFP-positive neurons is derived from transplanted LMO3-iPS-NPCs. Smaller black dot (arrow) reveals the expression of microtubules as a neuronal marker. **E**, Micrograph represents two synapses, and the synapse on the right is formed between the transplanted cell labeled with black dots as a presynaptic neuron and a host neuron (no black dot) as the postsynaptic neuron. **F**, Synapse formed between a presynaptic host neuron and a transplanted postsynaptic neuron. **G**, Synapse likely formed between two transplanted cells because large and small black dots existed in both sides of the synapse. Representative of brain samples from 7 animals.

cording (Fig. 10G,J,K). These results verified that facilitated transmitter release and a synaptic LTP-like effect were induced by excitation of transplanted cells that had been conditioned by the activation of LMO3 channels. The involved neurons were glutamatergic since their synaptic events were completely blocked by glutamate receptor antagonists NBQX (10 μ M) and D-AP5 (5 μ M) (Fig. 10J,K). Thus, transplanted LMO3-iPS-NPCs differentiated into glutamatergic neurons and integrated into existing circuits within and between cortical column structures, leading to enforced intrabarrel and interbarrel connectivity and neuronal synaptic plasticity.

Intranasally delivered CTZ stimulation of grafted LMO3-iPS-NPCs restored thalamocortical connections after stroke

To examine the thalamo-cortico-thalamic connectivity impaired after stroke, electrophysiological recordings were performed *in vivo* in the VPM nucleus of the thalamus to assess neuronal activities in the thalamo-cortico-thalamic pathway (Fig. 11A). One month after stroke, the single-unit recording detected neuronal activity in the VPM barreloid neurons (Fig. 11A–D), an indication of thalamo-cortico-thalamic connectivity involving afferent information from the brainstem and efferent signals from the barrel cortex (Temereanca and Simons, 2003). The frequency of this thalamo-cortico-thalamic activity was selectively increased by mechanical stimulation of contralateral whiskers, and this in-

crease in firing rate was much reduced by stroke (Fig. 11B,C) (L. Wei et al., 2004). In stroke mice of the combined treatment of LMO3-iPS-NPC plus CTZ, the firing rate of VPM neurons during whisker stimulation was protected (nonparametric Mann–Whitney’s U test; mean rank difference = -11.05 , $p = 0.0315$, $n = 5$ –8 per group) (Fig. 11D). This was not seen in the cell transplantation alone group, suggesting that CTZ stimulation of LMO3 in transplanted cells effectively restored the connectivity and propagation of neuronal signals along the whisker-thalamus-barrel cortex pathway. During the course of experimental procedures, no epileptic behavior/activity was observed in animals receiving LMO3-iPS-NPC transplantation and/or CTZ stimulation by eye surveillance or the MEA recording in brain slices.

Intranasally delivered CTZ stimulated grafted LMO3-iPS-NPCs and promoted functional/behavioral recovery after stroke

One month after stroke, mice that received LMO3-iPS-NPCs responded to intranasal CTZ administration with increased whisker-touching behaviors (nonparametric Mann–Whitney test, $p = 0.0437$ vs saline control, $n = 8$ and 6, respectively) (Fig. 12A). In contrast, stroke mice received iPS-NPCs expressing the ChR2 protein or LMO3-D248A-iPS-NPCs showed no response to CTZ administrations ($p = 0.3056$ vs saline control, $n = 8$ per group) (Fig. 12A). The CTZ triggered whisker-touching behavior

Figure 10. Neuronal circuit repair in the peri-infarct region of the barrel cortex. Stroke mice received LM03-iPS-NPCs at 7 d after stroke, followed by intranasal CTZ (2 mg/kg) daily treatment for 14 d. **A–E**, Three weeks after stroke, whole-cell voltage clamp was performed in brain slices to characterize the sEPSCs in endogenous neurons surrounding the fluorescence-positive LM03 cells. Exposure to 300 μ M CTZ in the bath solution significantly increased the frequency of sEPSCs by $\sim$ 2-fold, and no significant difference was found in the amplitude. The glutamate receptor antagonists NBQX (10 μ M) and D-AP5 (5 μ M) completely blocked the occurrence of sEPSCs. $n = 5$ brain slices from 3 animals in each group. **F**, MEA in brain slices recorded the fEPSPs. The distance between two electrodes was 200 μ m. Image represents the distribution of electrodes against the coronal brain section containing the ischemic core (white dotted line) of the barrel cortex and peri-infarct areas. Black dotted line outlines the edge of the slice. *The location of the electric stimulation electrode, which was 200–400 μ m away from the ischemic core. fEPSPs were triggered by electric pulse ($\pm$ 1500 mV, 0.1 ms, once every 30 s) and were stable in control recordings. After stabilization of fEPSP baseline recordings for at least 5 min, the brain slice was exposed to CTZ (100 μ M) for 5 min or light (473 nm, 10 Hz for 10 s, separated by 10 s intervals) for 4 min. fEPSPs were continuously monitored in 58 electrodes (minus one stimulation electrode and one reference electrode from total 60). Eighteen of 58 locations detected fEPSPs. Red plus sign represents reaction sites where fEPSPs were markedly upregulated by CTZ application. Green sign represents no-response sites (see corresponding heat map in **H**). **G**, Examples of fEPSP traces during CTZ exposure. The initial up and down sharp spikes were the stimulation artifacts, followed by the fEPSP response mediated by synaptic transmission. **H**, The fEPSP heat map shows the locations of changes of fEPSPs with CTZ exposure. The cortical regions showed strongest fEPSP enhancements (*Figure legend continues.*)

Figure 11. LMO3-iPS-NPC transplantation and CTZ treatment restored functional connections of the thalamo-cortico-thalamic pathway after stroke. The neuronal activity in the VPM that relays the afferent and efferent signals in the thalamocortical pathway was recorded using extracellular recordings 1 month after stroke (3 weeks after cell transplantation). **A**, Schematic diagram showing the neuronal connection and experimental setup. The VPM ipsilateral to focal ischemic stroke was recorded during whisker stimulation. **B**, Recording in a naive mouse. Top, Raster plot showing the single-unit response in the VPM to corresponding whisker stimulation. Colors represent different stimulation trials. Bottom, The average single-unit firing rate across all trials did not significantly change during ipsilateral whisker stimulation, which is a negative stimulation control in this test. **C**, Recording in a naive mouse. Top, Raster plot showing the single-unit response in VPM to contralateral whisker stimulation. Bottom, The average firing rate of neurons in VPM was noticeably increased during contralateral whisker stimulation. **D**, The fold change in average firing rates of ipsilateral VPM neurons during contralateral whisker stimulation. Compared with naive animals, stroke animals showed weak response during whisker stimulation, whereas stroke animals receiving the combined cell/CTZ treatment (Stroke + LMO3 + CTZ) significantly restored the neuronal response. $*p < 0.05$. $n = 5$ in control and 8 in experimental groups.

(Figure legend continued.) in responding to CTZ were sites 41 and 44, which were located in the same cortical column judged from the brain slice orientation in **F**. Several responding sites were 400–600 μm away from the stimulation site (*), implicating neurons in a different cortical column. For example, fEPSPs in sites 12 and 22 were noticeably facilitated by CTZ, possibly achieved by interbarrel connection and regulation. In multiple locations, there were downregulations of fEPSPs shown in the color change from light blue to dark blue. Inhibitory interneurons might regulate these sites. **I**, The ratio of increased slope and amplitude of fEPSPs before and during the CTZ exposure. $***p < 0.001$ (t test). $n = 7$ slices from 4 animals. **J**, **K**, Representative fEPSP traces at different time points before and after CTZ or light stimulation or in control recordings. Dot graphs underneath traces in **J** and **K** represent the time- and stimulation-dependent LTP-like enhancements of fEPSPs. 1, 2, and 3 indicate the recorded traces and their corresponding locations in the dot graphs. Progressive enhancements of fEPSPs developed, even after the termination of CTZ or light stimulation for at least 30 min. These events were blocked by glutamate receptor antagonists NBQX (10 μM) and D-AP5 (5 μM). Control recordings were performed with drug-free solution or low-frequency light stimuli, showing no significant change of fEPSPs. **L**, Bar graph represents the increased ratio of fEPSPs at 30 min after CTZ or light stimulation, normalized against the baseline level ($F = 32.68663$). $***p < 0.001$ (one-way ANOVA). $n = 7$ slices from 4 animals in CTZ and light groups, respectively. $n = 15$ in CTZ-free control and $n = 5$ in low-frequency light control.

of these mice was markedly different compared with stroke mice in the LMO3-iPS-NPCs group ($p = 0.0119$) (Fig. 12A). Similar results of the whisker-touching behavior were obtained 21 d after stroke in both male and female mice receiving the LMO3-iPS-NPCs/CTZ treatment (Fig. 12B).

In the adhesive removal test, stroke animals took a significantly longer time to detect (contact) and remove the adhesive stickers attached to the forepaw of the affected side (Fig. 12C,D). Cell transplantation alone significantly improved the performance in removing the adhesives tested 14 and 21 d after stroke (nonparametric Kruskal–Wallis test; 14 d: $p = 0.038$; 21 d: $p = 0.0095$). The combination of cell transplantation and CTZ treatment (Stroke + LMO3-iPS + CTZ) improved the sensorimotor function in both detecting and removing the adhesives (14 d contact: $p = 0.0054$; 14 d removal: $p < 0.0001$; 21 d contact: $p = 0.0692$; 21 d removal: $p < 0.0001$; Stroke vs Stroke + LMO3-iPS + CTZ) (Fig. 12C,D). In negative control experiments, stroke mice received vehicle or CTZ treatment alone showed no difference in their performance (Fig. 12E,F).

We examined coordinated motor function of the reach-to-grasp test that is more sensitive and clinically relevant (Marques and Olsson, 2010). In sham control and stroke animals, the suc-

Figure 12. LMO3 activation improved functional recovery after ischemic stroke. **A**, One month after stroke, animals in different groups were examined for the whisker-touching behavior triggered by intranasally administered CTZ, which is a specific acute examination of the functional integrity of the whisker-thalamus-barrel cortex neuronal pathway. CTZ (2 mg/kg) was intranasally delivered, and the time of the animals spent in touching their whiskers using forepaws was counted. The count was started 5 min after the administration of CTZ and ended 5 min later to avoid counting the animal reaction to a possible local skin assault. Only the animals that received LMO3-iPS-NPC transplantation responded with increased whisker-touching behavior. Stroke mice received iPS-NPCs expressing the optogenetic gene Chr2, which does not bind to CTZ or the mutant LMO3-D248A protein; both failed to show any response to CTZ administration. * $p < 0.05$ versus all groups (nonparametric Mann–Whitney test). # $p = 0.0119$ versus LMO3 group (nonparametric Mann–Whitney test). $n = 6–8$ per group. For detailed values in statistical analysis, see text. **B**, The responsive whisker-touching behavior in adult male and female mice of sham control and after stroke. Sham and stroke control mice (28 d after stroke) show no whisker-touching behavior after the CTZ delivery. Stroke mice (male and female) receiving LMO cells plus the daily CTZ treatment showed increased whisker-touching activities in responding to the acute application of CTZ. $n = 16$ for sham control group. $n = 13$ for stroke controls of male and female groups. $n = 18$ and $n = 16$ for stroke plus LMO cells and daily CTZ treatments of male and female, respectively. $F_{(4,71)} = 9.944$. * $p < 0.05$ compared with stroke vehicle controls (male or female). **C, D**, The sensorimotor function of animals in experimental groups was evaluated using adhesive removal test at 14 and 21 d after stroke. Stroke animals took a longer time to detect and remove the sticky dot on their left paw compared with sham animals. Stroke animals receiving the (Figure legend continues.)

cess rate of fetching the food pellet was 60% and 30%, respectively. Cell transplantation showed a trend of improving the success rate, whereas cell transplantation combined with CTZ treatment significantly improved the motor coordination to the normal level (one-way ANOVA with Bonferroni's correction; $p = 0.0013$ vs stroke control; $p = 0.032$ vs cell only group; no difference from sham controls, $p = 0.0865$) (Fig. 12G). In the open field test of anxiety behaviors, animals with stroke had the preference of staying in corners, suggesting increased anxiety and stress (Fig. 12H). This behavior was corrected by the cell/CTZ combination therapy so animals spent less time in the corner area ($p = 0.0013$ vs stroke controls; $p = 0.1008$ vs sham controls) (Fig. 12H).

Functional recovery after stroke in male, female, young adult, and aged mice

The above *in vivo* experiments were performed in young adult male mice. Next, we examined gender and age factors in responding to the optochemogenetics therapy. The corner test is a widely used functional assessment for unilateral sensorimotor cortical damage (Zhang et al., 2002; Choi et al., 2012). Normal mice make about equal left and right turns in their exploratory turning behavior. After the ischemic insult to the right sensorimotor cortex, both male and female animals showed biased turns consistent with the side of their brain damage (Fig. 13A,B). In male stroke mice of young adult (2 months old) receiving no cell transplantation, the CTZ intranasal multiday deliveries showed no effect of correcting this functional deficit, whereas LMO3 cells plus CTZ daily treatment restored the functional behavior to the normal level (one-way ANOVA and Bonferroni correction, $p = 0.010$ vs stroke control, $p = 0.0386$ vs cell only group) (Fig. 13A). In young female stroke mice, CTZ stimulation appeared to have some benefits, but only the combined cell/CTZ treatment significantly improved the behavior back to normal ($p = 0.038$ vs stroke control, $p = 0.045$ vs cell only) (Fig. 13A). The same functional benefits of LMO3 cell plus CTZ treatment were verified in aged male and female stroke mice (Fig. 13B; for statistics, see the figure legend).

←

(Figure legend continued.) cell/CTZ combined treatment performed significantly faster compared with stroke control and stroke plus cell groups. $^*p < 0.05$ versus sham (nonparametric Kruskal–Wallis test). $^{\#}p < 0.05$ versus stroke alone (nonparametric Kruskal–Wallis test). $^{\Delta}p < 0.05$ versus stroke plus LMO3-iPS-NPC transplantation group (nonparametric Kruskal–Wallis test). For detailed analysis values, see text. $n = 12$ –16 per group. **E, F**, The adhesive removal test was repeated in stroke animals receiving no cell transplantation. The same CTZ daily treatment in those animals showed no benefit in this negative control experiment. $n = 8$ per group. Performed at 7, 14, and 21 d after stroke, the two stroke groups with vehicle or CTZ alone performed similarly, verifying that CTZ by itself did not have any functional benefit. $^*p < 0.05$ versus sham controls (one-way ANOVA). $p > 0.05$ between vehicle and CTZ groups (one-way ANOVA). **G**, The reach and grasp test examined the coordinative motor function of accurate control of reaching and grasping food pellets placed 1.5 cm away from the animal. Stroke significantly reduced the success rate of the activity. Cell transplantation alone did not improve this challenging behavior, whereas cell/CTZ combined treatment brought the performance back to the normal level. $n = 14$ for sham controls. $n = 10$ for stroke controls. $n = 12$ for stroke plus LMO cells. $n = 11$ for stroke plus LMO cell/CTZ group. $F_{(3,40)} = 6.0$. $^*p < 0.05$ versus sham control (one-way ANOVA). $^{\#}p < 0.05$ versus stroke control (one-way ANOVA). $^{\Delta}p < 0.05$ versus cell alone (one-way ANOVA). **H**, The open field test was used to detect anxiety-like behavior that chronically develops in stroke models. Stroke animals exhibited enhanced anxiety behavior of staying in the corner. Again, the LMO cell/CTZ combined treatment reduced the anxiety level back to normal. $n = 6$ for sham group. $n = 18$ for stroke controls. $n = 12$ for stroke plus LMO cells. $n = 10$ for LMO cell/CTZ group. $F_{(3,34)} = 5.0$. $^*p < 0.05$ versus sham control (one-way ANOVA). $^{\#}p < 0.05$ versus stroke control (one-way ANOVA). $^{\Delta}p < 0.05$ versus cell alone (one-way ANOVA).

A unilateral injury to the motor cortex results in an asymmetry in the forelimb used for support during rearing, which can be measured using the cylinder test (Lee et al., 2016). Two weeks after stroke, the focal cerebral ischemia in male young adult mice induced significantly reduced use of the affected forelimb; CTZ alone did not show any effect on the deficit ($p > 0.999$ vs stroke control) (Fig. 13C). In female animals, the motor deficit was obvious after stroke; CTZ alone appeared to induce some improvement compared with stroke controls ($p = 0.021$ vs stroke control). The LMO3 cell transplantation plus CTZ group showed a complete improvement in the forelimb function ($p = 0.0146$ vs stroke control); the effect was significantly better compared with the CTZ group ($p = 0.008$ vs cell alone) (Fig. 13C). The same experiments were repeated in aged (18-month-old) animals to understand whether the therapeutic benefit was age dependent. Again, both male and female mice showed reduced use of their affected limbs. Mice in the CTZ alone group did not have significant improvement, whereas the LMO3-cell plus CTZ treatment partly recovered the motor deficit in aged male mice (Fig. 13D). In aged female mice, the combined treatment noticeably promoted the use of affected forelimb, whereas CTZ alone had no benefit (Fig. 13D; for statistics, see the figure legend).

Discussion

The present investigation created a stable iPS cell line expressing the novel optochemogenetic probe LMO3 of a bioluminescent luciferase and an excitatory opsin. Membrane depolarization of cells expressing LMO3 could be induced by either physical photostimulation or bioluminescence with the luciferase substrate, CTZ. This unique feature allows for optochemogenetic controls of iPS-derived cells *in vitro* as well as after transplantation into the brain via intranasally delivered CTZ. Stimulations of LMO3-iPS-NPCs created an enriched microenvironment of increased growth/trophic factors, leading to enhanced neuronal differentiation and neural network repair. In mice receiving LMO3-iPS-NPC transplantation after stroke, the CTZ stimulation of these cells resulted in activity-dependent benefits, including the establishment of synaptic connections, increased axonal outgrowth and myelination, formation of functional neuronal pathways, augmented neuronal plasticity, and improvements of functional/behavioral recovery after stroke. The MEA recording in brain slices and *in vivo* single-unit recordings affords novel evidence for reestablishments of cortical connections and the thalamo-cortico-thalamic pathway after stroke. The neuroplasticity of LTP-like modulation endorsed by the cell/CTZ therapy and acute CTZ stimulation implies an underlying mechanism for improved sensorimotor and behavioral functions after stroke as proposed in a working model of this study (Fig. 14).

Luminopsins possess unique features. The greatest advantage is the activation of the opsin by bioluminescence upon catalyzing its substrate. It is thus able to circumvent the invasive and bulky hardware requirements (optical fiber and light source) in optogenetics (Berglund et al., 2013, 2016a, b; Tung et al., 2015). Furthermore, the pharmacological approach provides neural excitation of transplanted cells in larger brain regions. This advantage is particularly important with the fact that transplanted cells are mobile and capable of migrating long distances (Kelly et al., 2004). Luminopsins retain the sensitivity to physical light, useful for interrogation of functional integration of transplanted cells (Boyden et al., 2005). Up to now, the only optochemogenetics report was a study of controlling “caged” tamoxifen analogs in gene expression (Lu et al., 2012). The term “optochemogenetics” is used in our investigation to distin-

Figure 13. Therapeutic effects of the optochemogenetics treatment in stroke mice of different genders and ages. The LM03-iPS-NPC transplantation and CTZ combination therapy were further examined in male and female of young adult (2-month-old) and aged (18-month-old) mice. After the focal ischemic stroke, animals received the cell transplantation at 7 d after stroke and 7 d CTZ intranasal treatment. **A, B**, The corner test 14 d after stroke demonstrated sensorimotor deficits in both male and female of young adult and aged mice (biased turning behavior deviated from 50% of right and left turns) (for young male: $p = 0.015$ vs sham controls; for young female: $p = 0.006$ vs sham controls; for aged male: $p = 0.009$ vs sham controls; for aged female: $p = 0.010$ vs sham controls). CTZ administration alone could not improve the functional deficit (for young male: $p > 0.999$ vs stroke controls; for young female: $p > 0.999$ vs stroke controls; for aged male: $p = 0.416$ vs stroke controls; for aged female: $p > 0.999$ vs stroke controls), whereas the LM03-cell plus CTZ treatment recovered the functional level to the normal equal turns in both young adult

guish from the conventional optogenetics as well as from chemogenetics, which often refers to the designer receptors exclusively activated by designer drugs (DREADDs) (Chen et al., 2016). The mechanism of action of DREADDs is different from luminopsin-induced activation. DREADDs use G-protein-coupled receptor signaling pathways that have widespread effects. The optochemogenetic, on the other hand, provides a unique tool and potential clinical treatments with highly selective control of neuronal cells. The present investigation is the first successful attempt to develop optochemogenetics tools in iPS transplantation therapy for the treatment of a brain disorder.

In our *in vivo* experiments, the LM03 activation was achieved by the noninvasive intranasal delivery of CTZ. The nasal-brain route has been used for delivering neuropeptides, trophic factors, and stem cells to the brain (Lioutas et al., 2015; Z. Z. Wei et al., 2015). Because of the noninvasive nature and the feasibility of repeated administrations, intranasal drug delivery reduces risks associated with exposures to anesthesia, surgical procedures, inflammation/infection, and stress. So far, intranasal delivery has been used clinically for a

← and aged mice (for young male: $p = 0.010$ vs stroke controls, $p = 0.038$ vs stroke plus CTZ; for young female: $p = 0.038$ vs stroke controls, $p = 0.045$ vs stroke plus CTZ; for aged male: $p = 0.031$ vs stroke controls, $p = 0.039$ vs stroke plus CTZ; for aged female: $p > 0.999$ vs stroke controls, $p > 0.999$ vs stroke plus CTZ; but $p > 0.999$ vs sham control). $n = 10$ for each group, except male stroke only control ($n = 14$). One-way ANOVA followed by Bonferroni correction: young male group: $F_{(3,40)} = 1.309$; young female group: $F_{(3,36)} = 3.520$; aged male group: $F_{(3,48)} = 3.164$; aged female group: $F_{(3,62)} = 6.432$. * $p < 0.05$ versus sham. # $p < 0.05$ versus stroke control. Δ $p < 0.05$ versus CTZ alone. **C, D**, The cylinder test detected the motor deficit of affected limb of stroke mice. Stroke induced much less use of the affected limb in 2-month-old male and female mice. CTZ showed no effect in male mice ($p > 0.999$ vs stroke controls) but some improvement in female mice ($p = 0.021$ vs stroke controls). Stroke mice received LM03 cells and CTZ treatment performed normally as sham controls (male: $p = 0.016$ vs stroke controls; female: 0.015 vs stroke controls), which was significantly better than CTZ alone (male: $p = 0.033$ vs stroke plus CTZ; female: 0.008 vs stroke plus CTZ). In aged mice of 18-month-old mice, CTZ or cell plus CTZ showed variable results, whereas the combination treatment promoted the use of the affected limb in aged female mice (aged male: $p > 0.999$ vs stroke controls, $p > 0.999$ vs stroke plus CTZ; aged female: $p = 0.049$ vs stroke controls, $p = 0.014$ vs stroke plus CTZ). One-way ANOVA followed by Bonferroni correction: young male: $F_{(3,41)} = 14.67$; young female: $F_{(3,36)} = 7.311$; aged male: $F_{(3,47)} = 4.432$; aged female: $F_{(3,54)} = 2.383$. $n = 11$ for sham and stroke control groups. $n = 13$ for stroke with CTZ alone group. $n = 10$ for stroke plus LM03-cells and CTZ. * $p < 0.05$ versus sham. # $p < 0.05$ versus stroke control. Δ $p < 0.05$ versus CTZ alone.

Figure 14. Illustration of the mechanisms and benefits of optochemogenetic stem cell therapy for ischemic stroke. Graph represents the experimental design in the investigation. It also outlines the hypothetical mechanisms and main therapeutic benefits induced by optochemogenetic stem cell therapy.

variety of brain disorders, such as epilepsy and neurodegenerative diseases (Freiherr et al., 2013; Tayebati et al., 2013; Novak et al., 2014; Spetter and Hallschmid, 2015).

Direct shining of laser light could open Chr2 or LMO3 channels within millisecond ranges, whereas CTZ application activates the LMO3 channel in cultured cells in seconds and even minutes after *in vivo* administration. While millisecond resolution is important for correlating stimulus with fast response, the chemogenetic paradigm in a slow pace is better suited for a regenerative treatment. The LMO3 protein provides the capabilities for the cells to be activated either in a way mimicking neuronal activities of fast activation or manipulations of the channel/cell in clinical treatments. The greater sensitivity of VChR1 to blue light shows higher coupling efficiency with GLuc and cyan bioluminescence, evoking more photocurrents compared with the same luciferase paired with Chr2 (Berglund et al., 2013; Berglund et al., 2016b). Using the cell line we engineered, activation of grafted iPS-NPCs shows significant synergistic benefits in neuronal differentiation and regenerative activities.

While optogenetic and chemogenetic activations possess multiple differences, the ultimate physiological effects converge on the same signaling pathways. Ca^{2+} imaging showed increased intracellular Ca^{2+} following LMO3 activation. Ca^{2+} can activate a series of Ca^{2+} -dependent molecules, such as CaMKII, cAMP, and protein kinase A, and trigger the expression and release of prosurvival/proregenerative genes, such as BDNF and NGF (Lonze and Ginty, 2002; Park and Poo, 2013), which shows beneficial paracrine and synaptogenesis effects (Park and Poo, 2013). BDNF is an activity-dependent factor that promotes axonal sprouting and extension (Mamounas et al., 2000). Stimulation through LMO3 upregulated presynaptic and postsynaptic markers, synapsin-1 and PSD95, suggesting the formation and stabilization of new synaptic contacts. Our electrophysiological recordings revealed robust synaptic activities in differentiated cells, indicating an activity-dependent maturation and synaptogenesis of iPS-derived neurons.

In the whisker-barrel cortex pathway, sensory signals are sent from the contralateral vibrissae to the primary somatosensory barrel cortex (S1) through the brainstem and the thalamus, which is in turn routed to the primary motor (M1) cortex to regulate whisker movements (Simons and Woolsey, 1979; Petersen, 2007). The tested stroke model allows for evaluation of circuit damage and repair through multiple approaches, including morphological, electrophysiological alterations, sensorimotor deficits determined by the adhesive removal task, and whisker-touching behaviors. Following focal injury in the barrel cortex, the reverberating secondary effects leads to remote disruption of thalamocortical activity (L. Wei et al., 1995; Mohamad et al., 2013; Paz et al., 2013; Fornito et al., 2015) and even retrograde neuronal degeneration of neurons in the VPM nucleus (L. Wei et al., 2004). Compared with naive animals, neuronal firing induced by whisker stimulation in the VPM ipsilateral to the stroke site showed diminished responses. This neurophysiological deficit was significantly ameliorated by the combined cell/CTZ therapy, suggesting a novel cell-based rehabilitation strategy, particularly for restoration of damaged neuronal connectivity. Overall, the present investigation supports and successfully demonstrates the importance of activity in the integrity and functional repair of neuronal connections in sensorimotor recovery after brain injury (Alawieh et al., 2017).

We also addressed gender and age factors in the optochemogenetics therapy. Our data show that iPS-NPC transplantation

and the combined cell/CTZ treatment are effective in most measurements of functional tests, although some beneficial effects may be less robust in aged male animals. Our rigorous control experiments *in vitro* and in animals provide compelling evidence that it is the selective LMO3 activation in iPS-NPCs responsible for the observed activities and benefits. Interestingly, in a few functional assessments, female animals seemed to show more or less response to CTZ alone (statistically significant in the corner test and cylinder test of young female mice). The observation needs to be verified in additional experiments of more functional tests; if it can be confirmed as a persistent phenomenon, a possible sex difference and underlying mechanism should be delineated.

It is important to achieve optimal functional recovery after stroke. To verify this issue, we examined functions that are essential and meaningful for daily activities of stroke patients. In these subacute and chronic assessments, the functionality of stroke animals recovered to normal levels after the cell/CTZ combinatory treatment. Clinical practice shows that even slight functional improvements, such as regaining movement of the palm/finger(s) or slow walking ability, can greatly improve the life quality of stroke patients. Accordingly, the minimal clinically important difference standard indicates that even 10% improvement in movement control can improve quality of life for patients with upper or lower limb impairments (Page, 2014; Pandian et al., 2016). In this regard, the functional recovery demonstrated in both genders and different ages is promising and of clinical significance.

In conclusion, the novel optochemogenetics approach provides a translational solution to take the advantage of selective activation of transplanted cells in a clinically feasible way to enhance regeneration, neural network repair, and functional recovery after stroke.

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