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Supplemental Information

A NeuroD1 AAV-Based Gene Therapy for Functional Brain Repair after Ischemic Injury through *In Vivo* Astrocyte-to-Neuron Conversion

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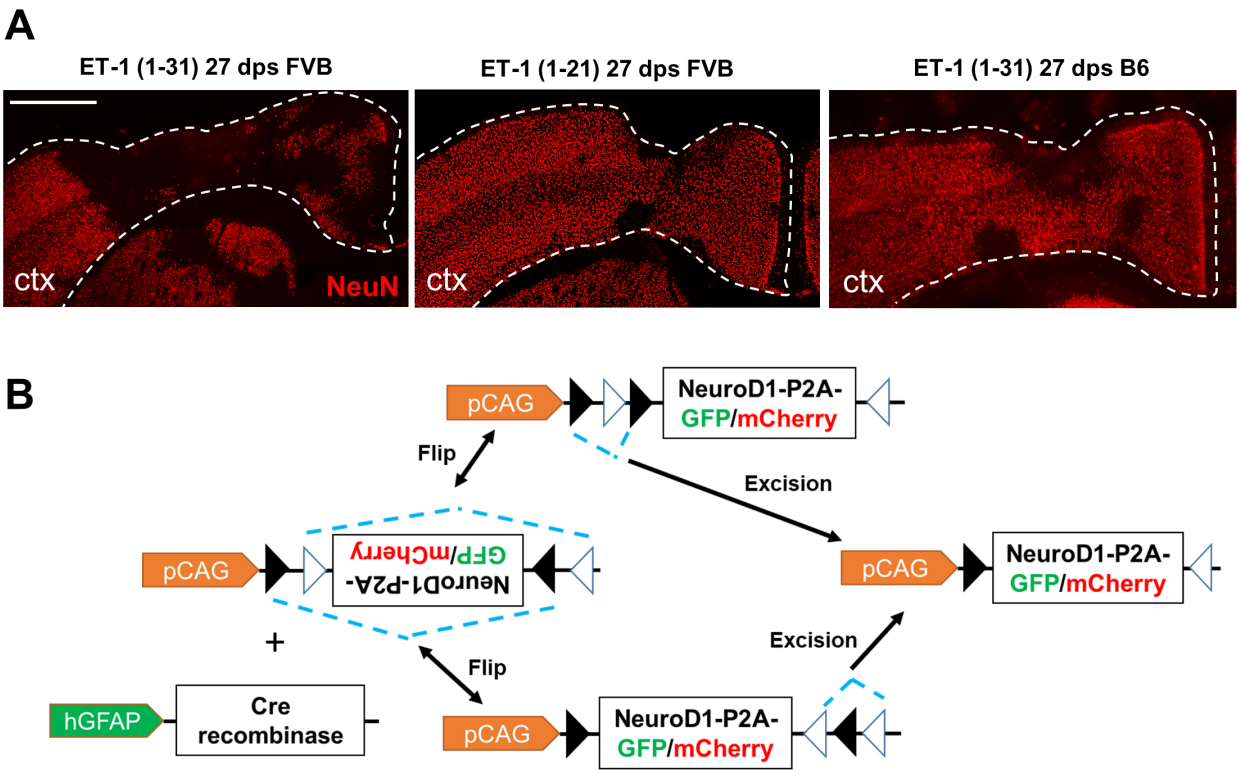
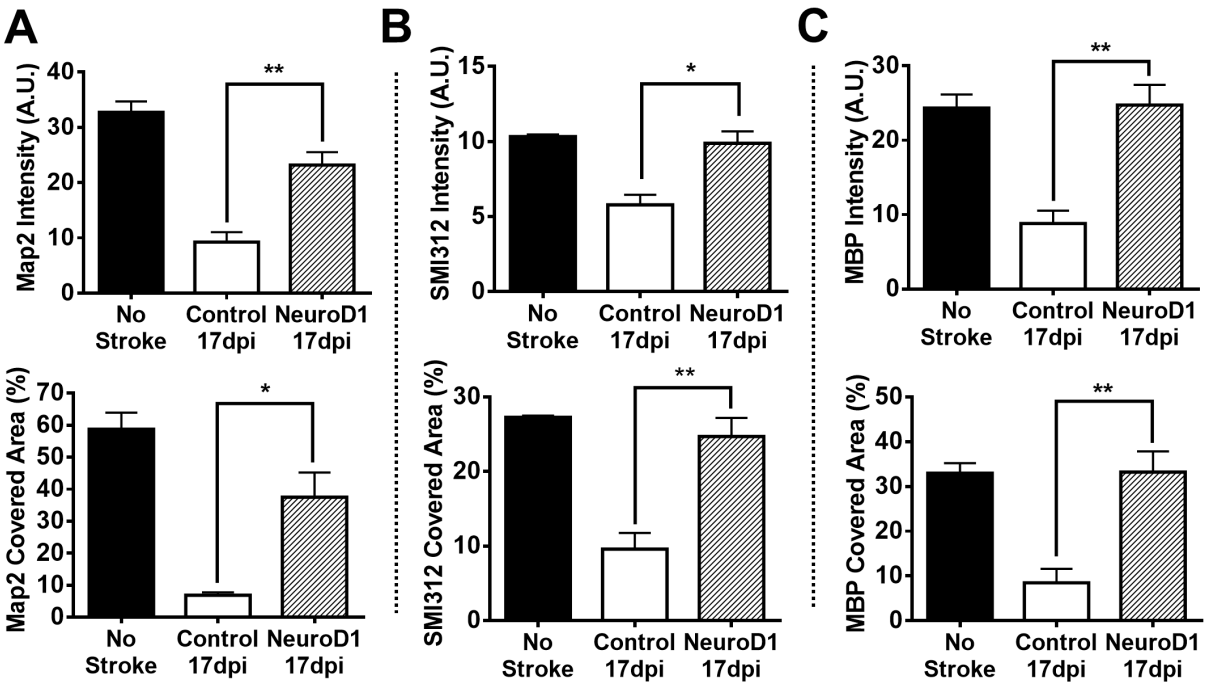
SUPPLEMENTARY FIGURES AND LEGENDS



Supplementary Figure 1 | Stroke model and engineered NeuroD1 Cre-FLEX system.

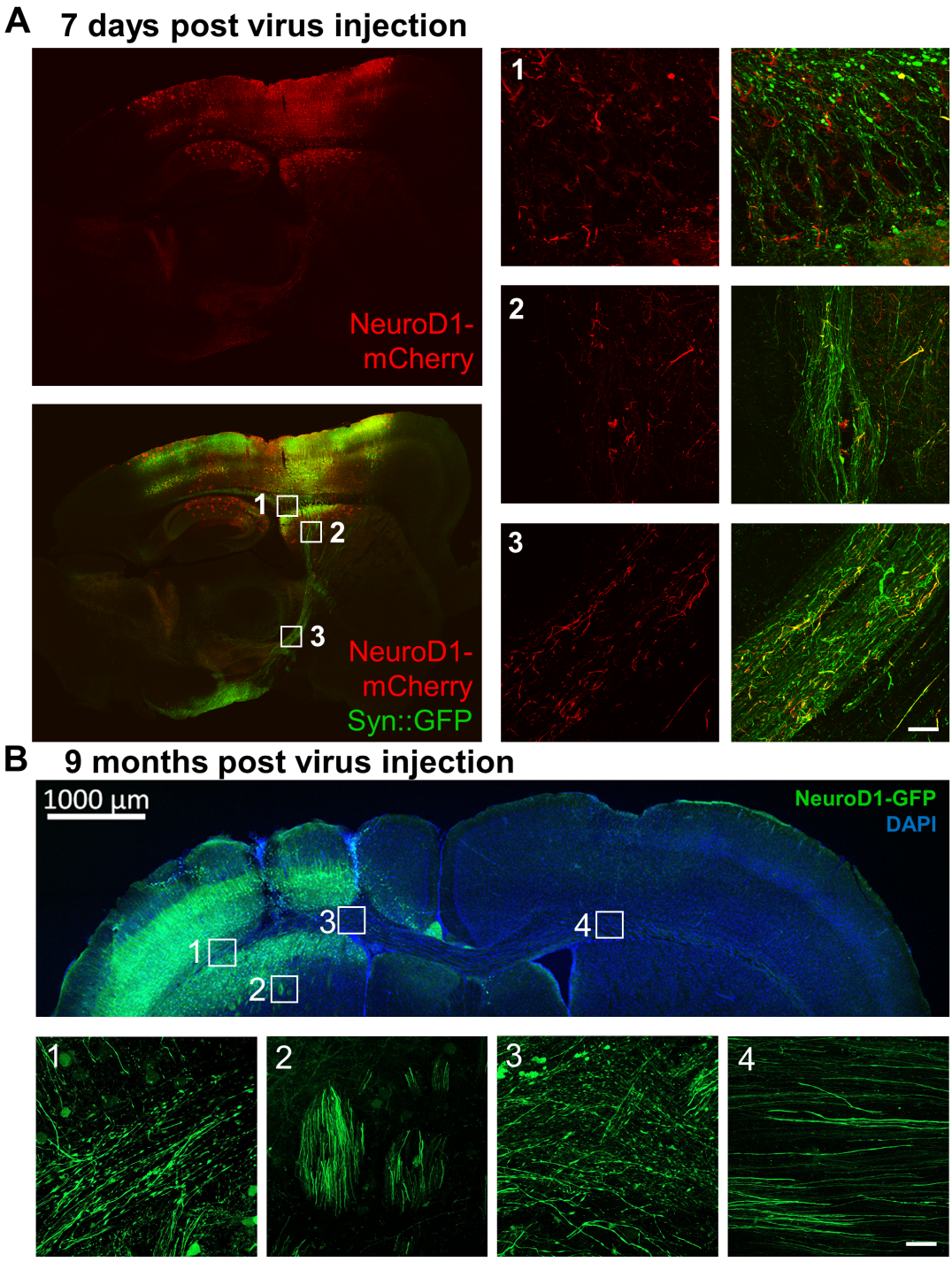
(A) Representative images illustrate severe ischemic injury induced by ET-1 (1-31) in FVB mice (left), but mild injury induced by ET-1 (1-21) in FVB mice or ET-1 (1-31) in B6 mice. NeuN immunostaining at 27 dps revealed that ET-1 (1-31) induced more NeuN loss and more severe cortical atrophy in FVB animals compared to the other two conditions. Scale bar, 800 μm.

(B) The Cre recombinase is expressed under the control of GFAP promoter in order to target astrocytes specifically. NeuroD1 and GFP (or mCherry) are flanked by double loxP sites in an inverted sequence under the control of CAG promoter. If expressing together in astrocytes, the Cre-mediated recombination will result in the expression of NeuroD1 and GFP (or mCherry) under the promoter of CAG.



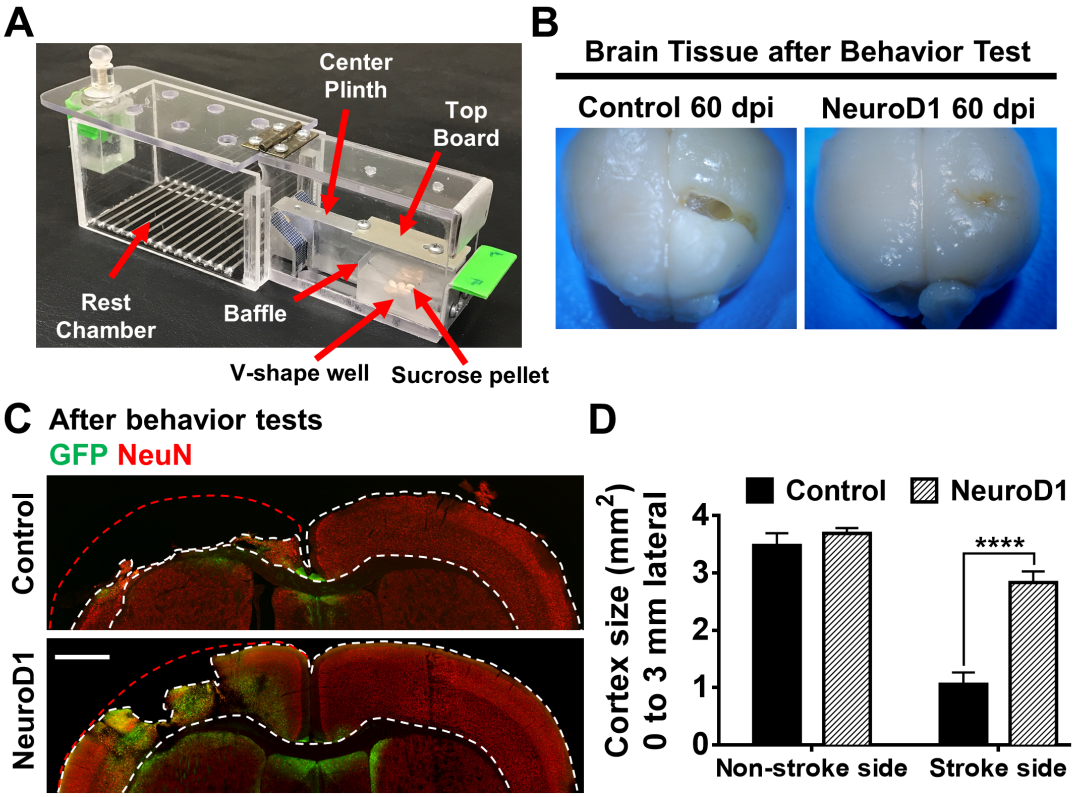
Supplementary Figure 2 | Neuronal recovery after NeuroD1-treatment.
Quantification of the signal intensity (A.U., artificial unit) and covered area (%) for dendritic marker Map2 (A), axon marker SMI312 (B) and myelination marker MBP (C).

* P < 0.05, ** P < 0.01. One-way ANOVA followed by Tukey multiple comparison test, n = 3 mice per group. Data are represented as mean ± s.e.m.



Supplementary Figure 3 | Long-range axonal projection after AtN conversion. (A) Newly generated neurons send out axons along preexisting axonal pathways. AAV9-pSyn::GFP was injected 7 days before ET-1 infusion to label the pre-existing endogenous neurons and their axonal pathways. AAV-NeuroD1-mCherry was injected

10 days post stroke and animals were sacrificed at 7 dpi. Note that there were limited number of axons labeled by mCherry, suggesting very few NeuroD1-converted neurons were sending out axons at this time point. On the other hand, the mCherry-labeled new axons were following GFP-labeled preexisting axonal pathways.
(B) Coronal section of mouse cortex at 9 months post NeuroD1 virus injection, showing long-range axon projection to the contralateral side through corpus callosum. Bottom panels illustrate enlarged view of axon projections inside the corpus callosum (box 1, 3, and 4) and projections through the striatum (box 2).
Scale bar at top row is 1000 μm, and the bottom row is 40 μm.



Supplementary Figure 4 | Assessment of Tissue Damage after Behavioral Tests. (A) Picture of the pellet retrieval device modified from staircase. The mouse in the rest chamber was trained to reach the sucrose pellet in the V-shape well after 18 hr food

deprivation. It is clear from our design that the mice had to spend a great effort with motor coordination and strength to successfully retrieve the food pellet (See Supplementary Video 1).

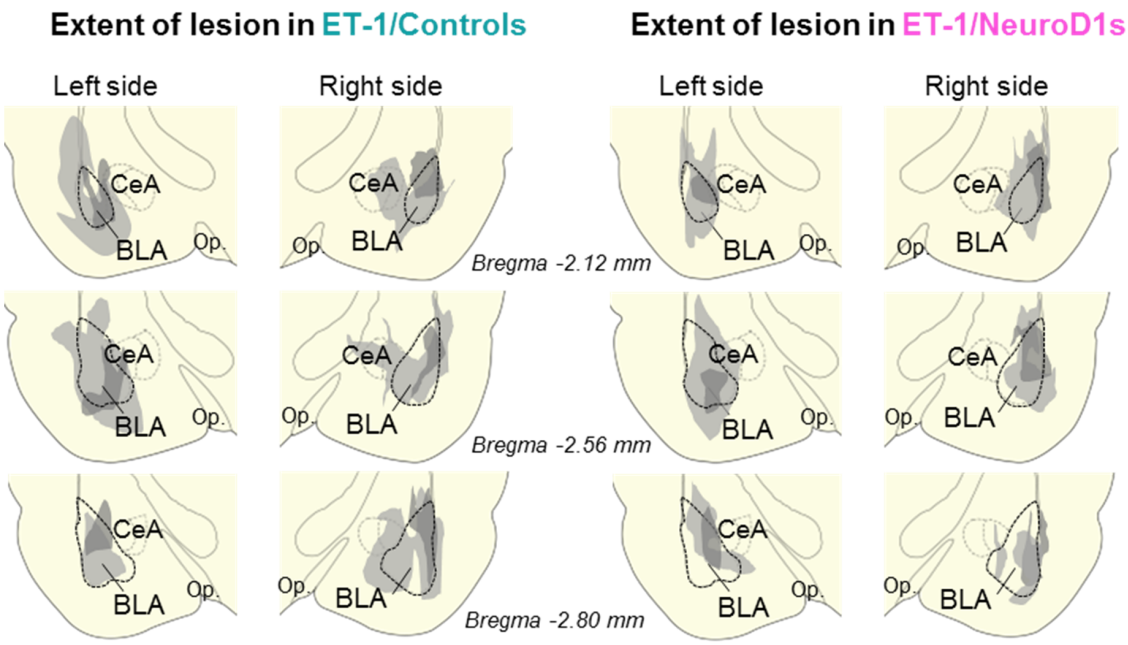
(B) Representative pictures of mouse brains after finishing behavioral tests (2 months post viral injection). The control group showed severe cortical tissue loss on stroke side after ET-1 (1-31)-induced focal stroke, whereas NeuroD1-treatment showed a significant rescue of the tissue loss.

(C) Representative images of the mouse cortex in coronal sections illustrating the severe tissue loss after ET-1 (1-31) induced ischemic stroke. Note that two ET-1 (1-31) injections were made in both motor cortex and sensory cortex on one side to induce more severe injury for long-lasting behavioral tests. Therefore, the tissue loss is also more severe than the single injection performed for most of the cell conversion studies. Nevertheless, the NeuroD1 repairing effect is still significant.

(D) Quantification of cortical tissue damage after behavioral tests. Two-way ANOVA followed by Sidak multiple comparison test, n = 6 mice per group. Data are represented as mean ± s.e.m.;

(E) Four months after NeuroD1-GFP AAV injection following stroke, c-Fos signal was detected after wheel running, and some were colocalized with NeuroD1 and GFP (arrowheads), suggesting functional integration of NeuroD1-converted neurons into the motor cortex.

Scale bars (C) 1000 μm; (E) 40 μm.



Supplementary Figure 5 | Estimated size of the amygdala lesion induced by the infusion of endothelin [ET-1(1-21)] in both ET-1/Control group (left) and ET-1/NeuroD1 (right). Gray areas represent the minimum (dark) and maximum (light) spread of the lesion at different antero-posterior levels of BLA (-2.12, -2.56, and -2.80 from bregma). BLA, basolateral nucleus of the amygdala; CeA, central nucleus of the amygdala, op., optical tract.

ADDITIONAL MATERIALS AND METHODS

Immunohistochemistry:

Mouse sample. Immunohistochemistry of floating frozen sections of mice brain was performed as described previously[1]. Briefly, animals were anesthetized with 2.5% Avertin and transcardially perfused with artificial cerebral spinal fluid (ACSF) to wash off the blood in the brain tissue. Then, brains were dissected out, trimmed, and put in 4% paraformaldehyde PFA for post-fixation at 4 °C overnight. After fixation, brain tissues were cut at 40 μm sections by a vibratome (Leica). Brain sections were permeabilized in 2% Triton X-100 in PBS for 1 hr, followed by incubation in blocking buffer (2.5 % normal goat serum, 2.5 % normal donkey serum, and 0.1% Triton X-100 in PBS) for 1 hr. The primary antibodies were added into the blocking buffer with brain sections and incubated overnight at 4 °C. After washing off primary antibodies in PBS, brain sections were incubated with secondary antibodies conjugated with different fluorophores (1:800, Jackson ImmunoResearch) for 1 hr at room temperature, washed in Triton-PBS, and then mounted onto a glass slide with an anti-fading mounting solution containing DAPI (Invitrogen). Images were acquired with confocal microscopes (Olympus FV1000 or Zeiss LSM800) and a Keyence microscope. To test for antibody specificity, primary antibody was withdrawn and only secondary antibody was used for immunostaining as a side-by-side control for all the primary antibodies. No specific signal was detected in these controls without primary antibodies. Primary antibodies are given in the Table1.

Rat sample. At the conclusion of behavioral experiments, rats were deeply anesthetized with sodium pentobarbital (450 mg/ml, i.p.) and perfused transcardially with 100 ml saline (0.9%), followed by 500 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were post fixed for 3 h in the same fixative solution and transferred to a solution of 30% sucrose in 0.1 M phosphate buffer at 4 °C for 2 nights. Brains were then frozen and a series of 40 μm sections were cut with a cryostat (Leica, CM 1850) in the frontal plane and collected at different levels of the BLA. Alternate sections were initially blocked in a solution of 2% normal goat serum (Vector Laboratories) plus 0.3% Triton X-100 (Sigma-Aldrich) in 0.12 M phosphate buffered

saline for 1 hour and then incubated overnight with anti-NeuN (1:200, conjugated with rabbit polyclonal Alexa Fluor 488; EMD Millipore). On the following day, slices were rinsed in potassium buffered saline, mounted on gelatin-coated slides, dehydrated and then coverslipped with anti-fading mounting medium (Vectashield, Vector Laboratories).

Electrophysiology. Brain slice recordings were performed similar to previously described[1,2]. Briefly, 2-3 months after AAV injection, the mice were anaesthetized with 2.5% avertin, and then perfused with NMDG-based cutting solution (in mM): 93 NMDG, 93 HCl, 2.5 KCl, 1.25 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 15 glucose, 12 N-Acetyl-L-cysteine, 5 sodium ascorbate, 2 Thiourea, 3 sodium pyruvate, 7 $MgSO_4$, 0.5 $CaCl_2$, pH 7.3-7.4, 300 mOsmo, bubbled with 95% O_2 / 5% CO_2. Coronal sections of 300 μm thickness were cut around AAV-injected cortical areas with a vibratome (VT1200S, Leica, Germany) at room temperature. Slices were collected and incubated at 33.0 ± 1.0 °C in oxygenated NMDG cutting solution for 10-15 minutes. Then, slices were transferred to holding solutions with continuous 95% O_2 / 5% CO_2 bubbling (in mM): 92 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 15 glucose, 12 N-Acetyl-L-cysteine, 5 sodium ascorbate, 2 Thiourea, 3 sodium pyruvate, 2 $MgSO_4$, 2 $CaCl_2$. After recovery at least 0.5 h at room temperature in the holding solution, a single slice was transferred to the recording chamber continuously perfused with standard aCSF (artificial cerebral spinal fluid) saturated by 95% O_2 / 5% CO_2 at 33.0 ± 1.0 °C. The standard aCSF contained (in mM): 124 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 Glucose, 1.3 $MgSO_4$, 2.5 $CaCl_2$. To detect action potential firing in NeuroD1-GFP-infected neurons, whole-cell recordings were performed with pipette solution containing (in mM): 135 K-Gluconate, 10 KCl, 5 Na-phosphocreatine, 10 HEPES, 2 EGTA, 4 MgATP and 0.3 Na_2 GTP, pH 7.3 adjusted with KOH, 280–290 mOsm. Depolarizing currents were injected to elicit action potentials under current-clamp model. To record spontaneous excitatory postsynaptic currents (sEPSCs) and spontaneous inhibitory postsynaptic currents (sIPSCs), pipette solution contained (in mM): 120 Cs-Methanesulfonate, 10 KCl, 10 Na-phosphocreatine, 10 HEPES, 5 QX-314, 1 EGTA, 4 MgATP and 0.3 Na_2GTP, pH 7.3 adjusted with KOH, 280–290 mOsm. To labeled recorded neurons, 0.5% biocytin was added to the pipette solution. The cell membrane

potentials were held at -70 mV (the reversal potential of $GABA_A$ receptors) for sEPSC recording, and 0 mV (the reversal potential of ionotropic glutamate receptors) for sIPSC recording, respectively. Data were collected with a MultiClamp 700A amplifier and analyzed with pCLAMP10 software (Molecular Devices).

CTB Retrograde tracing. Mice were injected with ET-1 in the motor cortex and then AAV9 (Cre-FLEX-NeuroD1-mCherry/GFP) was infused at same region 10 days after stroke. 40 days after viral injection, CTB-488/647 dye was injected into the thalamus area to retrogradely labeling cortical neurons. Animals were sacrificed for analysis 7 days after CTB injection.

RNA-sequencing Analysis. RNA quality check, mRNA enrichment, library construction and single-end 50 bp sequencing with HiSeq 3000 were performed at the UCLA Technology Center for Genomics & Bioinformatics. Quality check of the raw data was done using FastQC (v. 0.11.3) using default setting. The filtered reads were aligned against mouse reference genome mm10 using HISAT2 (v. 2.0.1)[3] and summarized using featureCounts (v. 1.5.0)[4]. Genes that had average expression levels greater than 5 counts were considered valid. Differential expression analysis was processed using DESeq2 (v. 1.16.1)[5], and genes with more than 3-fold differences, baseMean > 50 and adjusted p-value < 0.01 were called differentially expressed. Gene ontology (GO) analysis was performed on Gene Ontology Consortium (http://www.geneontology.org/).

Behavioral tests:

Mouse food pellet retrieval test. For all behavioral experiments in mice, ET-1 (1-31) was injected into two points of the forelimb motor cortex in order to induce severe motor deficits. The coordinates of the two points are: (i) +0.2 mm AP, +/- 1.35 mm ML; (ii) +0.38 mm AP, +/- 2.45 mm ML (+ or - ML was based on the opposite side of the dominant forelimb in pre-stroke pellet retrieval training). The food pellet retrieval chamber was modified from the staircase equipment for mice[6,7]. In brief, the staircase was changed to an arm with a single V-shape well and a small baffle at the end to

prevent food pellet dropped outside the well during the test. The well arm is 33 mm long and the lowest part of the well is 16 mm from the bottom of top board, as illustrated in the Figure S6A. Before pellet retrieval test, mice were food-deprived for 18 hrs to increase their motivation. During initial food deprivation training, mice were given 30 sucrose pellets (14 mg, TestDiet, Inc) in home cages for familiarization. For the first part of training, the top board of the center plinth was removed to reduce difficulty and encourage animals to try reaching the food. Five pellets were placed in the well either on the right side or left side to determine the dominant forelimb. Mice were given 5 min and tested twice on each side. The dominant side was determined at this stage by counting how many pellets were retrieved. Most animals could get ~5 pellets on one side and sometimes both sides. If the animals failed to get any pellet, they would be excluded from further test. For the second part of training and all the following testing, the top board was placed back onto the center plinth and 8 pellets were placed in the well at the dominant side. Animals were tested 3 consecutive times every day, each time 5 min for pellet retrieval. The animals would be used for further surgery and test if the average retrieval was more than 4 pellets on three consecutive training days. After training, ischemic stroke was induced in the contralateral motor cortex controlling the dominant forelimb. At 9 days after stroke and one day before viral injection, a test was performed to determine their functional levels. If the number of pellets retrieved were less than half of their pre-stroke level, these animals would be used for viral injection the next day (10 days post stroke). Following viral injection, pellet retrieval tests were performed at 20, 30, 50, and 70 dps to assess their functional recovery. For each time point, pellet retrieval tests were repeated on two consecutive days, with day 1 as training and day 2 as the actual test. Animals were coded and a second experimenter who was blind to the identity of the animals performed the test and pellet counting.

Mouse grid walking test. The grid-walking test was similar to previous reports[8,9]. We used a 1 cm^2 grid wire mesh of 24 cm long and 20 cm wide, elevated to 24 cm high. A video camera was placed below the grid, facing upwards with a 45° angle. Slow motion video footages were recorded to assess the animals' foot faults. Individual mouse was placed on top of the grid floor and allowed to freely walk for 5 min. The animals were coded and video footage was analyzed offline by a second researcher who was blind to

the animal identity. The total number of foot faults for dominant limb and the total number of non-foot-fault steps were counted. Foot fault percentage was calculated as: the number of foot faults / total number of steps × 100.

Mouse cylinder test. The cylinder test was based on previous work [7]. This test involved video recording of mice rising and touching the sidewall of a transparent cylinder with forelimbs (10 cm diameter, 15 cm tall). Each animal was placed into the cylinder on a transparent board and video-recorded with a camera from below. The experiment was stopped after the mouse attempted to rise and touch the sidewall for >30 times, which normally lasted ~3-4 minutes. 30 total attempts of rising and touching the wall were analyzed. A normal paw touch is defined as the animal placing both paws on the wall while rising, and using both paws to push against the wall while descending. An abnormal touch involves only using one of the paws to touch the wall, or one paw dragging along the wall after touching. Dragging behavior is defined as one paw, typically the injured one, sliding along the sidewall without holding steady against the wall. A non-touching behavior is defined as the mouse not using its injured paw but only using non-injured paw while rising and descending. Non-touching is considered a more severe injury phenotype since the mouse totally abandons the injured paw. The normal rising and touching behavior was quantified as: normal paw touches / total attempts × 100. The videos were analyzed offline by an experimenter blinded to the animal's identity.

Wheel running and c-Fos detection. 4 months after NeuroD1 virus injection following stroke, the animals were placed in a running wheel. Thirty minutes after actively running, the mice were placed back into the home cage and one hour later they were sacrificed and perfused for c-Fos immunostaining.

Rat fear conditioning test. A total of 41 male Sprague-Dawley rats (Harlan Laboratories) aging between 3-5 months and weighing 300-360 g underwent bar press training, auditory fear conditioning, and a fear retrieval test in standard operant chambers (12" W x 10" D x 12" H; Coulbourn Instruments), inside sound-attenuating

boxes (Med Associates). During all phase of the fear conditioning and tests, rats were able to press a bar to receive a sucrose pellet (VI 60 s reinforcement) in a dish located next to the bar. The floor of the chambers consisted of stainless steel bars that delivered a scrambled electric footshock. Conditioning was performed 3 weeks after intra-BLA infusion of saline or ET-1 (day 21), and consisted of habituation to five nonreinforced tones (75 dB, 30 s; habituation) followed by seven tones co-terminated with a footshock (0.5 s, 0.52 mA). On the next day (day 22), rats underwent a memory retrieval test in the same context (Test 1), in which they were exposed to two conditioned tones. Twenty-four hours later, rats previously infused with ET-1 were assigned to receive intra-BLA infusions (3 µl/side) of either control-virus or NeuroD1, whereas rats previously infused with saline received a second infusion of saline. Infusions were carried out using the same procedures described above. Control virus and NeuroD1 groups were chosen by matching freezing levels during Conditioning and Test 1. Three weeks were allowed for viral expression (day 23-44). Animals were then returned to the chamber (Test 2, day 45) for a fear retrieval test. All trials were separated by a variable interval of ~3 min. Between animals, grids and floor trays were cleaned with soap and water, and chamber walls were cleaned with wet paper towels. Sample size was determined as the minimal number of animals that would provide statistical power to detect a group difference (level of significance 0.05, power 0.9). Groups were assigned after matching for freezing levels during the conditioning session. Behavior was recorded with digital cameras (MicroVideo Products). Freezing was automatically scored using a commercially available video tracking system (Any-Maze, Stoelting). The amount of time spent freezing to the tone was expressed as a percentage of the tone presentation. Trials were averaged in blocks of two, and subjected to one-way analysis of variance (ANOVA), followed by Duncan's post-hoc test as appropriate (STATISTICA Statsoft).

Quantitative real-time PCR. The cortical tissues around the injury core (approximately 2 mm x 2 mm square) were taken after perfusion and flash-frozen in liquid nitrogen. The RNA extraction was conducted using Macherey-Nagel NucleoSpin RNA kit and the RNA concentration was measured by NanoDrop. The cDNAs were synthesized using

Quanta Biosciences qScript cDNA supermix. The reaction mixture was incubated at 25 °C for 5 min, 42 °C for 30 min, 85 °C for 5 min and held at 4 °C. Then the mixture was diluted 5-fold with RNase/DNase-free water and 2.5 µl was taken for qRT-PCR. The primers for qRT-PCR were designed using Applied Biosystems Primer Express software. The qRT-PCR was conducted using Quanta Biosciences PerfeCTa SYBR Green Supermix, ROX. GAPDH was used as an internal control and non-stroke cortical tissue from healthy mice was used as control. Comparative Ct method was used for calculation of fold change and Prism 6 was used for statistical analysis and bar graphs.

QUANTIFICATION AND STATISTICAL ANALYSIS

Cortical area size analysis. Cortical areas were quantified using the images taken with a 4x lens in a Keyence BZ-9000 Fluorescent microscope. Three slices around the injection point (+0.2 AP) with most obvious stroke injury were chosen for image-taking. DAPI and NeuN signals were used to identify the cortical upper boundary and the lower interface with cingulate cortex. Cortical areas from the midline to 3 mm lateral were measured by ImageJ.

Neuronal marker intensity and covered area analysis. Single layer confocal images of neuronal markers (Map2, SMI32, SMI312 and MBP) were used for quantification at 17 dpi. 40x lens was used for most markers except for SMI312, for which 63x lens was used. Three images were taken at virus-infected areas close to the stroke infarct (within 500 µm from injury core) and at the middle of cortical sections. One image was taken at healthy region without injury for normalization. The threshold for background signal was also set using the healthy area. Results from three images were used to get average data for each animal.

NeuN cell number analysis. NeuN cell number was counted on the images of NeuN immunostaining taken by the tile function of Zeiss LSM800 confocal microscope within the cortical areas from 500 µm to 2500 µm lateral of the midline. The confocal software Zen was used for cell counting. Slices around the injection point (+0.2 AP) with most obvious stroke injury were used for image analysis.

Electrophysiological analysis. The sEPSCs and sIPSCs were analyzed using Mini Analysis Program (Synaptosoft, New Jersey, USA). To avoid multiple detections of large events, the analysis results were further checked visually after auto-detection. To compare sEPSCs and sIPSCs frequency between different groups, over 200 events or at least 3-min recording periods were sampled for each cell before obtaining the average value.

Table 1 | Antibodies

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chicken polyclonal to GFP	Abcam	Ab13970
Mouse monoclonal to SATB2	Abcam	Ab51502
Mouse monoclonal to NeuroD1	Abcam	Ab60704
Chicken polyclonal to MAP2	Abcam	Ab5392
Rabbit polyclonal to Emx1	Abcam	Ab32925
Rat monoclonal to Ctip2	Abcam	Ab18465
Rabbit polyclonal to GFAP	Millipore	AB5804
Chicken polyclonal to GFAP	Millipore	AB5541
Rabbit polyclonal to NeuN	Millipore	ABN78
Rabbit polyclonal to Tbr1	Millipore	AB10554
Chicken polyclonal to MBP	Millipore	AB9348
Guinea pig polyclonal to VGLUT1	Millipore	AB5905
Rabbit polyclonal to GABA	Sigma	A2052
Mouse monoclonal to Parvalbumin	Sigma	P3088
Mouse monoclonal to SMI-32P	BioLegend	801701
Mouse monoclonal to SMI-312	BioLegend	837904
Guinea pig polyclonal to VGAT	SYSY	131004
Rat monoclonal to RFP	antibodies-online.com	ABIN334653
c-Fos	SySy	226 003

Table 2 | qRT-PCR primers

Primer	Forward sequence	Reverse sequence
m-*Gapdh*	GGAGCGAGACCCCACTAACA	ACATACTCAGCACCGGCCTC
m-*Rbfox3/Neun*	GCTGACTTGGGCTATTGCCT	CCTAACTGCTCCAGCCCCTT
m-*Robo2*	TCAGGAGGCCAATTAACCAGG	TCTCGGCAAGTCTGCATCATC
m-*Syn1*	CTGCTGAGCCCTTCATTGATG	TGGTCTTCCAGTTACCCGACA

Supplementary Movie 1 | Pellet retrieval test. A pre-stroke video is shown to illustrate how an animal retrieved 5 among 8 pellets in 5 min. Stroke was induced on this animal by ET-1 (1-31) injection and 9 days post stroke (dps) video shows its ability to retrieve pellets significantly reduced (retrieved 0 among 8 pellets). The AAV NeuroD1 was injected at 10 dps and the video at 60 days post viral injection (dpi) shows that NeuroD1-treatment helped recovery of pellet retrieval capability, with the mouse now getting 4 among 8 pellets. In comparison, another animal with similar pre-stroke capability (retrieved ~ 6 among 8) is also shown in the video at 9 dps and 60 dpi with control AAV injection. At 60 dpi, it retrieved significantly less pellets.

Supplementary Movie 2 | Grid walking test. Three time points (pre-stroke, 9 dps and 60 dpi) were shown for one animal which received ET-1 (1-31) injection followed by AAV-NeuroD1 injection. Some of the foot fault falls within the 5 minutes testing period were presented to illustrate the foot fault at different time points. Before stroke, this animal had a baseline of foot fault rate at 4.6%, which increased significantly to 11.4% after stroke prior to viral injection. Then at 60 dpi of injecting AAV NeuroD1, the foot fault rate reduced to 6.3%. A control animal is also shown at 60 dpi with foot fault rate of 11.2% (pre-stroke: 4.9%; 9 dps: 11.6%), suggesting a long-lasting deficit after stroke.

Supplementary Movie 3 | Cylinder test. Three time points (pre-stroke, 9 dps and 60 dpi) were shown for one animal which received ET-1 (1-31) injection followed by AAV NeuroD1 injection. Ten rising attempts among total thirty were presented to illustrate normal touching, dragging and non-touching behavior. Before stroke, this animal had a baseline of normal touching behavior at 86.7%, which decreased significantly to 23.3% after stroke prior to viral injection. At 60 dpi of injecting AAV NeuroD1, the normal touching behavior recovered back to 73.3%. A control animal is shown at 9 dps (33.3%) and 60 dpi after injecting control virus (40%), suggesting the control animal didn’t recover significantly.

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