SUPPLEMENTAL MATERIAL

Post-stroke shh agonist treatment improves functional recovery by enhancing neurogenesis and angiogenesis

Yongming Jin[1], PhD, Austin Barnett[1#], BS, Yifan Zhang[2#], PhD, Xin Yu[2], ScD, Yu Luo[1*], PhD

[1]Department of Neurological Surgery, Case Western Reserve University, Cleveland, USA

[2]Department of Biomedical Engineering, Case Western Reserve University, Cleveland, USA

Correspondence to

*Yu Luo, PhD, Department of Neurological Surgery, Case Western Reserve University, 2109 Adelbert Rd, Cleveland, OH, USA. Email: yxl710@case.edu, Phone: 01-216-368-4169,

[#], These authors contributed equally to this manuscript.

Cover title: shh agonist enhances post-stroke recovery

Supplemental methods:

Animals: All animal protocols were conducted under National Institutes Health (NIH) Guidelines using the NIH handbook *Animals in Research* and were approved by the Institutional Animal Care and Use Committee (Case Western Reserve University). The mice were housed in the animal facility of Case Western Reserve University on a 12-h light/dark diurnal cycle. Food was provided *ad libitum*. Male C57BL/6 mice (10-12 weeks old) were used in this study. To evaluate neurogenesis in stroke mice, the nestin-CreERT2-R26R-YFP strain was used in this study. The generation and characterization of animals used in this study were previously described [1, 2].

All the experimental groups are randomized and all outcome analysis was carried out by independent study team members blinded to the treatment condition. Details on experimental timeline, randomization and blinding of groups, animal numbers for each experiment, and survival rate/exclusions due to death are included in individual sections below.

Locomotor function: 28 mice were subjected to MCAo. All of the mice were subjected to Laser Doppler monitor, mice that did not have at least 80% of blood supply drop or without > 60 % reperfusion were excluded (n=2). On post-stroke day 3, the remaining 26 mice were subjected to a one-hour locomotion behavioral test in the automatic behavioral chambers. Mice were assigned into two equal groups according to their locomotion measurements. There is no difference in any the parameters measured in the two groups before or at post-stroke day 3 after MCAo (Supplemental TableI). The two groups were randomly assigned to either receive vehicle treatment or SAG treatment starting from post-stroke day 3 for 6 consecutive days. Locomotor function was measured in two animal groups again on post-stroke day 7, 21 and 30 after which animals were perfused and brains harvested. There were 3 more mice excluded (1 in vehicle and 2 in SAG-

treated group) due to death before the completion of the experiments on post-stroke day 30. Total of 12 vehicle-treated and 11 SAG-treated mice were used to analyze the final locomotor function data. Brain sections were used in subsequent immunostaining analysis as described in the following.

Cognitive function: Total of 36 mice were subjected to MCAo surgery, 4 mice died before the treatment started (post-stroke day 3). 32 mice were randomly assigned into 2 groups which received either vehicle or SAG starting post-stroke day 3 for 6 days. 4 mice from the vehicle and 2 mice from the SAG groups died before the test of Barnes maze on post-stroke day 30, resulting in a final of n=12 for vehicle and n=14 for SAG-treated groups for this behavioral test. To evaluate the effect of SAG treatment on Barnes maze performance on control mice, mice received SAG or vehicle treatment (10mg/kg for 6 days, n= 9 for each group) without surgery and were subjected to Barnes Maze test 21 days later.

MRI study: A subset of 18 mice randomly selected from the 36 mice described above in Barnes maze experiment received MRI at 2 days after MCAo and 3 mice died during the course of this experiment and 2 mice were excluded for further study because the MCAo volume is > than 60% of the half hemisphere on day2 after MCAo (average ischemic volume = 33.34$\pm$3.53%). The remaining 14 mice were randomly assigned into 2 groups (there is no difference in the ischemic volume between the two groups, vehicle =33.954$\pm$4.20%, SAG group= 32.64$\pm$6.7%, t-test p=0.868). 7 mice received vehicle treatment and 7 mice received SAG treatment from post-stroke day 3 for 6 days. These final 14 mice received a second MRI scanning for T2 and CBF on day 30 after stroke.

Neurogenesis evaluation: 20 Nestincre-ERT2-YFP mice were treated with TAM to induce YFP labeling of SVZ and SGZ NSCs as described in detail below. MCAo was induced in all 20 mice and 4 mice died before post-stroke day 3. 16 mice were randomly grouped into vehicle or SAG group and n=7 in each group survived till post-stroke day 30. Brain were harvested and analyzed for YFP, DCX and NeuN expression as described below.

BrdU pulse labeling: To evaluated whether SAG treatment affect NSCs proliferation in post-stroke mice., 20 mice were subjected to MCAo and 1 mouse was excluded due to lack of CBF drop. In the remaining 19 mice, 3 mice died before post-stroke day 3. The remaining 16 mice were assigned randomly into 2 groups each received either vehicle or SAG. 2 additional mice (1 in vehicle and 1 in SAG group) died before post-stroke day 7 when the mice were harvested (n=7 for each group). Mice received post-stroke vehicle or SAG treatment for 4 days from post-stroke day 3 and BrdU was injected on post-stroke day 6 (twice 0.1ml/10g BrdU labeling solution, Life technologies). All mice were perfused on post-stroke day 7 to evaluate cell proliferation by BrdU, PCNA or Ki67 immunostaining as described below.

Murine Model of Transient Focal Ischemia: Transient MCAo was induced in male 10-12 week old C57/BL6 mice (Jackson Laboratory, Bar Harbor, Maine USA) or male NestincreERT2-YFP mice (C57/BL6 congenic background, 10-12 week old) mice as described [3]. Briefly, transient MCAo was induced by an intraluminal suture method. Mice were anaesthetized with isoflurane and a midline neck incision was made to expose the right common carotid artery (rCCA). The isolated rCCA was temporarily ligated with a silk suture during whole period of occlusion. The right external carotid artery (rECA), and the right internal carotid artery (rICA) were also isolated by dissection of fascia. The rICA was clamped with an artery clamp above a loose suture tie around rICA. The rECA was ligated and cut to make a rECA stump. A nylon filament suture with a silicon

coated tip (Doccol co, Sharon, MA, USA) was inserted in rECA stump and further pushed along the lumen of ICA while the clamp was released and then the tie around rICA was tighten. After 35 minutes of occlusion, the nylon suture was gently removed from rMCA to reperfuse the rMCA territory. The rECA stump was permanently ligated and the temporary ligation in rCCA and rICA were removed to allow normal blood flow to the brain. During the surgery, mice were placed above a temperature controlled heating blanket and after the 35 minute occlusion, the skin wound was closed with suture and the mice were placed in a heated animal intensive care unit chamber until recovery. To ensure consistent and successful blockage of MCA, we monitored ischemia in all of our animals by Laser Doppler flowmetry (PeriFlux System 5000, Perimed, *Järfälla-Stockholm*, Sweden).

Tamoxifen administration: Male nestin-CreERT2-R26R-YFP mice (8-10 weeks old) were given tamoxifen dissolved in 10% EtOH/90% sunflower oil by gavage feeding at a dose of 180 mg/kg daily for 5 consecutive days. This dosing regimen was previously demonstrated to provide maximal recombination with minimal mortality and successfully monitored the activated SVZ NSCs by stroke [1, 2, 4]. For NSC fate mapping, TAM treated mice received MCAo surgery 10 days after the last TAM administration and mice were perfused at 30 days post stroke to harvest brain for immunostaining. The time frame was chosen to specifically to label NSCs in adult mice before the introduction of MCAo but also allow the clearance of TAM from mice at the time of MCAo to prevent labeling of reactive astrocytes which also upregulate nestin expression after stroke.

Behavioral assay: Locomotor function: Animals were placed in an Accuscan activity monitor (Columbus, OH, USA) one day before and 7, 21 and 28 days after MCAo for behavioral recording for 23 hours as previously described [5]. On post-stroke day 3, animals were monitored in the chambers for one hour to assign them into two equal groups before treatments started. The monitor

contained 16 horizontal and 8 vertical infrared sensors spaced 2.5 cm apart. Each animal was placed in a 42×42×31 cm Plexiglas open box for 60 min or 23 hours with food and water supply. Motor activity was calculated using the automated Versamax software (Accuscan, Columbus, OH, USA). The following variables were measured: (A) horizontal activity (the total number of beam interruptions that occurred in the horizontal sensors), (B) total distance traveled (the distance, in centimeters, traveled by the animals), (C) total movement number (the total number discrete horizontal movements), (D) total movement time (the total time, in seconds, that animals are moving), (E) Vertical activity (the total number of beam interruptions that occurred in the vertical sensors) and (F) vertical movement number (the total number discrete vertical movements). This behavioral test is automatically monitored by the computer and software therefore avoided observer bias.

Barnes maze test: Barnes Maze (Stoelting company, Wood Dale, IL, USA) was used to test mouse spatial memory at post-stroke week 4. A 91.5–cm diameter circular platform with 20 holes along the perimeter was placed at 91.5 cm above the ground. Mice were discouraged to idle around aimlessly by blowing fans and a bright light above the platform. Mice were trained for 4 trials in 2 sessions on the day before testing trail to locate the escape tunnel placed under one of the holes. One trial was run on the testing day and the test was video-taped till the mouse getting into the escaping hole or stopped at 5 minutes when the mouse could not locate the escaping hole. Time spent to locate the escaping hole and error numbers in finding the hiding hole made by the mouse were read from the video by an observer who was blinded to the treatment groups.

MRI method: Two imaging sessions were repeated at 2 and 30 days after stroke by staff that were blinded to the treatment groups.

MRI studies were performed on a horizontal Bruker 9.4 T scanner with a 3-cm birdcage coil. Multi-slice, T_2-weighted, axial images were acquired using a rapid acquisition with relaxation enhancement (RARE) sequence[6] to quantify ischemic edema volume. Imaging parameters were: TE/TR, 15/2000 ms; RARE factor, 8; NAV, 4; matrix size, 256×256; slice thickness, 1 mm; number of slices, 13; field of view (FOV), 2.4×2.4 cm. One Bregma-containing slice was selected for CBF quantification by arterial spin labeling (ASL). ASL images were acquired with a flow-sensitive alternating inversion recovery (FAIR) preparation sequence followed by fast imaging with steady-state precession (FISP) readout[7]. The inversion pulse used a 3-ms hyperbolic secant adiabatic pulse, with the slice thickness set to three times that of the imaging slice thickness. FISP acquisition was implemented at 1420 ms following the inversion pulse with the follow parameters: flip angle, 60°; TR, 2.4 ms; TE, 1.2 ms; NAV, 20; matrix size, 128×128; FOV, 2.4×2.4 cm; slice thickness, 1.5 mm. A proton density image (M_0) with no inversion preparation was also acquired. A T_1-apparent map with the same spatial resolution was acquired with a FISP-based Look-Locker sequence with the following parameters: flip angle, 10°; TR, 4.0 ms; TE, 2.0 ms; NAV, 20. T_2 map of the same slice was also acquired using the Carr-Purcell-Meiboom-Gill sequence with echo times varying from 30 to 240 ms[8]. Other acquisition parameters were: TR, 500 ms; number of echoes: 16, NAV, 2; matrix size, 180×180.

Image reconstruction and analysis were performed offline using in-house developed, MATLAB-based (Natick, MA, USA) software. ROIs of ischemic edema volume and brain tissue were drawn from T_2-weighted images. Consequently, the percentage of ischemic edema volume was calculated. CBF was calculated using the method proposed by Pell et al[9], assuming a blood T_1-apparent of 2.4 s. CBF in the ischemic edema volume was compared against that in the contralateral brain.

Immunohistochemistry: At different time points after stroke, mice were perfused transcardially with a solution of 4% paraformaldehyde (PFA, pH 7.2) in 0.1 M phosphate buffer (PB, pH 7.2). Brains were removed from the skull, post-fixed in 4% PFA overnight at 4 °C, and sequentially transferred to 30% sucrose in 0.1M phosphate buffer, pH 7.2 solutions overnight. Brains were frozen on dry ice and sectioned on a cryostat to obtain coronal sections of 25 μm in thickness.

The sections were then incubated with blocking buffer for one hour. The primary antibodies were prepared in the blocking buffer and the sections were incubated in the solution overnight. The antibodies used were rabbit anti-GFP (Green fluorescent protein) (1:1000; Invitrogen), guinea pig anti-DCX (1:500, Millipore), mouse anti-NeuN (1:500, Millipore), rat anti-BrdU (1:1000, Life Technologies), mouse monoclonal anti-PCNA (1:400; Dako), rabbit anti-Ki67 (1:1000, Fisher Scientific), mouse anti-α-SMA (smooth muscle antibody) (1:400, Dako) and rat anti-CD31 (1:200, Biolegend). For BrdU immunohistochemistry (IHC) staining, sections were incubated in denaturing solution and 2N HCL before blocking and incubation with primary antibody solution. For PCNA and α-SMA IHC staining, sections were subjected to antigen retrieval before blocking as described previously[10]. After incubation with primary antibody solution, the sections were washed and incubated for four hours at room temperature in diluted secondary antibody prepared with blocking solution (secondary antibody conjugated with Alexa 488 or Alexa 555, 1:1000; Life Technologies, Carlsbad, CA, USA). The slides were then washed with T-TBS and coverslipped. Images were acquired using an Olympus microscope (Shinjuku, Tokyo, Japan). Omission of primary or secondary antibodies resulted in no staining and served as negative controls. Group and treatment information are all blinded to image analyzer.

Quantification of cell proliferation, neurogenesis and angiogenesis:

For cell proliferation evaluation, brains were harvested 24 hours after pulse BrdU labeling on post-stroke day 6 from both vehicle and SAG-treated (post-stroke day 3-6) mice, and BrdU and PCNA positive cells were quantified on 3-4 sections per animal using a Leica DM5000B microscope (Leica Microsystems, Bannockburn, IL) equipped with a motorized stage and Stereo Investigator image software (MBF Bioscience, Williston, VT). Dorsal and ventricle SVZ were defined as described. Proliferating cells in the DG of hippocampus were quantified by counting each BrdU or Ki67 positive cells in at least 5 sections that contained SGZ at similar coronal levels. Both contralateral and ipsilateral side of the SVZ and the SGZ were quantified. For quantification of neurogenesis and migration of SVZ NSC-derived cells into striatum and cortex, stereological quantification of YFP+, DCX+ or NeuN+ cells was achieved using the optical fractionator probe. A counting area was drawn around the striatum and cortex rear of the infarcted hemisphere with a SRS (systematic random sampling) grid of 150x150um and a counting frame of 67.2x75um. YFP, DCX or NeuN labeled cells were individually counted with this method, with a mounted thickness of 30um and a section interval of 6 for each animal. Typically, 8-10 sections were counted for each animal. Gundersen coefficients of error for m=1 were all less than 0.10. Determination of the percentage of co-labeled YFP and DCX cells was achieved by utilizing the optical fractionator workflow to randomly select counting areas within the striatum to count the DCX+, YFP+, NeuN+ and co-labeled cells within the counting frame of 67.2x75um and a SRS grid of 200x200um until a minimum of 100 each of DCX+ and YFP+ cells were recorded per animal. Percentage of YFP positive cells that are DCX positive or NeuN positive was calculated as: (100 X Total number of double positive cells/ Total number of YFP+ cells). For quantification of angiogenesis, histological images were acquired using an Infinity3 camera (Lumenera Corp, Ottowa, Ontario, Canada) and NIKON 80i microscope (Nikon Corp, Tokoyo, Japan). Three to four representative

sections from each animal were quantified for the density of arteries or microvessels. Quantification of arteries in the infarct area were carried out by counting a-SMA positive vessels as described in [10]. The number of a-SMA positive vessels in the infarct area were quantified by Nikon NIS-Elements software (Tokoyo, Japan) and averaged from 3 sections. CD31 positive microvessel density was quantified by first drawing the region of interests at the boarder of infarct area. CD 31+ blood vessels were then highlighted by using NIS-Elements and the density of CD31+ blood vessels were automatically calculated by the software and expressed as object area fraction in the ROI. Four sections near the largest infarct area were quantified and averaged for each animal. All immunohistochemical measurements were done by blinded observers.

RT-PCR: To examine gene expression in SVZ NSCs, additional 16 nestin-CreERT2-R26R-YFP mice were subjected to MCAo and SAG or vehicle treatment (post stroke day 3-8) and SVZ tissue microdissected using method described in [11] for quantitative RT-PCR. Total of four mice died after MCAo before the tissue harvest, resulted a total of 12 mice for RT-PCR experiment. SVZ tissues were harvested for qRT-PCR analysis at the last day of SAG or vehicle treatment (post stroke day 8). Mice were euthanized and the brains were immediately removed and chilled on ice. The SVZ of both contralateral and ipsilateral side was dissected and total RNA was extracted following the instructions from the manufacturer (RNAqueous, Ambion) as described previously [12]. Total RNA (1 μg) was treated with RQ-1 Rnase-free Dnase I and reverse transcribed into cDNA using random hexamers by Superscript III reverse transcriptase (Life Sciences). cDNA levels for HPRT1 (hypoxanthine phosphoribosyltransferase 1), Hmbs (hydroxymethylbilane synthase) and various target genes were determined, using specific universal probe Library primer probe sets (Roche), by quantitative RT-PCR using a Roche Light Cycler II 480. Relative expression level was calculated using the delta Ct method compared to Hbms as a reference gene. Primers and 6-

carboxyfluorescein (FAM) labeled probes used in the quantitative RT-PCR for each gene are listed in Supplemental Table III.

Supplemental Tables:

Table I. No differences in locomotor parameters in vehicle and shh pathway agonist (SAG) treated mice before and 3 days after stroke.

	Pre-stroke (23 hrs)					*Post stroke day 3 (1hr)*				
	vehicle		*SAG*			*vehicle*		*SAG*		
	mean	*s.e.m.*	*mean*	*s.e.m.*	**p value*	*mean*	*s.e.m.*	*mean*	*s.e.m.*	**p value*
HACTV	96088.4	4134.5	10219.4	5481.4	0.44	8540.1	1031.6	9533.9	512.6	0.41
TOTDIST	19982.7	1568.2	20612.5	1665.8	0.74	1560.7	250.1	1485.9	127.5	0.80
MOVNO	5314.3	247.0	5422.4	253.6	0.76	399.2	51.1	454	22.7	0.35
MOVTIME	2446.1	171.7	2532.5	181.9	0.72	206.5	34.4	193.6	16.9	0.75
RESTIME	83953.9	171.7	83867.5	181.9	0.72	3393.5	34.4	3406.4	16.9	0.75
VACTV	10894.3	696.7	11846.2	847.2	0.42	538.2	95.6	606.1	90.0	0.61
VMOVNO	2871.4	207.1	3072.2	122.0	0.33	162.5	22.9	180.6	23.4	0.58
VTIME	7425.2	344.3	7582.2	487.3	0.81	342.5	49.0	396.2	58.7	0.48

*p value for difference between vehicle and SAG-treated mice within pre-stroke or post-stroke condition (ANOVA).

HACTV (horizontal activity) = total number of beam interruptions that occurred in the horizontal sensors.
TOTDIST (total distance travelled) = the total distance (cm) travelled.
MOVNO (number of movements) = the number of separate horizontal movements executed.
MOVTIME (movement time) = the amount of time (second) in ambulation.
RESTIME (rest time) = the amount of time (second) in rest.
VACTV (vertical activity) = total number of beam interruptions that occurred in the vertical sensors.
VMOVNO (vertical number of movements) = the number of separate vertical movements executed.
VTIME (vertical movement time) = the amount of time (second) in vertical activity.

Table II. Differences and p values in locomotor parameters in vehicle and shh pathway agonist (SAG) treated mice at different post-stroke days.

	Post-stroke day 7			*Post-stroke day 21*			*Post-stroke day 28*		
	vehicle	*SAG*		*vehicle*	*SAG*		*vehicle*	*SAG*	
	mean	*mean*	**p value*	*mean*	*mean*	**p value*	*mean*	*mean*	**p value*
HACTV	74738.8	73841.5	0.892	73557.5	93034.6	0.004	87033.0	101051.1	0.03
TOTDIST	10771.1	13296.4	0.186	11922.7	18644.0	<0.001	14605.3	18350.9	0.05
MOVNO	3338.7	3779.0	0.22	3553.8	4974.5	<0.001	4191.5	5020.2	0.02
MOVTIME	1373.5	1685.2	0.2	1535.2	2431.3	<0.001	1856.8	2372.7	0.03
RESTIME	85026.5	84714.8	0.2	84864.8	83968.7	<0.001	84543.1	84027.3	0.03
VACTV	8291.7	9070.6	0.5	8261.7	10932.2	0.02	10353.8	10886.2	0.65
VMOVNO	1984.6	2027.0	0.83	1975.3	2644.6	0.002	2408.8	2613.3	0.32
VTIME	6076.8	6676.7	0.35	5653.7	7030.9	0.03	6274.9	6890.3	0.34

*p value for difference between vehicle and SAG-treated mice at different time point (ANOVA, post-hoc Bonferroni t-test for repeated measure, pair wire comparison).

HACTV (horizontal activity) = total number of beam interruptions that occurred in the horizontal sensors.

TOTDIST (total distance travelled) = the total distance (cm) travelled.

MOVNO (number of movements) = the number of separate horizontal movements executed. MOVTIME (movement time) = the amount of time (second) in ambulation.

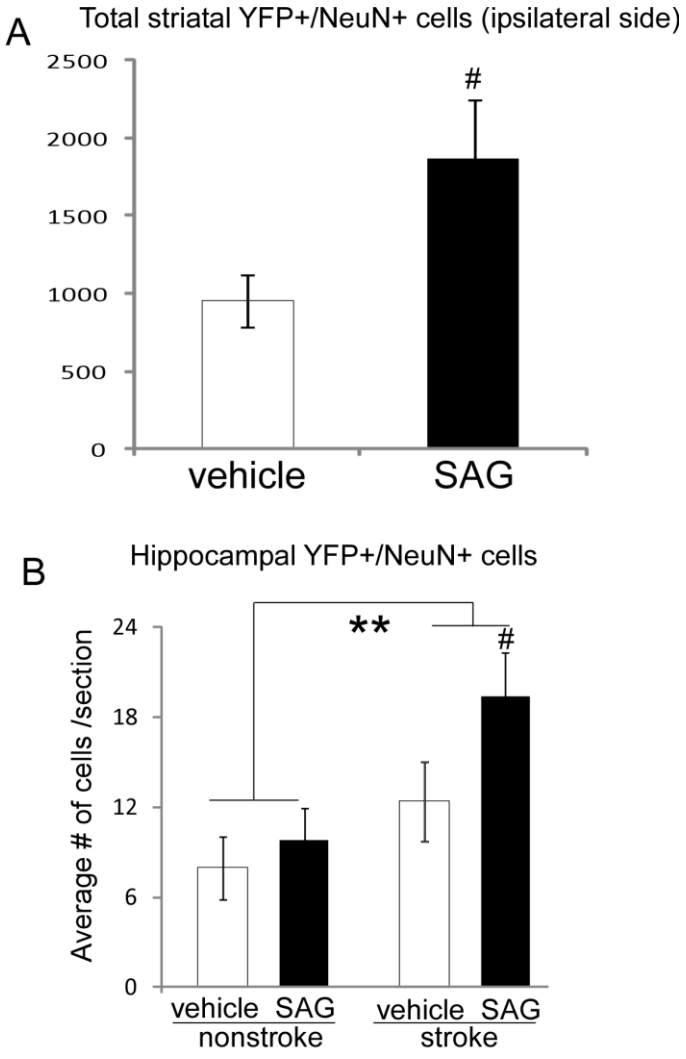
RESTIME (rest time) = the amount of time (second) in rest.

VACTV (vertical activity) = total number of beam interruptions that occurred in the vertical sensors. VMOVNO (vertical number of movements) = the number of separate vertical movements executed. VTIME (vertical movement time) = the amount of time (second) in vertical activity.

Table III: Primer/ probe sets used in qRT-PCR.

Primer/probe set:		
Hmbs:	Forward Primer:	5'-TCC CTG AAG GAT GTG CCT AC-3'
	Reverse Primer:	5'-ACA AGG GTT TTC CCG TTT G-3'
	Probe:	Universal Probe Library: Probe 79 - Roche
HPRT1:	Forward Primer:	5'-TGA TAG ATC CAT TCC TAT GAC TGT AGA-3'
	Reverse Primer:	5'- AAG ACA TTC TTT CCA GTT AAA GTT GAG-3'
	Probe:	Universal Probe Library: Probe 22 - Roche
smo	Forward Primer:	5'- GCA AGC TCG TGC TCT GGT-3'
	Reverse Primer:	5'- GGG CAT GTA GAC AGC ACA CA-3'
	Probe:	Universal Probe Library: Probe 3 - Roche
Gli1	Forward Primer:	5'-CTG ACT GTG CCC GAG AGT G-3'
	Reverse Primer:	5'-CGC TGC TGC AAG AGG ACT-3'
	Probe:	Universal Probe Library: Probe 84 - Roche

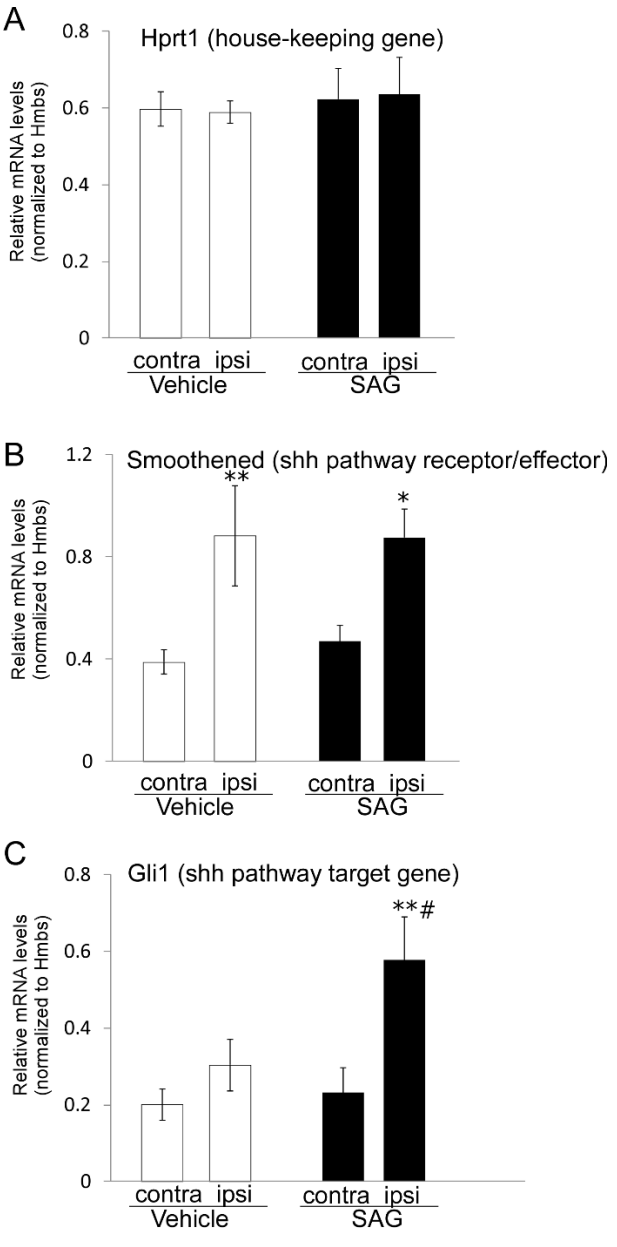
Supplemental Figures:



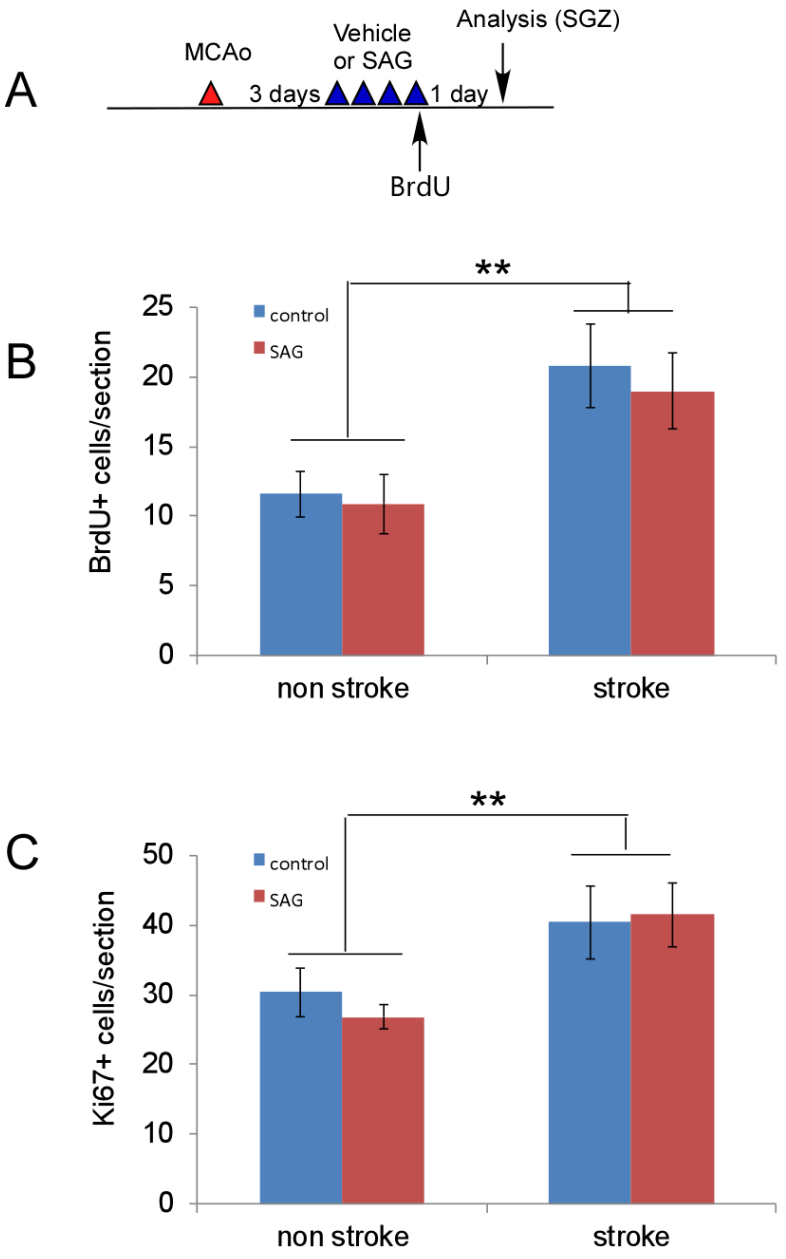
293

294 Supplemental Fig I. Post-stroke SAG treatment increased the number of surviving NSCs derived
295 NeuN positive cells in both ipslateral striatum and hippocampus. YFP+ and NeuN+ double
296 positive cells migrate into striatal and cortical area in ischemic hemisphere in vehicle (open bar)
297 or SAG (solid bar) treated mice at 30 days post stroke were quantified using StereoInvestigator
298 and results show (A) SAG increased the total number of YFP/NeuN positive cells (#, $p<0.05$,
299 Student's t-test, n=7). There is no visible YFP+/NeuN+ cells in the strital or cortical region in the
300 contralateral hemisphere. (B) Quantification of YFP+/NeuN+ /YFP+ double positive cells in the
301 contralateral and ipslateral side of the hippocampus at 30 days post stroke. MEAN± SEM. **

p<0.01, nonstroke vs. stroke. #, p<0.05 compared to vehicle-treated mice, ANOVA. n= 7 each group.



Supplemental Fig II. **Gene expression alterations in SVZ of stoke mice treated with vehicle or SAG.** Gene expression was analyzed using qRT-PCR after vehicle or SAG treatment in stroke mice using fluorescent microdissected SVZ tissue. All gene expression levels were normalized to Hmbs house-keeping gene expression for each sample. (A) There is no difference in expression levels of a second house-keeping gene Hprt1. (B) Smoothened expression is upregulated in ipsilateral side of the SVZ in both vehicle and SAG-treated group. (C) In addition, SAG-treated group showed increased Gli expression levels suggesting in vivo post-stroke SAG treatment at 10mg/kg was able to activate the stroke-induced smoothened receptor and activate downstream signaling pathway. MEAN$\pm$ SEM. * or **, $p<0.05$ or $p<0.01$ compared to contralateral side, # indicates $p< 0.05$ compared to vehicle-treated groups, ANOVA, Student-Newman-Keuls post-hoc tests. n=5-7)



318

319 Supplemental Fig III. SAG post-stroke treatment does not affect the enhanced proliferation of SGZ

320 NSCs in stroke mice. (A). Experimental time line. (B). BrdU + cells and Ki67+ cells in SGZ of

321 hippocampus after stroke. MEAN± SEM. **, $p<0.01$, ANOVA. (n=5-7).

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