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Supplemental Information

Relative Levels of *Gli1* and *Gli2* Determine the Response of Ventral Neural Stem Cells to Demyelination

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SUPPLEMENTAL FIGURES AND LEGENDS:

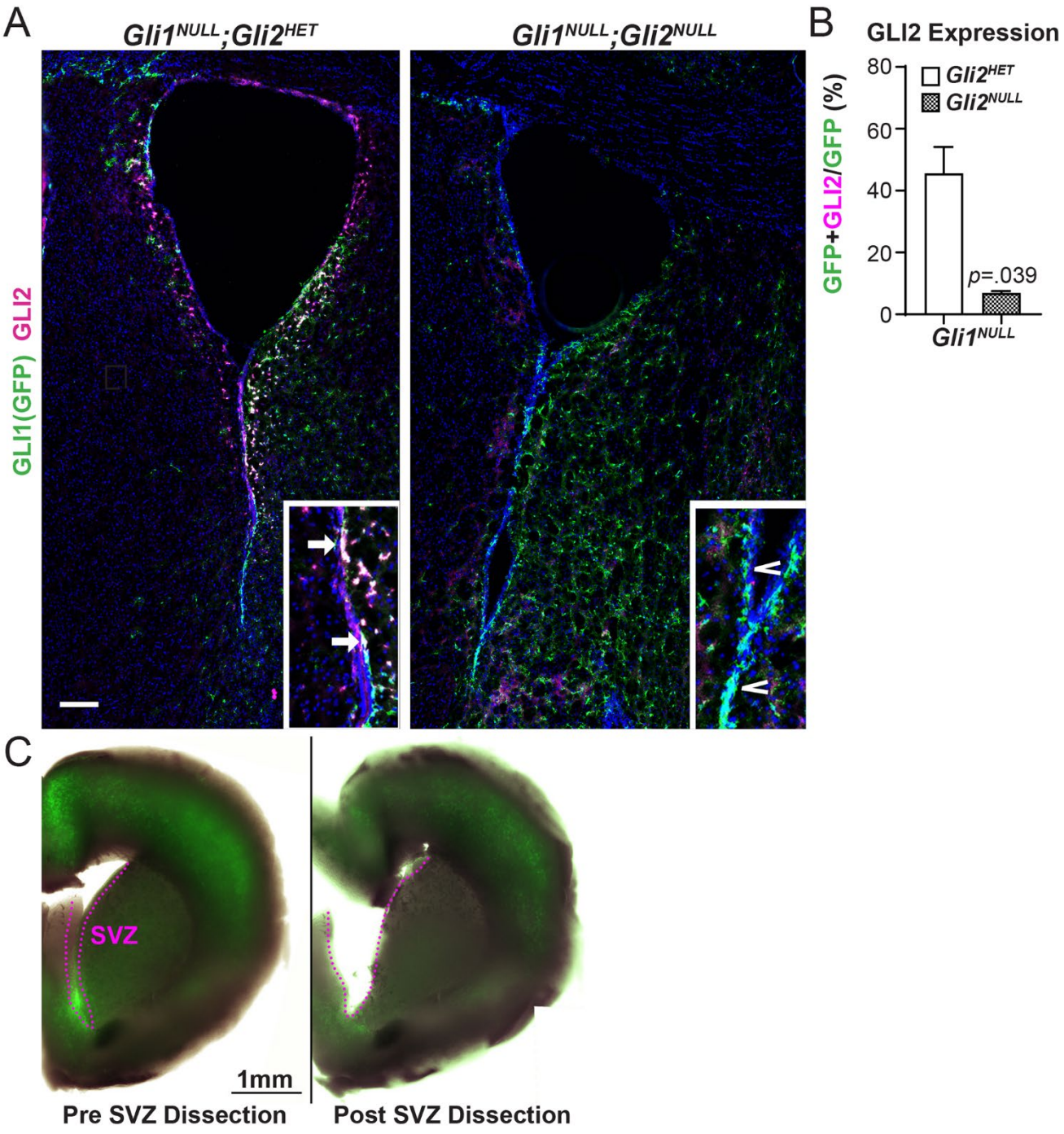

Figure S1 – Recombination efficiency of *Gli2* in GLI1 vNSCs and SVZ dissection (Related to Figure 2 and Methods).

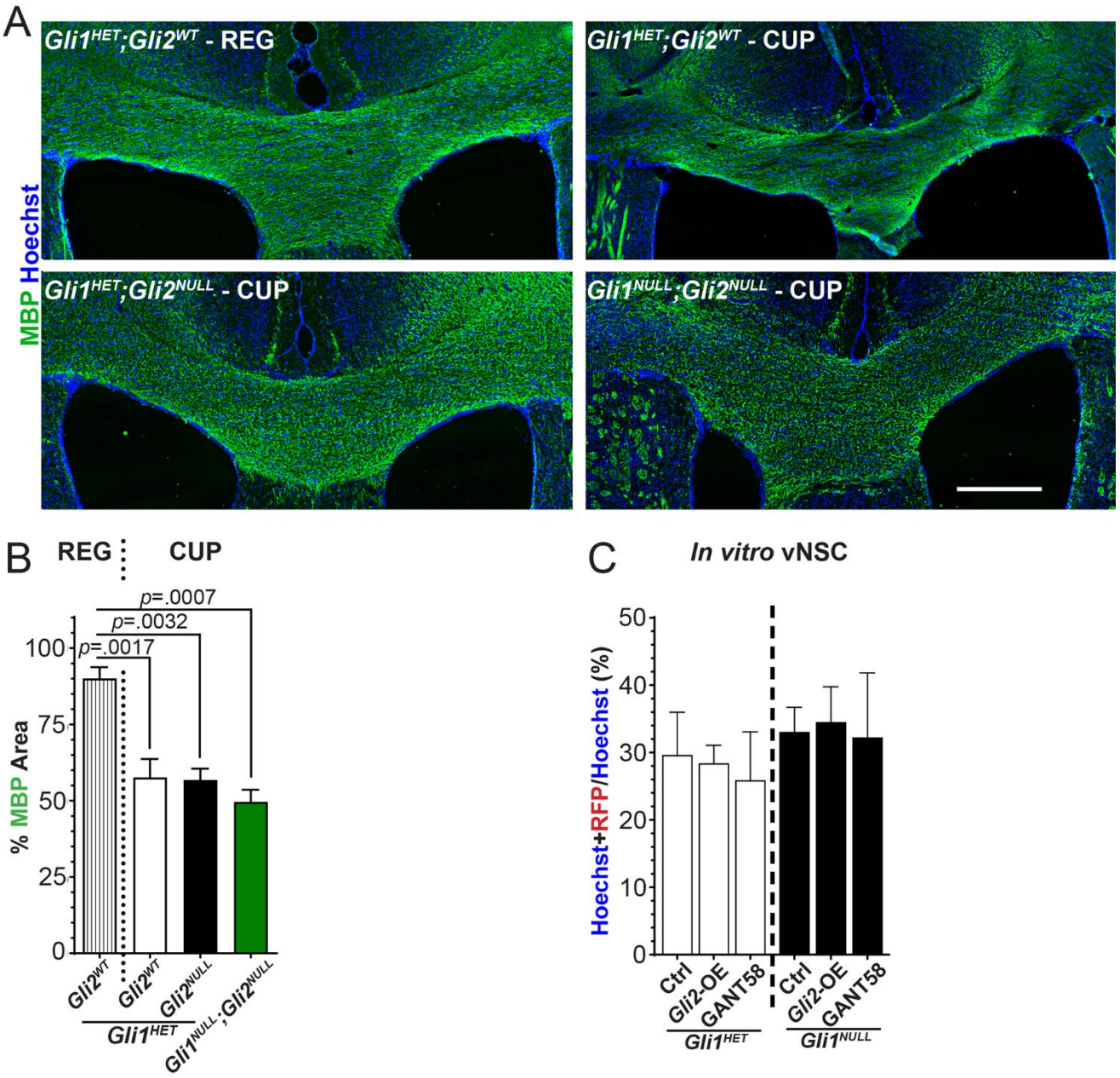

Figure S2 – Demyelination extent in the CC with *in vitro* mRNA and fate-mapping analysis. (Related to Figure 2).

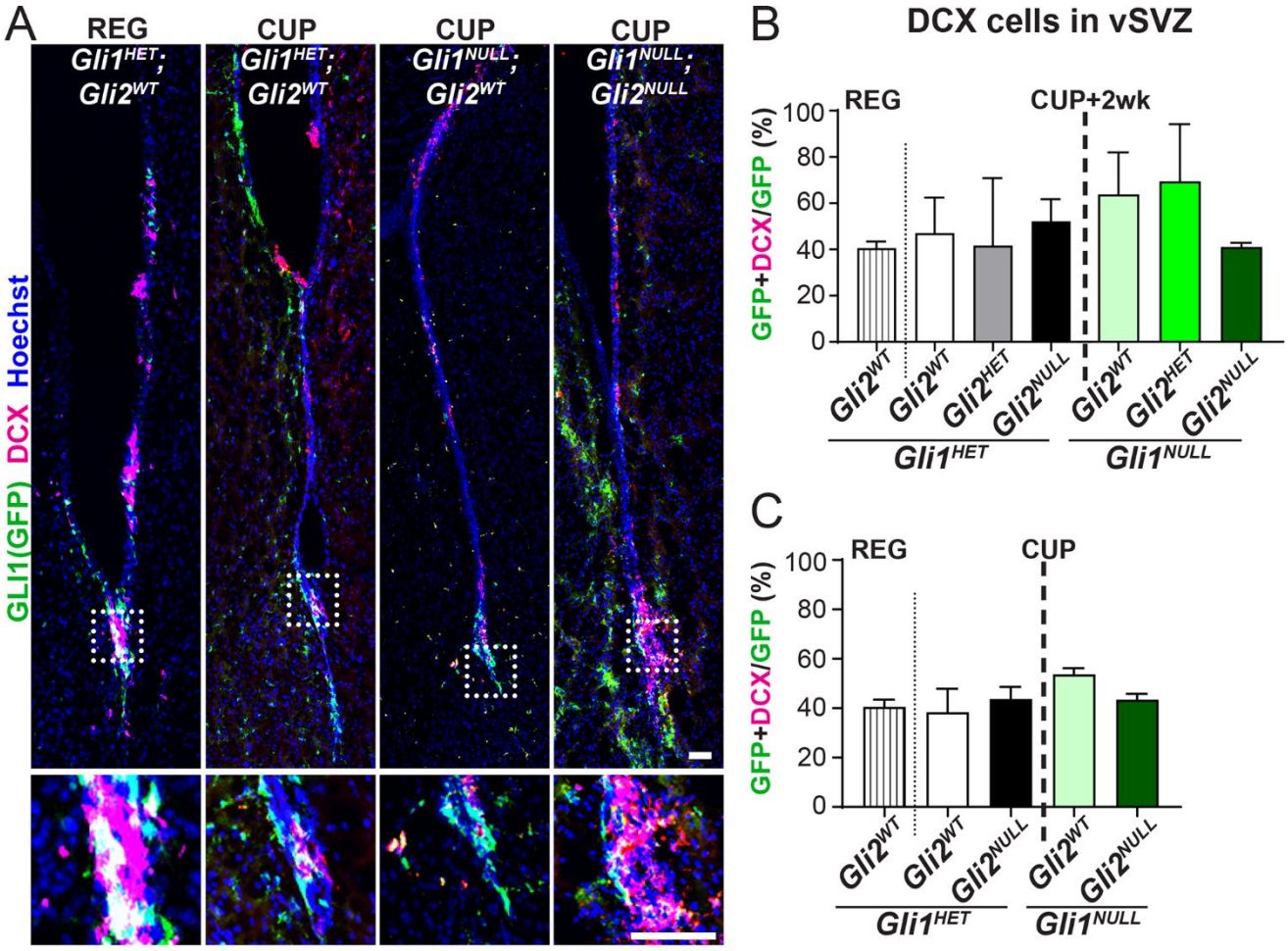

Figure S3 – Differentiation of GLI1 vNSCs into neuroblasts in the SVZ following demyelination (Related to Figure 3).

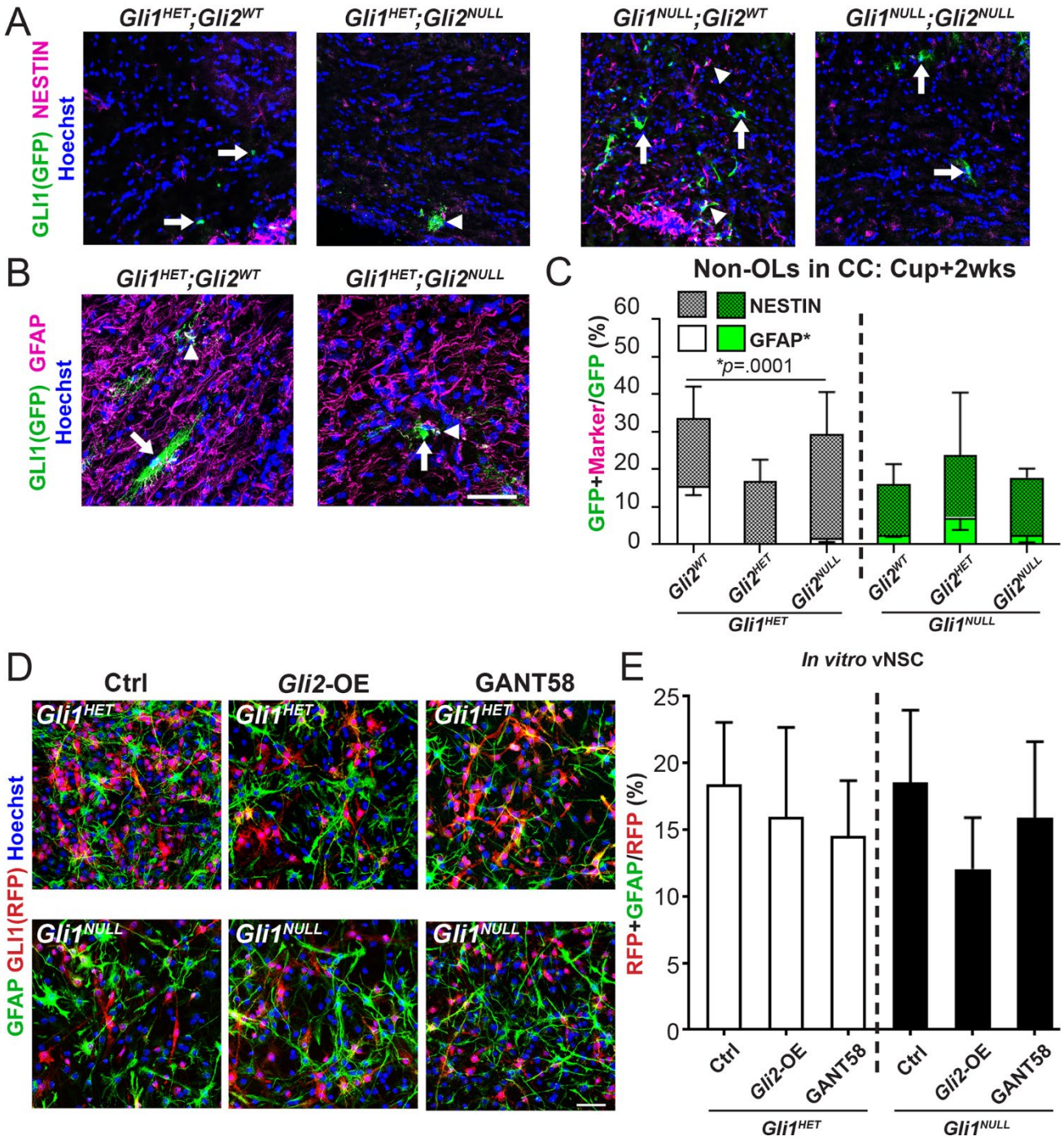

Figure S4 – Astrocyte differentiation from vNSCs (Related to Figure 4).

Figure S1 – Recombination efficiency of *Gli2* in *Gli1* vNSCs and SVZ dissection (Related to Figure 2 and Methods).

A) Immunofluorescence for GFP+ *Gli1* fate-mapped cells (green) and GLI2 expression (magenta) in the subventricular zone (SVZ) showing GFP+GLI2+ cells (left inset, arrows) in $Gli2^{HET}$ vNSCs and GFP+GLI2- cells (right inset, arrowheads) in $Gli2^{NULL}$ vNSCs. Scale bar=250µm.

B) Quantification of (A) shows a reduction in the percentage of *Gli1* vNSCs expressing GLI2 in the $Gli2^{NULL}$ SVZ. Unpaired t-test comparing $Gli2^{HET}$ and $Gli2^{NULL}$ animals; all data presented as mean ± SEM; n=3 mice/genotype.

C) Fluorescent image overlaid on brightfield image of a slice from *Gli1* (GFP, green) fate-mapped mouse before dissecting the SVZ (left) and after removal of the SVZ (right)(magenta outline).

Figure S2 – Demyelination extent in the CC with *in vitro* mRNA and fate-mapping analysis. (Related to Figure 2).

A) Immunofluorescence for MBP+ myelin in the CC of mice on regular diet or cuprizone diet.

B) Quantification of the MBP+ area throughout the CC in (A) showed a significant decrease at 5wks of cuprizone diet.

C) Quantification of the number of RFP+ *Gli1* fate-mapped vNSCs *in vitro* showed no change on overexpression or inhibition of *Gli2*.

All tests are 1-way ANOVAs with Tukey's post-hoc t-tests. Data presented as mean ± SEM, n=3; scale = 200µm. Regular Diet (REG), Cuprizone diet (CUP), Ventral neural stem cells (vNSCs), *Gli2*-overexpression (Gli2-OE).

Figure S3 – Differentiation of *Gli1* vNSCs into neuroblasts in the SVZ following demyelination (Related to Figure 3).

A) Immunofluorescence for DCX+ neuroblasts (magenta) and GFP+ fate-mapped *Gli1* vNSCs (green) in the SVZ of mice on regular diet or at 2wks of recovery from cuprizone diet.

B) Quantification of the number of GFP+DCX+ cells in the SVZ at 2wks of recovery from cuprizone diet in (A).

C) Quantification of the number of GFP+DCX+ cells in the SVZ at peak demyelination (5wks of cuprizone diet).

One-way ANOVA with post-hoc t-tests; data presented as mean ± SEM; n=3, scale=25µm. Regular Diet (REG), Cuprizone diet (CUP), Ventral sub-ventricular zone (vSVZ).

Figure S4 – Astrocyte differentiation from vNSCs (Related to Figure 4).

A) Immunofluorescence for GFP+ fate-mapped vNSCs (green) in $Gli1^{HET}$ and $Gli1^{NULL}$ CC co-expressing the progenitor marker NESTIN (magenta). n= 3 mice/genotype

B) Immunofluorescence for GFP+ fate-mapped $Gli1^{HET}$ vNSCs (green) and the astrocytic marker GFAP (magenta). n= 3 mice/genotype.

C) Quantification of the proportion of GFP+ fate-mapped $Gli1^{HET}$ (white) and $Gli1^{NULL}$ (green) vNSCs co-expressing NESTIN (A) and GFAP (B) shows a decrease in astrocytes in the $Gli1^{HET}$ CC with loss of one or both copies of *Gli2*. n= 3 mice/genotype

D) Immunofluorescence for RFP+ fate-mapped vNSCs (red) from $Gli1^{HET}$ and $Gli1^{NULL}$ SVZ co-expressing the astrocytic marker GFAP (green) after 7 days of differentiation *in vitro*.

E) Quantification of RFP+GFAP+ cells in (D) shows no difference with *Gli2*-overexpression (Gli2-OE) or inhibition with GANT58. n=3 replicates

1-way ANOVAs with Tukey's post-hoc t-tests; all data presented as mean ± SEM; Scale bars=50µm. Ventral neural stem cells (vNSCs), corpus callosum (CC), Cuprizone diet (CUP), *Gli2*-overexpression (Gli2-OE).

EXPERIMENTAL PROCEDURES

In vivo **Immunofluorescent image analysis:** Mice were perfused with 4% PFA and coronal sections were acquired starting from the olfactory bulb rostrally to the anterior hippocampus caudally. For each analysis, we used 10 sections through the corpus callosum, each section was 20um thick with 200um distance between 2 sections, thus sampling the entire rostrocaudal span of the corpus callosum. Epifluorescent images were obtained as Z-stacks of 5µm optical sections using a Keyence BZ-X700 microscope with inbuilt automatic deconvolution at 20x magnification. For quantification of CC1, PDGFRα, GFAP, MBP and OB GFP immunostaining, the Keyence BZ-X Analyzer Hybrid Cell Count tool was used to count cells that were positive for Hoechst nuclei and the antibody labeled cells. A threshold was determined for each antibody and CNS location, then images were cropped to include either the cingulum of the CC, a 30□m section around the SVZ, or the entire olfactory bulb. Images from all animals were batch counted to avoid biases in manual cell counting, and results were collated in Microsoft Excel before analysis in GraphPad Prism. We also acquired z-stack images with the Leica confocal microscope to confirm the co-expression of different markers and manually counted a subset of images in ImageJ. We did not detect a difference between the manual and automatic cell counts. Publication quality images were obtained as Z-stacks of 1µm optical sections using a confocal laser-scanning microscope (Leica TCS SP8) and processed using Adobe Photoshop. The investigators were blinded to allocation during experiments and outcome assessment.

Primary NSC culture: The brains were harvested from mice after euthanasia and placed in an acrylic mouse brain mold (BS-A-5000C - Braintree Scientific) and 1mm slices were acquired from the caudal aspect of the olfactory bulbs to the dorsal hippocampus. Brain slices were moved to dissection media composed of DMEM/F-12 (#11320033 – Gibco) with 1x Antibiotic-Antimycotic (#15240112 – Gibco) and the SVZ tissue lining the ventral and lateral aspects of the lateral ventricles was microdissected from 3 consecutive slices (Fig. S1C). The dissected SVZ tissue was minced and collected by centrifugation for 5min x 400g. The tissue was then digested with 0.05% Trypsin-EDTA (#25300062, Gibco) and incubated at 37˚C for 5min with agitation every 60-90secs to form a cell suspension. After neutralizing the Trypsin-EDTA with 1mg/mL Soybean Trypsin Inhibitor (#17075029, Gibco), the cells were needle triturated and resuspended in Mouse NSC Proliferation Media (1x Anti-Anti, 10ng/mL bFGF (#78003, Stem Cell Tech, Vancouver), 20ng/mL mEGF (#78016 Stem Cell Tech), 0.0002% Heparin Sulfate (#H7640, Sigma), 1x Mouse Proliferation Supplement in NeuroCult Media (#5702 Stem Cell Tech Kit). The dissociated cells were then grown as floating neurospheres in proliferation media at 37˚C and 5% CO_2. Primary NSCs were supplemented with fresh bFGF and mEGF at day 3 and day 5 after plating. Neurospheres were passaged at day 7 to generate secondary neurospheres. Secondary neurospheres were dissociated into single cells and plated on Matrigel (#356234 Corning, concentration tested by WiCell) coated coverslips in differentiation media (1x Anti-Anti, 1ng/mL bFGF, 2ng/mL mEGF, 0.0002% Heparin, 1x Differentiation Supplement in Neurocult Media (#5704 Stem Cell Tech Kit). Cells were maintained in Differentiation Media for 7 days with half media changes every other day, then harvested for RNA or fixed in ice-cold methanol for 15min for immunolabelling.

In vitro **Immunofluorescent image analysis:** Images were acquired using Keyence BZ-X700 epifluorescent microscope from 10 fields per coverslip, for total 3 coverslips per marker per experimental group. Each experiment was repeated 3 times and the cell counts were averaged together. Total RFP+ (fate-mapped) cells were counted using Keyence software, while NG2+ (OPCs), MBP+ (OLs), and GFAP+ (Astrocytes) cells were counted manually using ImageJ.

qRT-PCR: SVZ was dissected (Fig. S1C) by slicing the brains into 1mm coronal sections and cutting the SVZ from each slice using 1mm spring scissors under a dissecting microscope. The SVZ was defined as the CNS tissue between the lateral ventricles and striatum (identified by white matter bundles) in the 3mm, 4mm, and 5mm slices caudal to the olfactory bulb. mRNA was extracted from three mice per genotype using RNeasy kit (Qiagen) and reverse-transcribed to complementary DNA using iScript cDNA Synthesis Kit (BioRad). ITaq Universal SYBR Green Supermix (BioRad) was used to perform qPCRs in a Biorad CFX Connect thermal cycler. Primers used were Gli2 (forward, 5'-AGA GAC AGC AGA AGC TAT GCC CAA-3'; reverse, 5'-TGG GCA GCC TCC ATT CTG TTC ATA-3') and GAPDH (forward, 5'-GGT GTG AAC GGA TTT GGC CGT ATT G-3'; reverse, 5'-CCG TTG AAT TTG CCG TGA GTG GAG T-3'). The $2^{-\Delta\Delta Ct}$ method was used to analyze the relative gene expression, each Gli1 genotype was normalized to regular diet.

GANT58 Treatment: GANT58 (#14193, Cayman Chemicals) was dissolved in DMSO at a concentration of 50mM and added to differentiating vNSCs beginning 2-3hrs after plating at a final concentration of 50uM. Fresh GANT58 was added every other day coinciding with media changes. An equal volume of DMSO was added to cells as a control.

Gli2 **overexpression:** $2x10^6$ dissociated NSCs were Nucleofected with the *pCS2-MT GLI2 FL* plasmid (a gift from Erich Roessler, Addgene plasmid # 17648) using the Lonza Mouse Neural Stem Cell Nucleofector Kit (#VPG-1004, Lonza) with Amaxa Nucleofector II.

EdU labeling: On day 7 of NSC differentiation, 10mM 5-ethynyl-2'-deoxyuridine (EdU) (#20518 Cayman Chemicals, MI) was dissolved in DMSO and added to cells in differentiation media at a final concentration of 10uM for 1hr before fixation. EdU was detected with the Click-IT EdU Kit – Alexa 647 (#C10340 Invitrogen) following the manufacturer's instructions.

Table S1: Antibodies used for immunofluorescence

Antibody	Concentration	Company	Catalog number
Rabbit anti-GFP	1:1000	Thermo Fisher Scientific	A-11122
Chicken anti-GFP	1:1000	Thermo Fisher Scientific	A-10262
rat anti-PDGFRα	1:200	BD Biosciences	562171
Mouse anti-CC1	1:400	Millipore	OP-80
Mouse anti-GFAP (GA-5)	1:400	Novus Biologicals	NBP2-29415
Rabbit anti-GFAP	1:400	Thermo Fisher Scientific	PA1-10019
Mouse anti-MBP	1:500	Biolegend	808402
Mouse anti-LacZ	1:2000	Sigma Aldrich	G8021
Rabbit anti-NG2	1:200	Millipore	AB5320
Rat anti-RFP	1:1000	Chromatek	5f8
Goat anti-Mouse Alexa 488 secondary Ab	1:1000	Thermo Fisher Scientific	A32723
Goat anti-Mouse Alexa 568 secondary Ab	1:1000	Thermo Fisher Scientific	A11004
Goat anti-Mouse Alexa 488 secondary Ab	1:1000	Thermo Fisher Scientific	A11008
Goat anti-Rabbit Alexa 568 secondary Ab	1:1000	Thermo Fisher Scientific	A11036
Goat anti-Guinea Pig Alexa 488 secondary Ab	1:1000	Thermo Fisher Scientific	A11073
Goat anti-Chicken Alexa 488 secondary Ab	1:1000	Thermo Fisher Scientific	A32932
Goat anti-Rat Alexa 488 secondary Ab	1:1000	Thermo Fisher Scientific	A11006
Goat anti-Rat Alexa 568 secondary Ab	1:1000	Thermo Fisher Scientific	A11077