YMTHE, Volume *29*

Supplemental Information

Gsx1 promotes locomotor functional recovery after spinal cord injury

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Supplemental Information

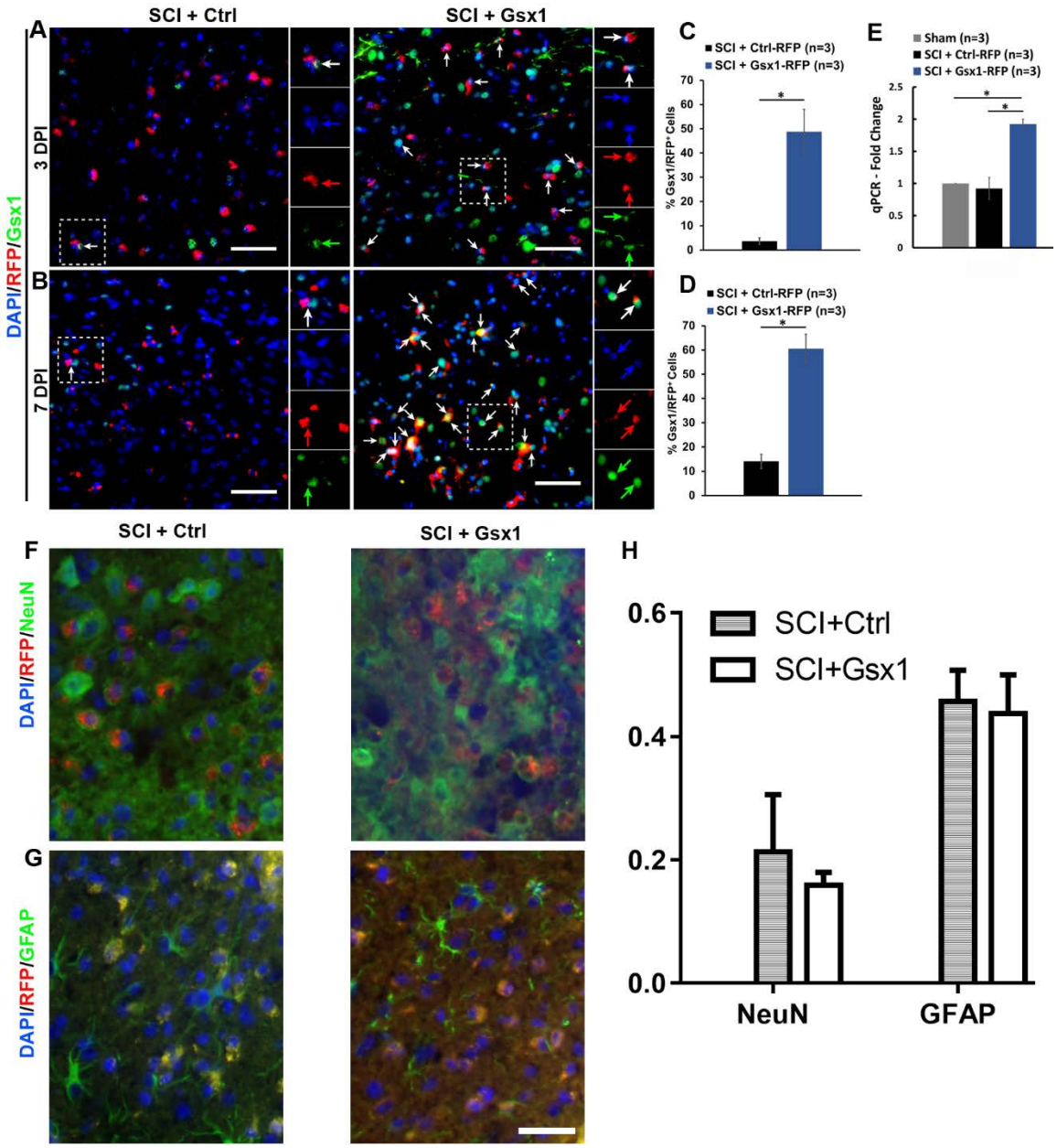

Figure S1. Transduction of lenti-Gsx1-RFP is successful in delivering and overexpressing Gsx1 after SCI

Hemisection SCI was performed on 8-12 weeks old mice around T9-10. Immediately after lentivirus injection encoding Control (Ctrl or empty vector) or Gsx1 gene along with RFP reporter. Animals were harvested at 3 DPI (**A**) and 7 DPI (**B**). Sagittal sections were immunostained with antibodies against Gsx1 (**A** and **B**), NeuN (**F**) and GFAP (**G**). Arrows indicate co-expression of RFP and Gsx1 (green). Montage on the right of each of the image indicates small region (white box) of sagittal sections with separate channels (DAPI, RFP, and Gsx1) to indicate co-expression. Scale bars = 50 µm. Quantification of virally transduced cells co-labeled with Gsx1 at 3 DPI (**C**) and 7 DPI (**D**). (**E**) Histograms show the RT-qPCR analysis of

Gsx1 mRNA expression at 3 DPI, normalized to the Sham. n = 3; Mean ± SEM; * = $p < 0.05$ indicates statistical signifance; Students' T-test (**C**-**D**); one-way ANOVA and Tukey post-hoc analysis (**E**). Sections of spinal cord samples at 3 DPI were also immunostained with antibodies against NeuN (**F**) and GFAP (**G**). Quantification of virally transduced cells co-labeled with NeuN and GFAP (**H**). DPI = days post injury.

A *RNA-Seq Sample Size*

	SCI+Ctrl	SCI+Gsx1
3 DPI	n = 3	n = 3
14 DPI	n = 3	n = 3
35 DPI	n = 4	n = 4

B *Differentially Expressed Genes (DEGs)*

	Total DEGs	Upregulated DEGs	Downregulated DEGs
3 DPI	475	180	295
14 DPI	1447	577	870
35 DPI	2273	600	1673

C

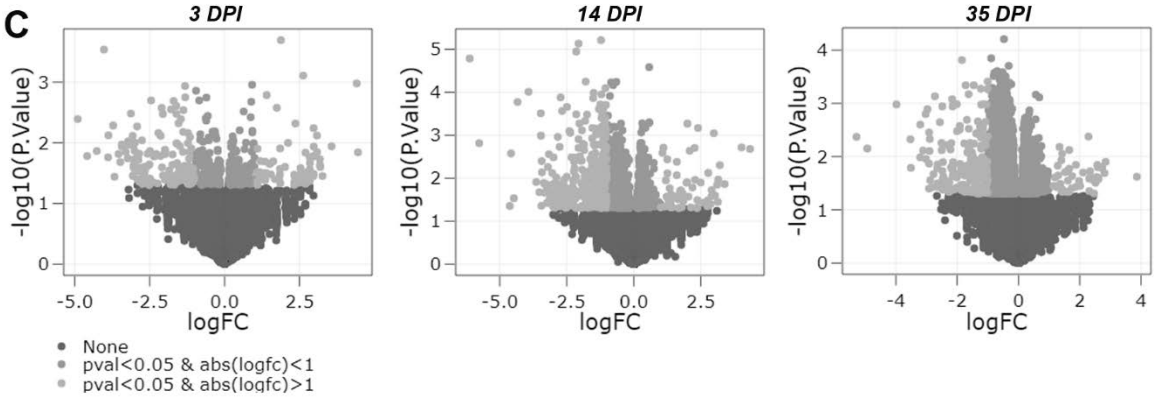

Figure S2. Summary of RNA-seq analysis

(**A**) List of the number of biological replicates used for each group (SCI+Ctrl and SCI+Gsx1) at 3, 14, and 35 DPI for RNA-seq analysis. (**B**) List of the total number of differentially expressed genes (DEGs; $p<0.05$) that were upregulated and downregulated at 3 DPI, 14 DPI, and 35 DPI. (**C**) Volcano plots depicts the differentially expressed genes at 3, 14, and 35 DPI.

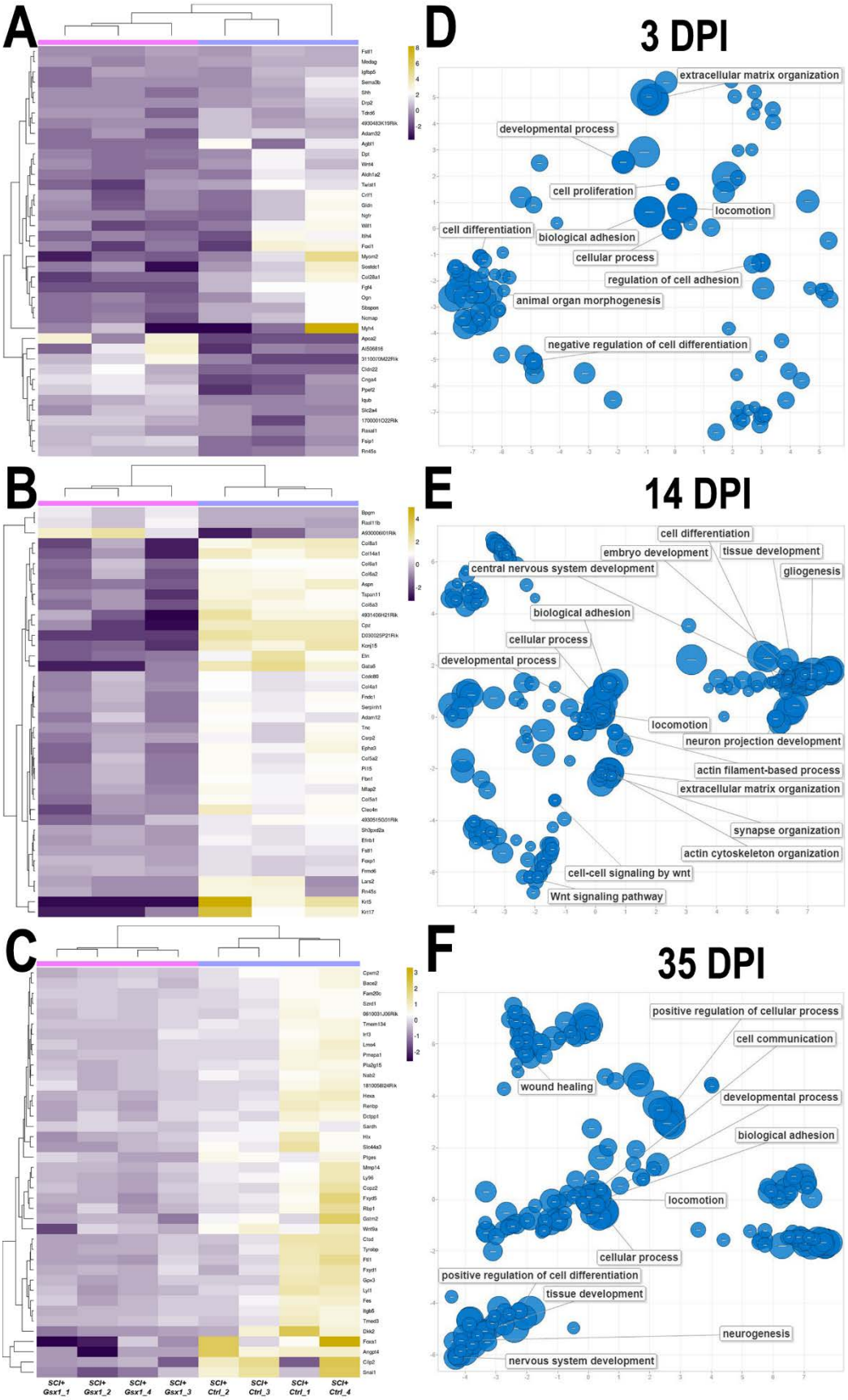
A
B
C
D
3 DPI
extracellular matrix organization
developmental process
cell proliferation
locomotion
cell differentiation
biological adhesion
cellular process
regulation of cell adhesion
animal organ morphogenesis
negative regulation of cell differentiation
E
14 DPI
cell differentiation
embryo development
tissue development
central nervous system development
gliogenesis
biological adhesion
cellular process
developmental process
locomotion
neuron projection development
actin filament-based process
extracellular matrix organization
synapse organization
actin cytoskeleton organization
cell-cell signaling by wnt
Wnt signaling pathway
F
35 DPI
positive regulation of cellular process
cell communication
wound healing
developmental process
biological adhesion
locomotion
cellular process
positive regulation of cell differentiation
tissue development
neurogenesis
nervous system development
SCI+ Gsx1_1
SCI+ Gsx1_2
SCI+ Gsx1_4
SCI+ Gsx1_3
SCI+ Ctrl_2
SCI+ Ctrl_3
SCI+ Ctrl_1
SCI+ Ctrl_4

Figure S3. Top 40 Gsx1-induced differentially expressed genes (DEGs) and functional enrichment of gene ontology (GO) terms

Heatmaps of the top 40 DEGs between the SCI+Ctrl and SCI+Gsx1 groups at 3 DIP (**A**), 14 DPI (**B**), and 35 DPI (**C**). Purple indicates downregulation and yellow indicates upregulation of the gene expression. Scatter plots of enriched terms for biological process using REVIGO at 3 DPI (**D**), 14 DPI (**E**), and 35 DPI (**F**). Circle size indicates the log10(p-value) of the GO terms. For 3 DPI, n=3; 14 DPI, n=3; and 35 DPI, n=4.

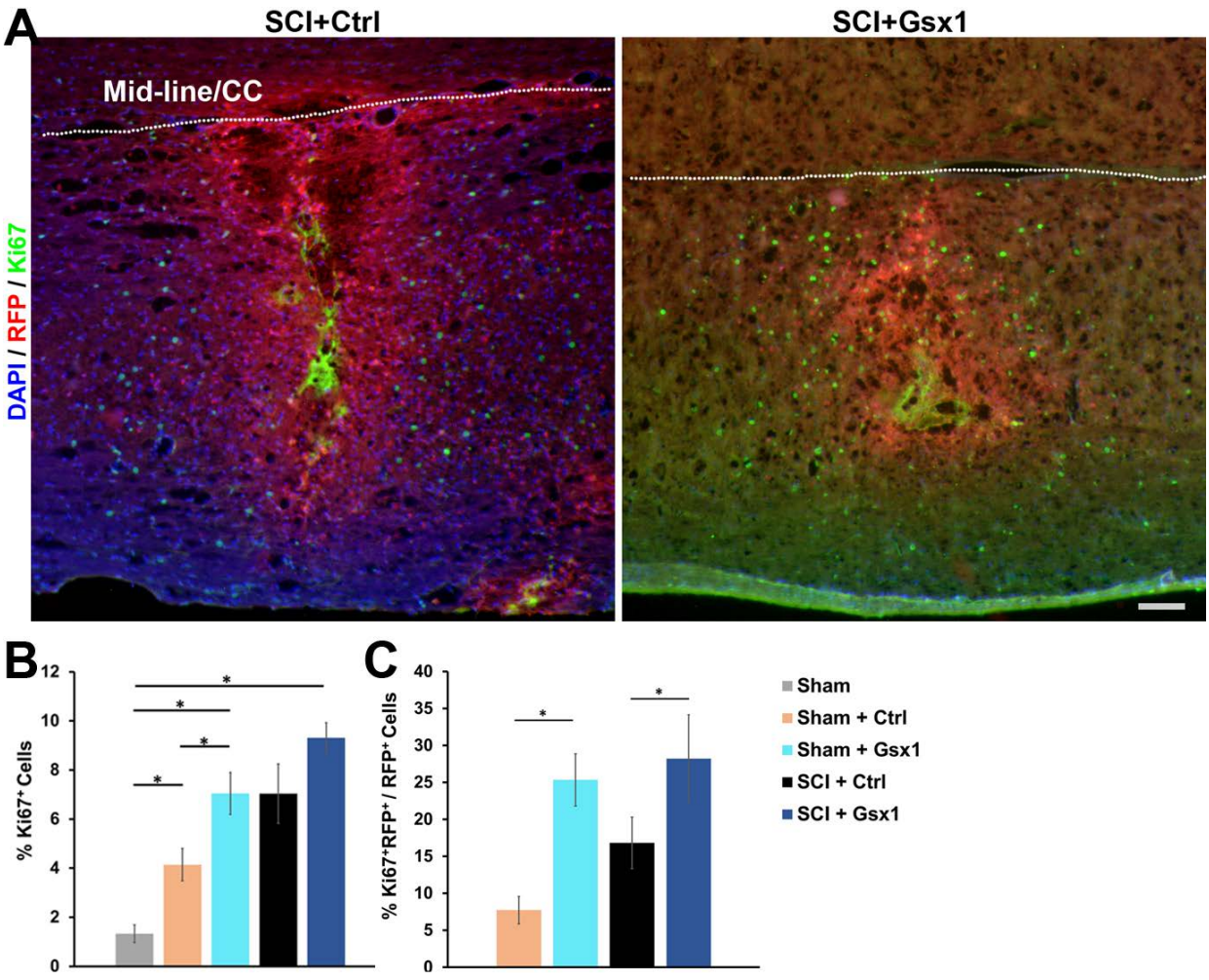

Figure S4. Gsx1 promotes cell proliferation in both the injured and sham mice.

(**A**) Representative low magnification images of sagittal sections through T9-10 spinal cord at 3 DPI showing the expression of viral reporter RFP and cell proliferation marker Ki67. Scale bar=100 µm. White dotted line indicates the midline and the central canal of the spinal cord. Red dots/signals show virally transduced cells and green dots show Ki67+ cells at the lesion site. Histograms show the quantification of Ki67+ cells (**B**) and Ki67+/RFP+ co-labeled cells (**C**) among RFP+ cells. n = 3; Mean ± SEM; * = $p < 0.05$ indicates statistical signifance; one-way ANOVA and Tukey post-hoc analysis.

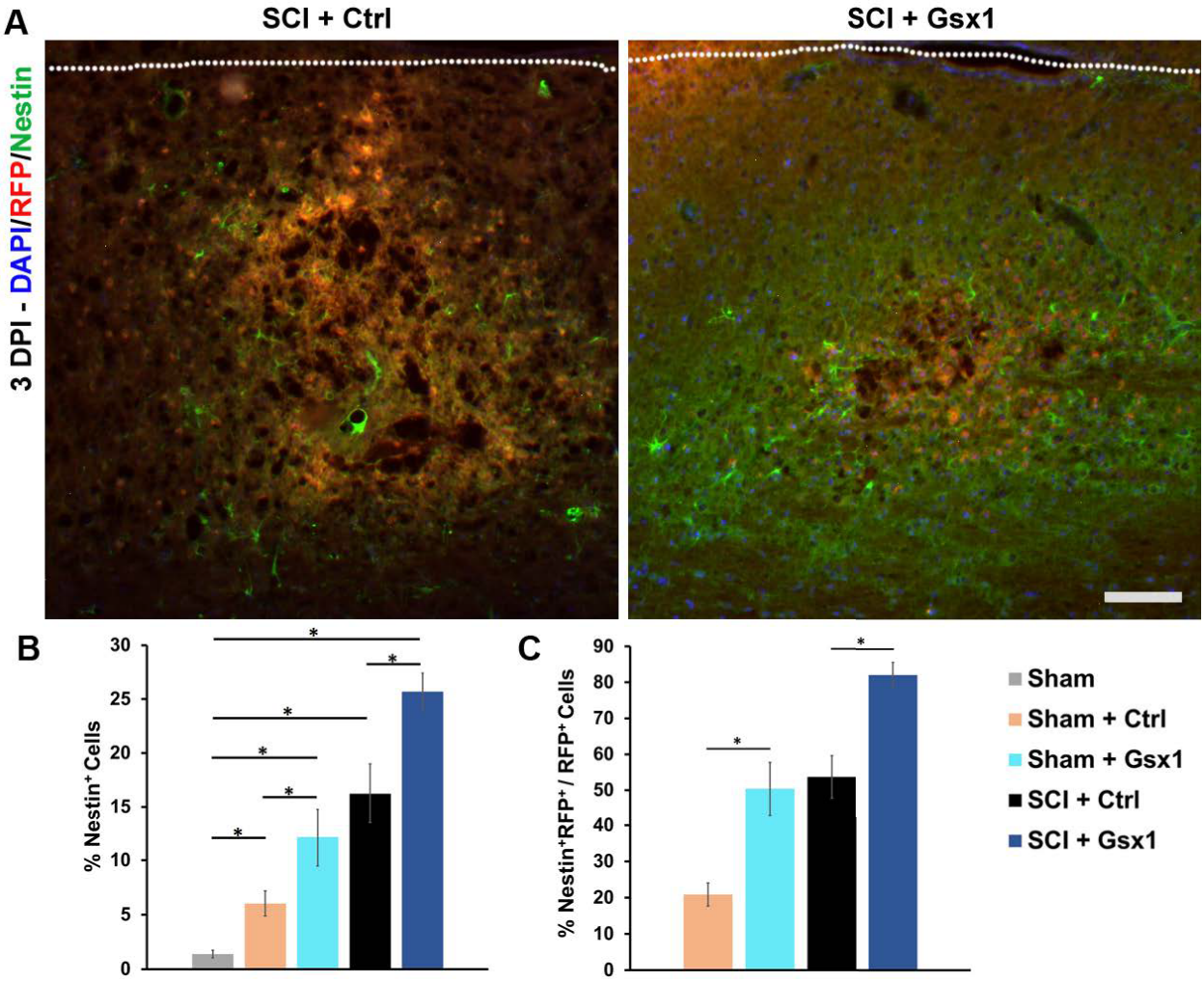

Figure S5. Gsx1 increased the number of NSPCs in both injured and sham mice.

(**A**) Representative low magnification images of sagittal sections through T9-10 spinal cord at 3 DPI showing the expression of viral reporter RFP and NSPC marker Nestin. Scale bar=100 μm. White dotted line indicates the midline and central canal of the spinal cord. Red dots/signals show virally transduced cells and green dots show Nestin+ cells. Histograms show the quantification of Nestin+ (**B**) and Nestin+/RFP+ co-labeled cells (**C**) among RFP+ cells. n = 3; Mean ± SEM; * = $p < 0.05$ indicates statistical signifance; one-way ANOVA and Tukey post-hoc analysis.

A *Key Genes in Notch signaling*

ID	Log2(Fold Change)
Hes7	1.161
Rbpj	0.717
Hes1	-0.522

B *Genes known to Positively Regulate NSPCs*

ID	Log2(fold Change)
Twist1	1.681
Fgf4	1.332
Igf2	0.692
Foxc1	0.64
Slit2	0.566
Wnt5a	0.362
Notch2	0.263
Ndrg2	-0.24

C *Genes known to Negatively Regulate NSPCs*

ID	Log2(fold Change)
Ngfr	1.547
Sfrp4	1.07

D

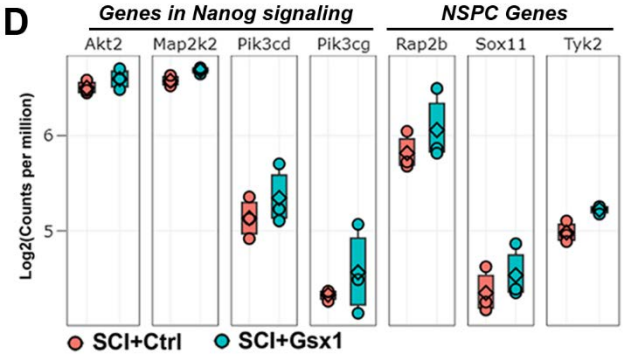

Figure S6. Gsx1 upregulates NSPC signaling pathways.

Lists of differentially expressed genes that are known to promote Notch signaling (**A**), and regulate NSPCs (**B**-**C**) identified by RNA-seq (DESeq2) analysis at 3 DPI. (**D**) Gene expression box plots of the genes associated with Nanog signaling pathway and NSPC genes. Each dot represents the gene expression as log2(count per million) for one biological replicate sample. n=3 for all data points; Mean ± SEM; * = $p < 0.05$ indicates statistical signifance; Students' t-test.

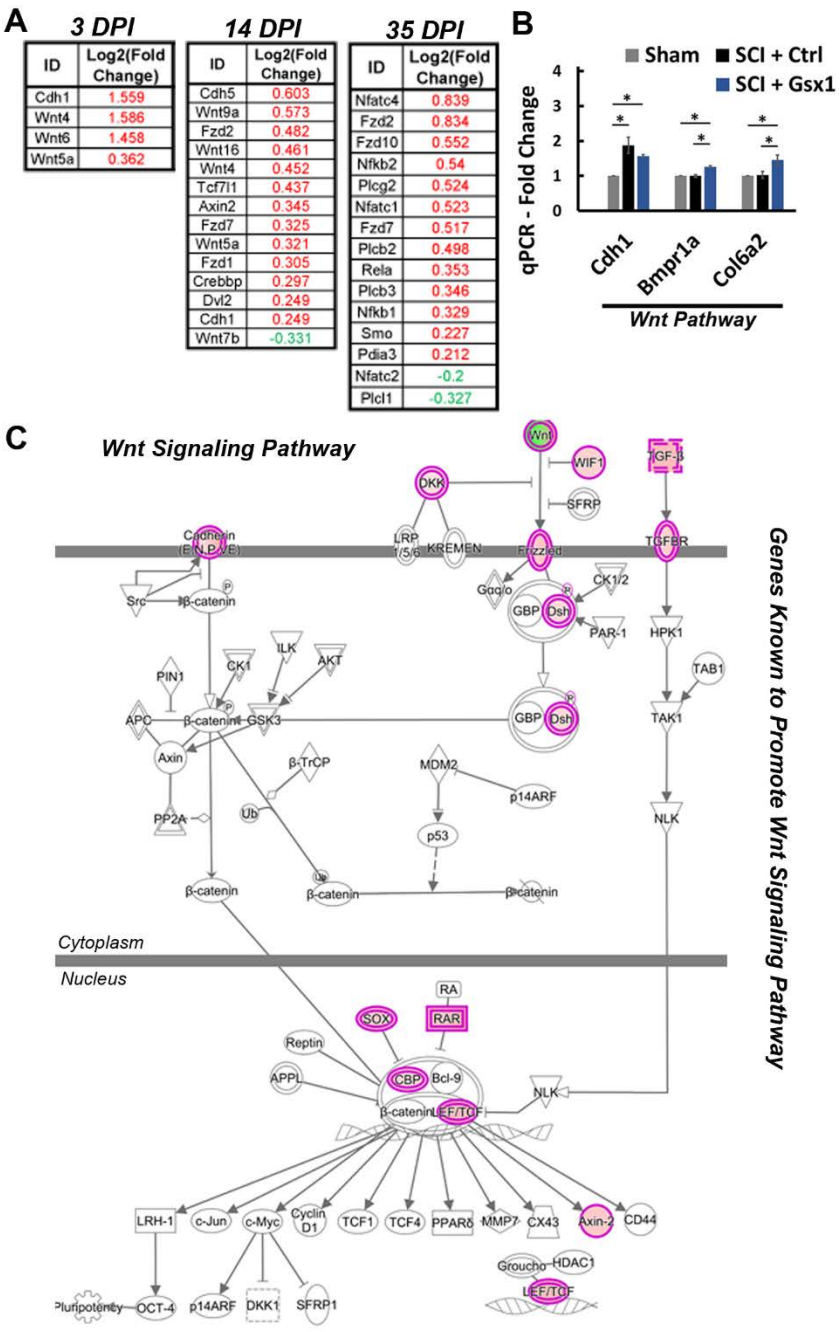

Figure S7. Gsx1 upregulates Wnt signaling pathway in the injured spinal cord.

(**A**) Lists of differentially expressed genes that are involved in Wnt signaling at 3, 14, and 35 DPI from RNA-seq analysis. (**B**) A histogram shows the RT-qPCR analysis of the genes involved in the Wnt signaling pathway (Cdh1, Bmpr1a and Col6a2). N=3; Mean ± SEM; * = $p < 0.05$ indicates statistical signifance; One-way ANOVA followed by Tukey post-hoc test. (**C**) A diagram depicts the upregulated Wnt signaling pathway by Gsx1 expression revealed by IPA.

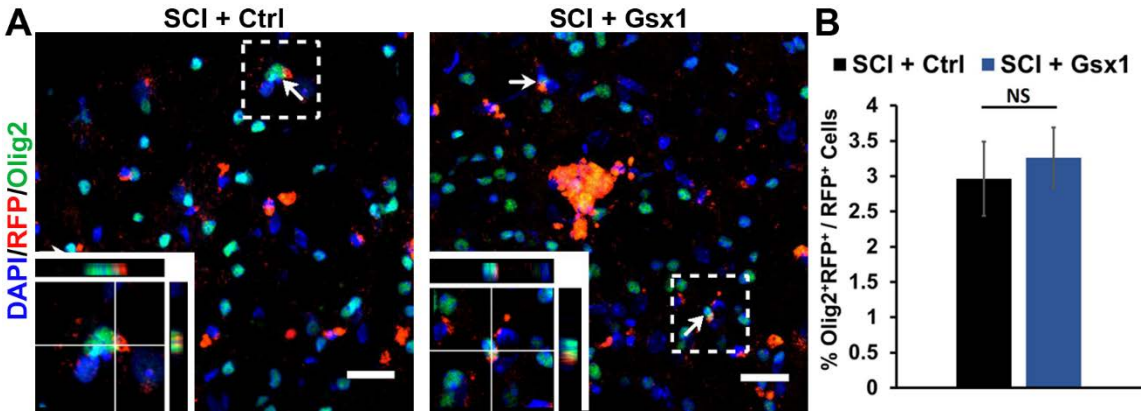

Figure S8. Gsx1 treatment does not change the number of oligodendrocytes after SCI
Hemisection SCI was performed on 8-12 weeks old mice around T10 followed by lentivirus injection encoding Ctrl or Gsx1 gene along with RFP reporter. Animals were harvested 56 DPI and sagittal sections are immunostained with oligodendrocyte marker, Olig2 (**A**). Bottom left of the image includes the higher magnification z-stack view of the area denoted by a dashed white line to indicate co-expression. Scale bar = 20 µm. (**B**) A histogram shows the quantification of Olig2+/RFP+ among RFP+ cells at 56 DPI. n = 6; Mean ± SEM; * = $p < 0.05$ indicates statistical signifance; Students' t-test.

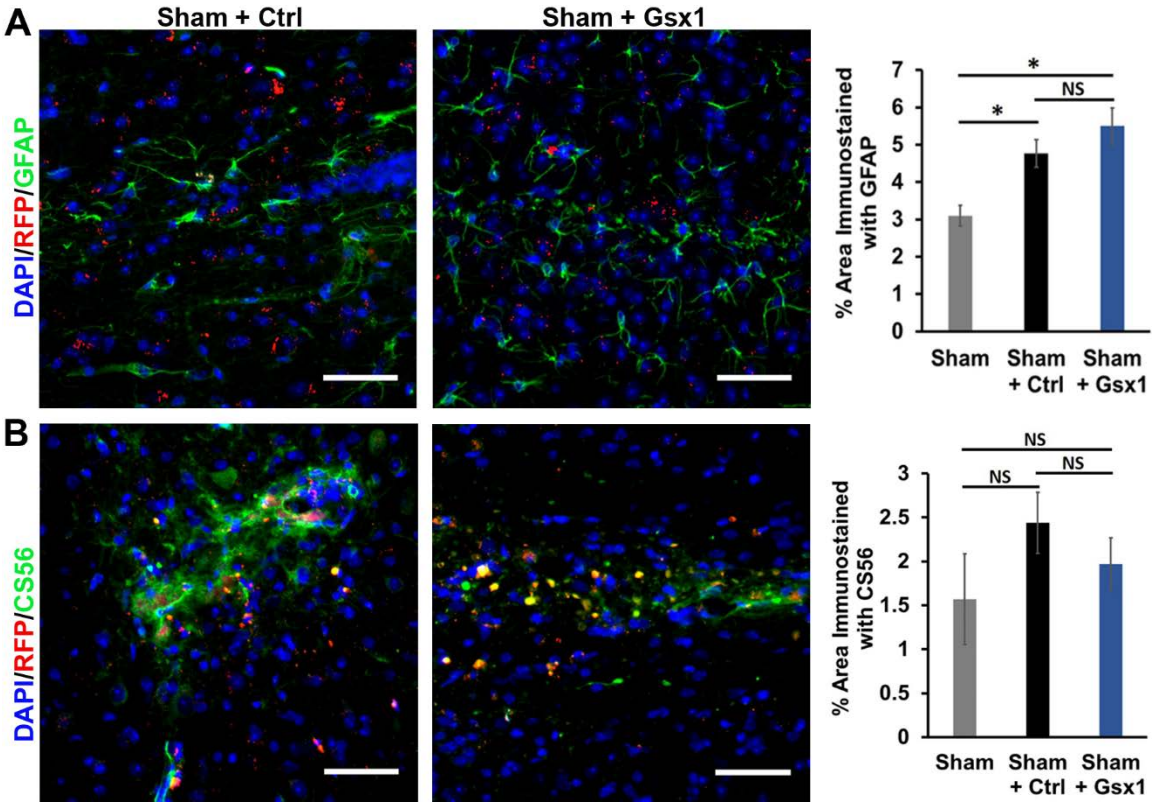

Figure S9. Effects of Gsx1 on astrogliosis and glial scar formation in the uninjured spinal cord is not significant

Representative fluorescence images of sagittal sections through the lesion site in the spinal cord at 56 DPI show the expression of viral reporter RFP, GFAP (**A**) and chondroitin sulfate proteoglycan (CSPG, stained with CS56) (**B**), and the quantification of the immunostained area with anti-GFAP and anti-CS56 around the injury site is shown on the right. Scale bar =50 µm, n=4 for all three groups: Sham, Sham+Ctrl and Sham+Gsx1. Mean ± SEM; * = $p < 0.05$ indicates statistical signifance; One-way ANOVA followed by Tukey post-hoc test.

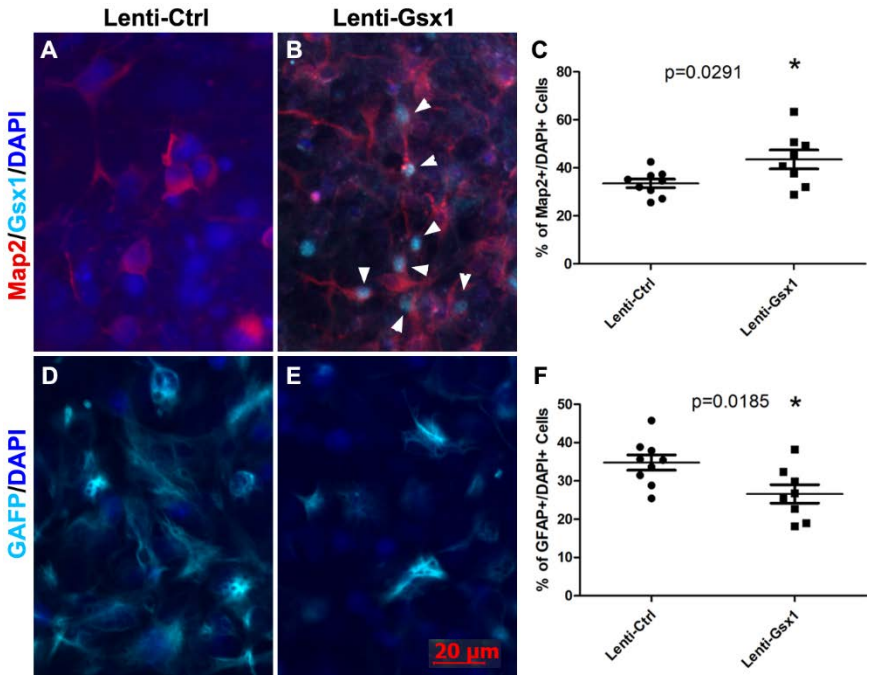

Figure S10. Gsx1 expression promotes neurogenesis and inhibits astrogliosis *in vitro*
Neural stem cells, NE-4C (ATCC), were cultured for 3 days post transduction before inducing differentiation with 10^{-7} M retinoic acid. The effect of Gsx1 on neural differentiation was performed by lentivirus transduction, a control lentivirus (Lenti-Ctrl) (**A, D**) and lentivirus carrying Gsx1 (Lenti-Gsx1) (**B**, **E**) were transduced into NE-4C cells. Cells were selected with 0.5 µg/mL Puromycin for 48-hours 3 days after viral transduction and cultured for 14 more days and followed by immunocytochemistry assay. Arrowheads indicate Gsx1-labeled cells in cyan color confirming lentivirus-mediated Gsx1 expression in virally transduced cells. Cell nuclei were labeled with DAPI in blue. (**C**, **F**) histograms of the percentages of MAP2+ neurons and GFAP+ astrocytes over the total number of DAPI+ cells. N=9; Data shown as Mean ± SEM. Students' T-test. p-value < 0.05 indicates statistical significance.

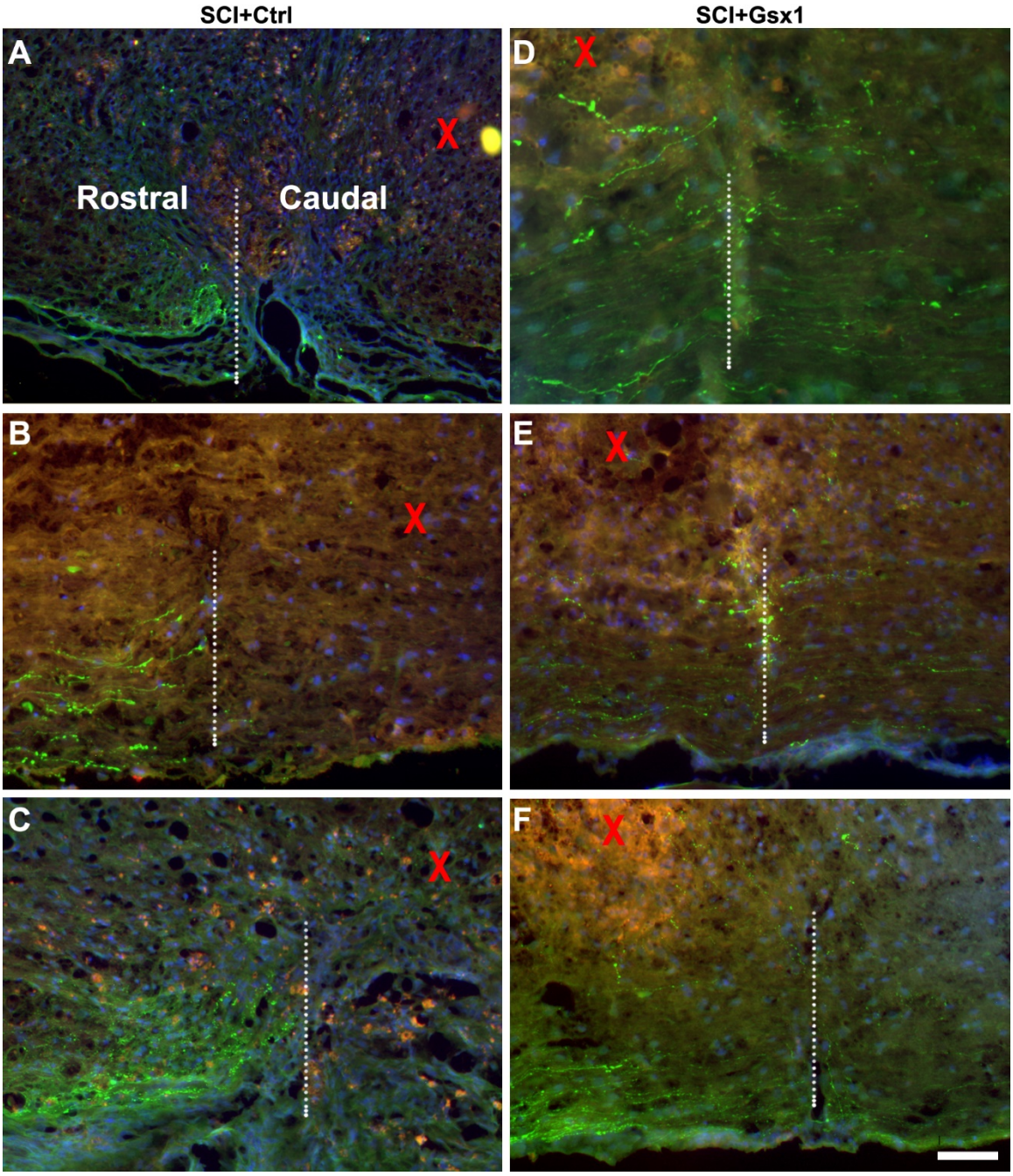

Figure S11. Gsx1 expression promotes 5-HT neuronal activity
Hemisection SCI was performed on 8-12 weeks old mice around T10 followed by lentivirus injection immediately after SCI. Representative images of sagittal section of the spinal cord from the SCI+Ctrl (n=3: **A**-**C**) and SCI+Gsx1 (n=3: **D**-**F**) groups at 35 DPI. The white dotted line indicates the hemisection site. "X" indicates the virus injection site. In the SCI+Ctrl group, 5-HT immunostained axons stopped rostral to the lesion site (**A**-**C**). In the SCI+Gsx1 group, 5-HT stained axons were detected caudally to the lesion site (**D**-**F**). Scale bar = 20 µm.

Supplementary Tables S1-3. IPA reports of the DEG-associated signaling pathways induced by Gsx1 expressoin (see attached Microsoft Excel files)**.**

Supplementary Table S4. Top 20 upregulated and 20 downregulated DEGs determined by RNA-seq analysis

	3 DPI		14 DPI		35 DPI	
Top 20 Upregulated Genes	ID	Log2(Fold Change)	ID	Log2(Fold Change)	ID	Log2(Fold Change)
	Ppef2	1.7456	Bpgm	0.6457	9330175M20Rik	0.9304
	Cnga4	1.5203	Snca	0.6409	Gm2897	0.9209
	Tmem51as1	1.4642	Fam46c	0.5624	4930525G20Rik	0.8941
	AI506816	1.3532	Gjc2	0.5451	Zfp819	0.8928
	Cdsn	1.3006	Nkx2-9	0.5346	Ripply2	0.8629
	G530011O06Rik	1.2743	Ppp1r14a	0.5226	Peg12	0.8311
	Fsip1	1.2560	Rasl11b	0.5223	Mum1l1	0.8199
	3110070M22Rik	1.2345	Prr18	0.5131	1500015L24Rik	0.8142
	Fbxw10	1.2303	Klk6	0.5103	1110015O18Rik	0.8049
	Galr3	1.2074	Ptgs1	0.5076	4930441O14Rik	0.8042
	Rn45s	1.2041	Ptp4a1	0.4919	Sox14	0.7864
	C130026I21Rik	1.1821	S100b	0.4672	Zfp804b	0.7683
	Erv3	1.1741	Bin2	0.4563	Mir331	0.7637
	Afp	1.1694	Tmem88b	0.4517	Cacna1f	0.7612
	A330048O09Rik	1.1366	Mbp	0.4422	Fgf5	0.7601
	Xlr3a	1.1299	Atp10b	0.4393	Mipol1	0.7477
	1700001O22Rik	1.1218	Isg20	0.4377	Mir149	0.7471
	Apoa2	1.1206	AI848285	0.4364	Tmem232	0.7392
	Mir466i	1.1092	Arhgef37	0.4320	Gpr88	0.7370
	Crybb1	1.0996	Plp1	0.4288	6430584L05Rik	0.7369
Top 20 Downregulated Genes	ID	Log2(Fold Change)	ID	Log2(Fold Change)	ID	Log2(Fold Change)
	Col28a1	-2.4223	Col1a1	-2.0110	Snai1	-1.4631
	Ogn	-2.0659	Aspn	-1.8378	Wfdc17	-1.4354
	Wif1	-1.9547	Col1a2	-1.7949	Dkk2	-1.4094
	Sbspon	-1.7291	Col6a3	-1.6645	Cilp2	-1.3829
	Itih4	-1.6936	Col5a1	-1.4541	H19	-1.3015
	Twist1	-1.6811	Kcnj15	-1.4052	Asgr2	-1.2978
	Sostdc1	-1.6651	Mfap5	-1.3046	Atp6v0a4	-1.2820
	Plekha4	-1.6475	Thbs1	-1.2104	Foxa1	-1.2655
	Ncmap	-1.6447	Serpinh1	-1.1806	Apoc2	-1.2628
	Aqp1	-1.6233	Ppic	-1.1093	Angpt4	-1.2556
	Gldn	-1.6205	Loxl1	-1.0945	Fam180a	-1.2413
	Prx	-1.6059	Tnc	-1.0594	Cd8b1	-1.2400
	Wnt4	-1.5861	Ltbp2	-1.0186	Twist1	-1.2251
	Cdh1	-1.5587	Cpz	-1.0108	Pi16	-1.2225
	Ngfr	-1.5471	Scara5	-0.9871	Gstm2	-1.2204
	Slc43a1	-1.5467	Scara3	-0.9867	Wnt9a	-1.1770
	Foxd1	-1.5382	Rcn3	-0.9815	Dpt	-1.1667
	Kcnj13	-1.5141	Mrc2	-0.9796	Col6a2	-1.1481
	Crlf1	-1.4928	Sh3pxd2a	-0.9693	Gpnmb	-1.1465
	Dpt	-1.4878	Tspan11	-0.9674	Ms4a7	-1.1420

Supplementary Table S5. List of primary and secondary antibodies used for immunohistochemistry

	Vendor, Catalog	Host Species	Type	RRID	Dilution
Primary Antibody					
Gsx1	Millipore Sigma, SAB2104632	Rabbit	Polyclonal	AB_10667904	1 : 200
Ki67	Abcam, ab15580	Rabbit	Polyclonal	AB_443209	1 : 1000
Nestin	Abcam, ab6142	Mouse	Monoclonal	AB_305313	1 : 200
Caspase3	Cell Signaling, 9661S	Rabbit	Polyclonal	AB_2341188	1 : 300
DCX	Santa Cruz Biotechnology, sc-8067	Goat	Polyclonal	AB_2088491	1 : 100
PDGFRa	Abcam, ab61219	Rabbit	Polyclonal	AB_2162341	1 : 100
NeuN	Millipore Sigma, MAB377	Mouse	Monoclonal	AB_2298772	1 : 300
GFAP	Millipore Sigma, G3893	Mouse	Monoclonal	AB_477010	1 : 400
Olig2	Millipore Sigma, AB9610	Rabbit	Polyclonal	AB_570666	1 : 500
vGlut2	Millipore Sigma, AB2251-I	Guinea Pig	Polyclonal	AB_2665454	1 : 1000
ChAT	Millipore Sigma, SAB2500236	Goat	Polyclonal	AB_10603616	1 : 300
GABA	Millipore Sigma, A-2052	Rabbit	Polyclonal	AB_477652	1 : 3000
CS56	Millipore Sigma, C8035	Mouse	Monoclonal	AB_476879	1 : 200
Map2	Invitrogen, MA5-12826	Mouse	Monoclonal	AB_10976831	1 : 500
GFAP	Invitrogen, PA1-10019	Rabbit	Polyclonal	AB_1074611	1 : 1000
Secondary Antibody					
Alexa Fluor 488 Donkey anti Mouse	Jackson Immuno Research, 715-545-150	-	Polyclonal	AB_2340846	1 : 200
Alexa Fluor 488 Donkey anti Rabbit	Jackson Immuno Research, 711-545-152	-	Polyclonal	AB_2313584	1 : 200
Alexa Fluor 488 Donkey anti Goat	Jackson Immuno Research, 705-545-003	-	Polyclonal	AB_2340428	1 : 200
Alexa Fluor 488 Donkey anti Guinea Pig	Jackson Immuno Research, 706-545-148	-	Polyclonal	AB_2340472	1 : 200
Alexa Fluor 647 Donkey anti Mouse	Jackson Immuno Research, 715-605-150	-	Polyclonal	AB_2340862	1 : 200
Alexa Fluor 647 Donkey anti Rabbit	Jackson Immuno Research, 711-605-152	-	Polyclonal	AB_2492288	1 : 200
Alexa Fluor 647 Donkey anti Goat	Jackson Immuno Research, 705-605-003	-	Polyclonal	AB_2340436	1 : 200
Alexa Fluor 647 Donkey anti Guinea Pig	Jackson Immuno Research, 706-605-148	-	Polyclonal	AB_2340476	1 : 200

Supplementary Table S6. List of primers for qRT-PCR analysis

Gene	Forward (5' -> 3')	Reverse (5' -> 3')
Gsx1	CTTCCCTCCCTTCGGATCG	GTCCACAGAGATGCAGTGAAA
Cd68	GGACCCACAACTGTCACTCAT	AAGCCCCACTTTAGCTTTACC
Itgam	ATGGACGCTGATGGCAATACC	TCCCCATTCACGTCTCCCA
Cd86	TGTTTCCGTGGAGACGCAAG	TTGAGCCTTTGTAAATGGGCA
Il1b	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
Tnf	CCTGTAGCCCACGTCGTAG	GGGAGTAGACAAGGTACAACCC
Ki67 (Mki67)	ATCATTGACCGCTCCTTTAGGT	GCTCGCCTTGATGGTTCCT
Nestin	CCCTGAAGTCGAGGAGCTG	CTGCTGCACCTCTAAGCGA
NeuN (Hrnbp3)	AACCACGAACTCCACCCTTC	GACCTCAATTTTCCGTCCCTC
vGlut (Slc17a6)	TGGAAAATCCCTCGGACAGAT	CATAGCGGAGCCTTCTTCTCA
Th	GTCTCAGAGCAGGATACCAAGC	CTCTCCTCGAATACCACAGCC
Tph1	AACAAAGACCATTCCTCCGAAAG	TGTAACAGGCTCACATGATTCTC
Chat	CCATTGTGAAGCGGTTTGGG	GCCAGGCGGTTGTTTAGATACA
Gfap	CGGAGACGCATCACCTCTG	AGGGAGTGGAGGAGTCATTCG
Lcn2	GCAGGTGGTACGTTGTGGG	CTCTTGTAGCTCATAGATGGTGC
Serpina3n	ATTTGTCCCAATGTCTGCGAA	TGGCTATCTTGGCTATAAAGGGG
Notch1	CCCTTGCTCTGCCTAACGC	GGAGTCCTGGCATCGTTGG
Nrarp	AAGCTGTTGGTCAAGTTCGGA	CGCACACCGAGGTAGTTGG
Jag1	CCTCGGGTCAGTTTGAGCTG	CCTTGAGGCACACTTTGAAGTA
Jag2	CACTGTCCGTCAGGATGGAAC	TAGCCGCCAATCAGGTTTTTG
Dll1	CCCATCCGATTCCCCTTCG	GGTTTTCTGTTGCGAGGTCATC
Hes1	TCAGCGAGTGCATGAACGAG	CATGGCGTTGATCTGGGTCA
Cdh1	CAGGTCTCCTCATGGCTTTGC	CTTCCGAAAAGAAGGCTGTCC
Bmpr1a	TGCAAGGATTCACCGAAAGC	TGCCATCAAAGAACGGACCTAT
Col6a2	GCTCCTGATTGGGGGACTCT	CCAACACGAAATACACGTTGAC
Ctnna1	AAGTCTGGAGATTAGGACTCTGG	ACGGCCTCTCTTTTTATTAGACG
Ntng1	TGCTAAACACAGTCATTTGCGT	GCACACATTCTCATCGTCCAG
Syn1	AGCTCAACAAATCCCAGTCTCT	CGGATGGTCTCAGCTTTCAC

Supplementary Videos. Open field locomotor behavior observation of the sham; SCI+Ctrl, and SCI+Gsx1 animals, related to Figure 6H.