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Supplemental Information

VCAM1 Labels a Subpopulation of Neural Stem Cells in the Adult Hippocampus and Contributes to Spatial Memory

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Supplemental Information

Supplemental Figures

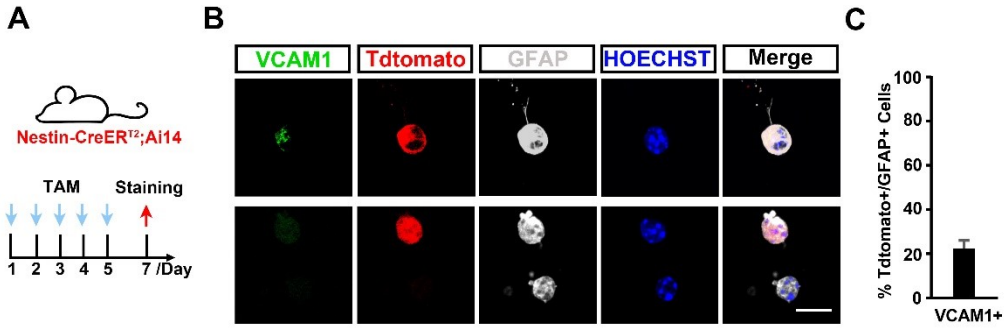

Figure S1. Result from acute isolated single cell staining, related to Figure 1.

(A) Strategy was illustrated that tamoxifen have been injected into Nestin-creERT2; Ai14 mice to induce Nestin+ NSCs expressing tdTomato fluorescence.

(B) Represent images in acute staining of DG cells from Nestin-creERT2; Ai14 mice.

(C) Statistic result show VCAM1+ cells ratio in tdTomato+GFAP+ NSCs.

Scale bars: (B) 10 μm. Data represent mean ± SEM. (C) at least 1,000 cells were counted.

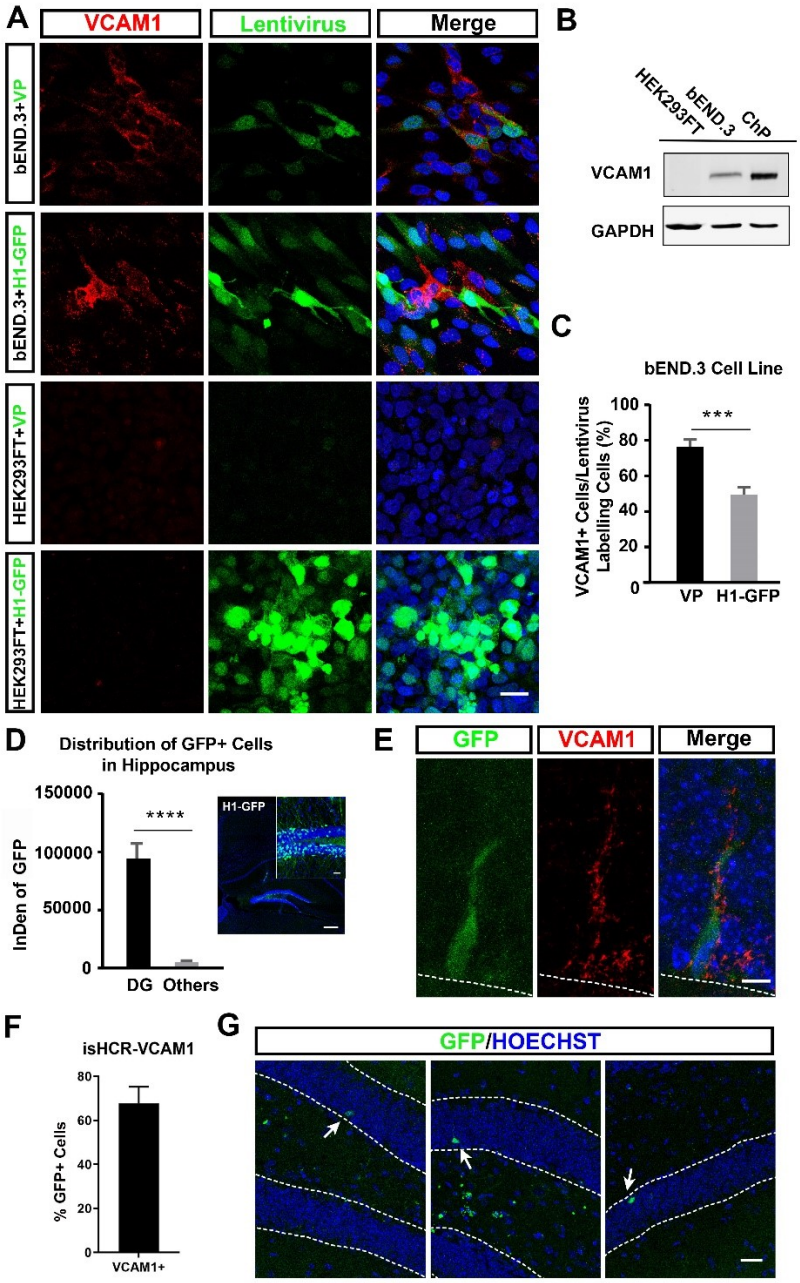

Figure S2. Identification of the specificity of VP-lentivirus and the infection range of viral injection. Related to figure 2.

(A) Representative images of VCAM1 staining (red) 5 days after infecting VP and H1-GFP lentivirus on bEND.3 and HEK293FT cell lines. Virus labelling cells are indicated in green.

(B) Western blot data showing VCAM1 expression level in bEND.3 cells and HEK293FT cells.

(C) Quantitative analysis of VCAM1+ cells in lentivirus labelling cells in bEND.3 cells 5 days after viral infection.

(D) Quantitative analysis the distribution of H1-GFP+ infected cells in the adult hippocampus at 5 dpi. The image shows the section from H1-GFP injected mouse brain.

(E-G) High power images (E) of VP-GFP (green) co-labelling with VCAM1 (red) detected by isHCR method in the adult DG at 5 dpi. (F) Quantitative data show the ratio of isHCR-VCAM1+ cells in VP-green cells. Low power images of VP-GFP+ cells (green) in the adult DG at 5 dpi. Arrows indicate positive cells.

Nuclei were stained with Hoechst (blue). Scale bars: (A), (G) 25 μm; (D) low power 100 μm, high power 25 μm; (E) 7.5 μm. Data represent mean ± SEM. Value represent: (C) 11 repeats for VP group and 6 repeats for H1-GFP group; (D) 11 mice for each group. Student's t-test for (C), (D). ****$p < 0.0001$; ***$p < 0.001$. ChP: Choroid Plexus.

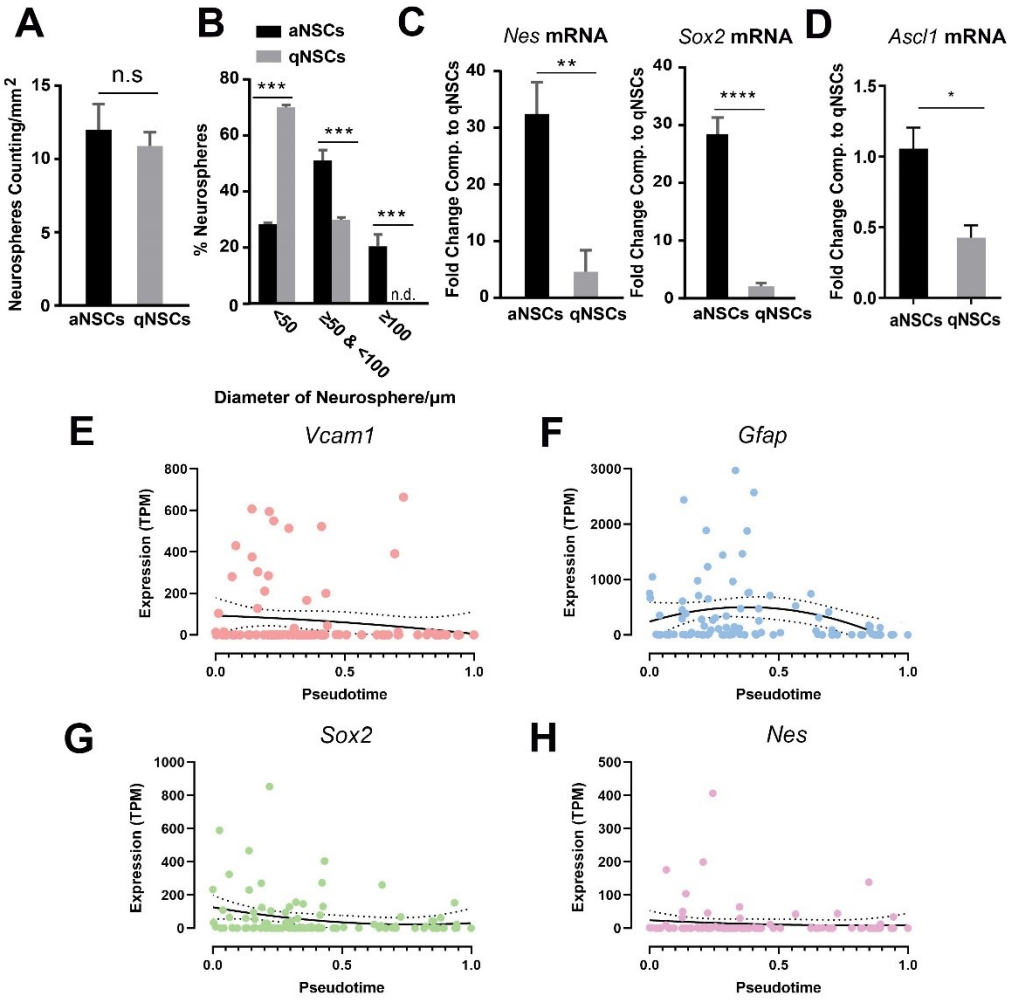

Figure S3. Cellular and molecular character of aNSCs and qNSCs. Related to Figure 3.
(A-B) Quantitative analysis shows the number (A) and the size (B) of neurospheres between induced aNSCs and qNSCs from the adult DG.
(C-D) The transcript changes of *Nes*/*Sox2*(C) and *Ascl1* (D) in aNSCs versus qNSCs at the checking time revealed by qPCR.
(E-H) Result from single cell RNA-seq data analysis. Representative expression profiles of NSCs marker (*Vcam1*, *Gfap*, *Sox2*, *Nes*) during adult neurogenesis, data analysis from published paper on single cell RNA-seq (Shin et al., 2015). Each data point represents the gene expression level presented by transcripts per million (TPM). Data points are fitted with local polynomial regression fitting (black lines) with 95% confidence interval (dashed line area). ****$p < 0.0001$; ***$p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

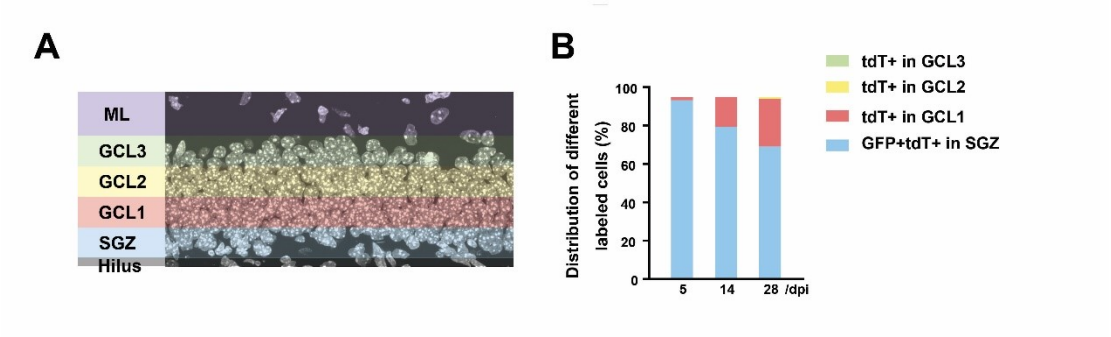

Figure S4. Distribution of VCAM1-expressing NSCs in subregions of the adult DG. Related to Figure 4.

(A) The scheme depicts the subdivisions of the DG.

(B) Quantification of the distribution of VCAM1-expressing NSCs within the subregions of the adult DG of Ai14 mice at 5, 14, 28 dpi.

Data represent mean. Value represent: (B) 5 dpi: 59 cells, 14 dpi: 61 cells, 28 dpi:103 cells. SGZ: subgranular zone, GCL: granular cell layer, ML: molecular layer.

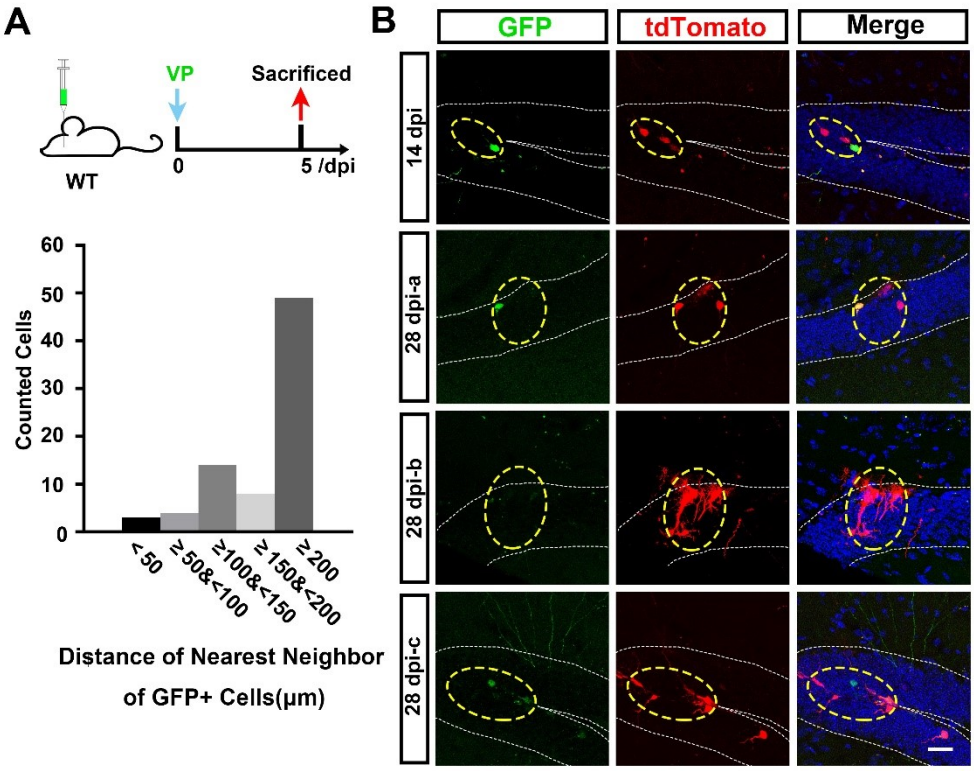

Figure S5. Clonal analysis of VCAM1-expressing NSCs in the adult DG. Related to Figure 5.
(A) Experimental design shows VP lentiviral injection in the adult DG of WT mice. Histogram shows the distance counting between nearest GFP+ cells in the adult DG.
(B) Representative images of the individual clones in the adult DG of Ai14 mice after VP lentiviral injection. Time points are indicated in panels on the left. GFP in green, and tdTomato in red. An oval dotted boxed indicates a clone.
Nuclei were stained with Hoechst (blue). Scale bar: (B) 25 μm. Data represent as counting number.

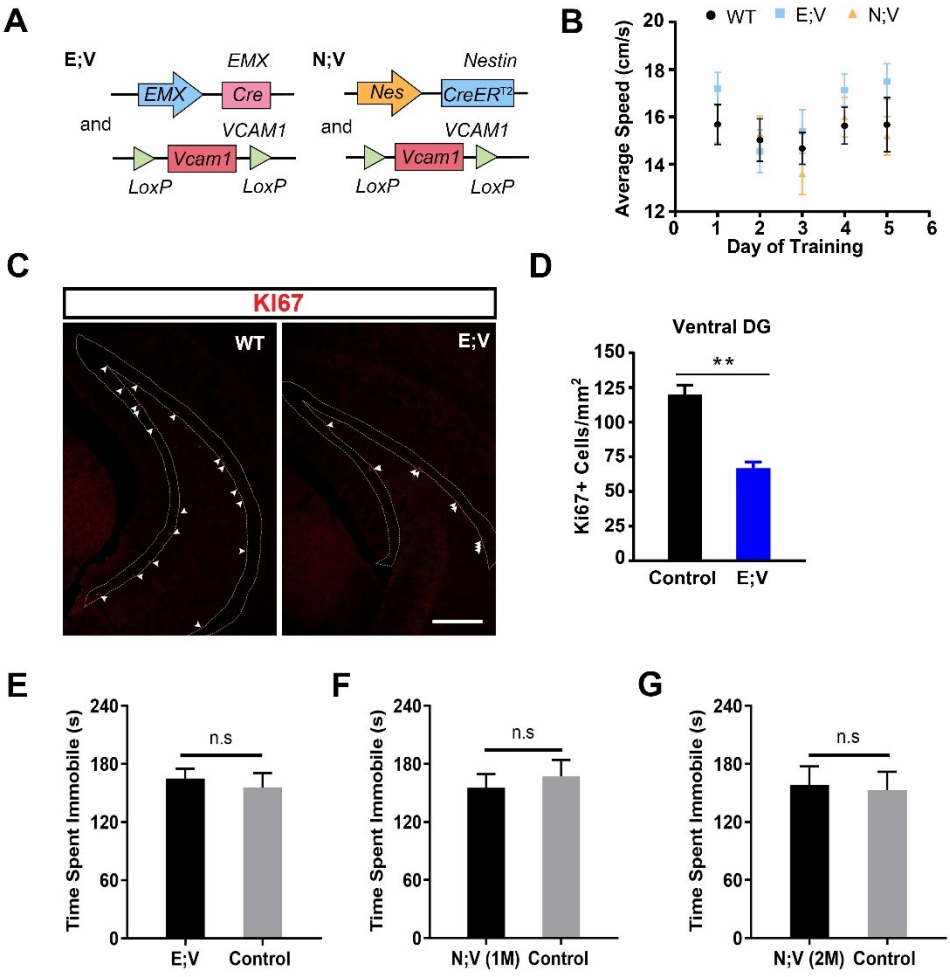

Figure S6. Loss of VCAM1 embryonically does not affect motor ability but reduces adult aNSCs. Related to Figure 6.

(A) schematic graph describes the strategy for VCAM1-cKO construction (E;V and N;V).

(B) The graph illustrate the mean speed of swimming during Morris water maze task between E;V, N;V and Control mice.

(C) Representative images (C) and quantitative analysis (D) for Ki67 in ventral DG between adult E;V and Control mice. Arrowheads indicate Ki67 positive signals along SGZ.

(E-G) quantitative analysis of time spend immobile in ventral DG related behavior test (forced swimming) were showed in (E) E;V cKO mice and (F-G) in N;V cKO mice.

Scale bars: (C) 250 µm. Data represent mean ± SEM. Value represent: (B) E;V: 13 mice, Control: 7 mice; (E-G) E;V and N;V: 4 mice, Control: 3 mice. Two-way ANOVA for (B); student's t-test for (D-G). **p<0.01.

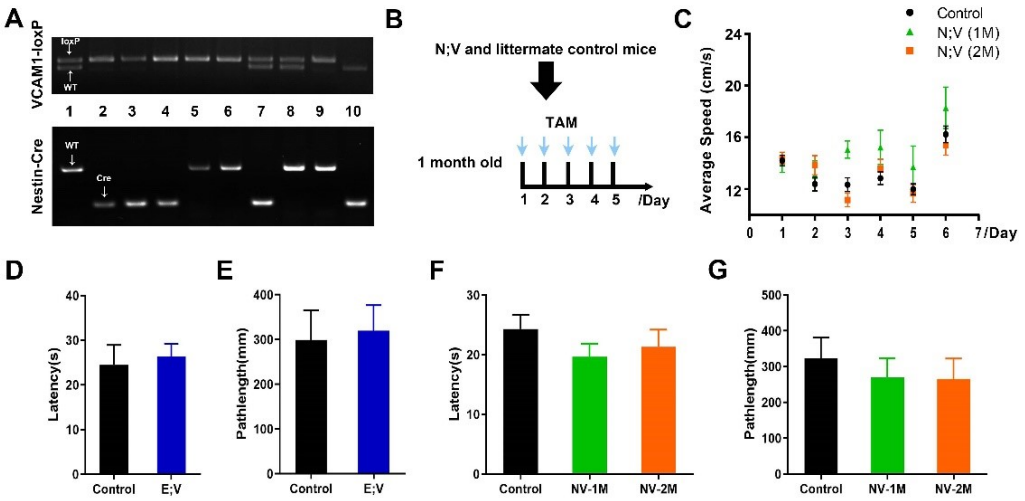

Figure S7. Loss of VCAM1 during adulthood impaired learning behavior not result from visible defect of cKO mice. Related to Figure 7.

(A) Genotyping for Nestin-CreER^{T2};VCAM1$^{fl/fl}$ (N;V) mice. Top gel image shows WT band and *loxp* site for *VCAM1-loxP* locus, and bottom shows band for wt *Nestin* and *Cre*. Lanes 2, 3, 4 represent Nestin-CreER^{T2};VCAM1$^{fl/fl}$ mice. Lane 10 represents Nestin-CreER^{T2}; VCAM1$^{+/+}$ mice. Lane 7 represents Nestin-CreER^{T2};VCAM1$^{fl/+}$ mice. Lanes 1, 8 represent VCAM1$^{fl/+}$ mice. Lanes 5, 6, 9 represent VCAM1$^{fl/fl}$ mice. Nestin-CreER^{T2}; VCAM1$^{+/+}$, Nestin-CreER^{T2};VCAM1$^{fl/+}$, VCAM1$^{fl/+}$ and VCAM1$^{fl/fl}$ mice were their littermate control (Control).

(B) Experimental outline of TAM administration in N;V and their Control mice.

(C) The graph illustrates the mean speed of swimming during Morris water maze task among N;V-1M, N;V-2M and Control mice.

(D-G) graphs show the statistic results of latency to visible platform and pathlength toward visible platform in watermaze test for different cKO mice.

Data represent mean ± SEM. Value represent: (C) N;V-1M: 4 mice, N;V-2M: 11 mice, Control: 13 mice; (D-G) E;V; N;V-1Mand N;V-2M: 3 mice, Control: 3 mice.

Supplemental Experimental Procedures

Animals

The animals were maintained under a 12 h day/night cycle and had free access to food and water. Both male and female mice were included in this study, ranging from 1- to 3-months-old at the onset of experiments.

VCAM1 conditional knockout mice were generated by breeding Emx-Cre (B6.129S2-Emx1tm1(cre)Krj/J, Stock No: 005628)(Gorski et al., 2002) or Nestin-CreERT2 (C57BL/6-Tg(Nes-cre/ERT2)KEisc/J, Stock No: 016261) (Lagace et al., 2007) mice with floxed Vcam1 transgenic mice (B6.129(C3)-Vcam1tm2Flv/J, Stock No: 007665) (Koni et al., 2001), in which the genomic region encompassing the cytokine-responsive promoter and exon 1 of the Vcam1 gene was flanked by a loxP site, creating EMX-Cre;VCAM1fl/fl (E;V) or Nestin-CreERT2;VCAM1fl/fl (N;V) mice, respectively. Wild-type (WT) and Ai14 (Stock No: 007908) (Madisen et al., 2010) mice were also used in this study. All of the mice were maintained on a C57BL/6 genetic background, and were genotyped on postnatal day 21 by PCR using genomic DNA. The primer pairs used for genotyping are listed in table S1, and the PCR products were run on a 2% agarose gel.

isHCR

Firstly, HCR amplification buffer was prepared (5X sodium chloride citrate buffer, 0.1% Tween-20, and 10% dextran sulfate in ddH2O). Frozen sections were blocked in 5% BSA-PBST (0.1M PBS and 0.3%

Triton X-100) at 25°C for 1 h and incubated overnight at 4 °C with primary antibodies diluted in blocking solution. The brain sections were incubated in HCR amplification buffer for 30 min at room temperature, followed by biotinylated secondary antibodies for 2 h. The sections were then incubated in 1 μg/ml streptavidin for 30 min at room temperature, followed by 0.5 μM DNA-biotin HCR initiators at room temperature for 30 min. Two DNA–fluorophore HCR amplifiers were snap-cooled separately. Then, the sections were incubated in HCR amplification buffer containing both HCR amplifiers overnight at room temperature. An additional graphene oxide step was added for background suppression. The sections were then counterstained with nuclear and mounted.

Lentiviral Vector Infection of Cell

HEK293FT and bEND.3 cells were obtained from ATCC. The cells were thawed, passaged, and frozen according to standard procedures. HEK293FT and bEND.3 cells were plated on PDL-coated coverslips in 24-well plates, respectively Cells were plated in coverslips in 24-well plate precoated with PDL, respectively. Following this, 1 μl VP or H1GFP lentivirus was added into the cell medium to infect the HEK293FT or bEND.3 cells, and they were then allowed to grow for 3 days

Tamoxifen (TAM) Treatment

Firstly, 20 mg⁄ml stock solutions of TAM (Sigma-Aldrich) were prepared using 90% corn oil (Sigma-Aldrich) and 10% ethanol. One-month-old N;V mice and their littermate controls were i.p. injected with TAM (180 mg/day/kg weight) once a day for five consecutive days to induce VCAM1 deletion.

Brain Sectioning

Mice were anesthetized and perfused transcardially with 37 °C saline followed by 4 °C 4% paraformaldehyde (PFA) in 0.1 M PBS. Their brains were removed, post-fixed in 4 °C 4% PFA overnight, and then dehydrated in 30% sucrose in 0.1 M PBS for 2 days. The brains were sectioned at a thickness of 30 μm in the coronal plane through the hippocampus (between -1.60 mm and -3.64 mm from bregma) using a freezing microtome.

Additionally, for BrdU immunostaining, the sections were pre-treated with 2N HCl for 30 min at 37 °C to denature DNA and were then neutralized with borate buffer (pH 8.5) for 20 min at room temperature. The sections were then blocked, incubated with an anti-BrdU primary antibody and corresponding secondary antibody, counterstained with Hoechst 33342, and mounted.

Acute and Cell Staining

The DG was dissected from adult mice and dissociated into single cells as previously described in the "Adult Neural Stem Cell Culture" subsection. These cells were then seeded onto coverslips precoated with PDL. After the cells had adhered onto the coverslips (~ 4 hours after plating), an anti-VCAM1 antibody was added into the culture medium for 30 min. Following this, the cells were fixed with cold 4% PFA for 30 min. To stain cells adhered to coverslips, cells were fixed with cold 4% PFA for 30 min 3 days after lentivirus infection. The coverslips were blocked in 5% BSA-PBST at room temperature for 1 h and incubated with primary antibodies at 4 °C overnight. The coverslips were then incubated with corresponding secondary antibodies for 2 h at room temperature. Following this, the coverslips were counterstained with Hoechst 33342 for 10 min, and were reversely mounted onto glass slides with anti-fade fluoromount G.

Antibodies

The following primary antibodies were used in this study: rat monoclonal anti-CD106/VCAM1 (1:50, B&D Systems, Cat# 550547), mouse monoclonal anti-GFAP (1:1000, Millipore, Cat#NE1015), rabbit polyclonal anti-GFAP (1:1000, Abcam, Cat#ab7260), mouse monoclonal anti-rat 401/Nestin (1:40, Sigma Cat#MAB353), rabbit polyclonal anti-SOX2 (1:500, Abcam, Cat# ab97959), rabbit polyclonal anti-Olig2 (1:500, Abcam,Cat#ab109186), mouse monoclonal anti-BrdU (1:40, Abcam, Cat# ab8039), rabbit polyclonal anti-KI67 (1:100, Abcam, Cat# ab16667), rabbit polyclonal anti-NeuN (1:500, Millipore, Cat#ABN78), and rabbit polyclonal anti-S100β (1:2000, Abcam, Cat#ab41548). Alexa-Fluor conjugated (1:1000, Invitrogen, Cat# A-21240; Cat# A-21244; Cat# A-21042; Cat# A-21123) and Cy3-conjugated (1:800, Jackson ImmunoResearch, Cat#615-165-214; Cat#111-165-045) secondary antibodies were also used in this study.

Viral Vector Production

Following lentivirus plasmids were used in the study: VP plasmid expressing GFP protein and Cre recombinase under a Human *VCAM1* promoter, H1-GFP plasmid expressing GFP under a SV40 promoter, and two packaging plasmids pLnV and pVSVG, which are helper plasmids in a three-plasmid system to

generate viral stocks. VP and H1-GFP lentivirus were generated, respectively. In brief, all plasmids were firstly expanded in DH5α (Tiangen, China). And lentivirus was packaged subsequently in HEK293FT cell line by means of PEI transfection process (McSweeney and Mao, 2015), with the combination of VP, pLnV and pVSVG or H1-GFP, pLnV and pVSVG. Virus-containing supernatant was harvested 72h after transfection and concentrated by one round of ultracentrifugation (26,000g, 4h, 4 °C). Resuspend viral pellet in aseptic phosphate buffer (PBS) overnight and pipette up and down to fully dissolve. Virus suspensions were stored at -80 °C until use and were briefly centrifuged and kept on ice immediately before usage.

Stereotaxic Surgery

Before the stereotaxic injections were performed, the animals were deeply anaesthetized with a mixture of Hypnorm (0.5 mg/kg/ml) and Dormicum (5 mg/kg/ml). The mice were placed on a stereotactic apparatus and kept on a heating pad to maintain body temperature during surgery. The lentivirus was then bilaterally injected into the DG (coordinates relative to bregma: -2.10 mm anterioposterior, ±1.10 mm mediolateral, and -2.50 mm dorsoventral). For each injection, 1 µl virus solution was injected into the target site with a microinjector connected to a customized borosilicate glass microcapillary tip and an automated injection pump (rate = 0.2 µl/min). After surgery, the mice were placed on a heating pad until they awoke and were monitored and weighed daily.

Gene Expression Analysis

Total RNA of proliferative and quiescent NSCs were purified with The RNeasy Plus Mini Kit (QIAGEN,USA), and cDNA was then synthesized using the FastQuant RT Kit (with gDNase) (Tiangen, China) according to the manufacturer's protocol. For all target genes analysis, qPCR was performed on 8-well tubes. The amplification mixture consisted of PowerUp SYBR green master mix (Applied Biosystems, USA), 1 µM forward primer, 1 µM reverse primer, approximately 10 ng of cDNA template and ddH_2O. Thermal cycling was carried out with a 10 min denaturation step at 95 °C, followed by 40 two-step cycles: 15 s at 95 °C, 60 s at 62 °C. Finally, melt curve analysis was carried out to confirm the specific amplification of a target gene and absence of primer dimers. All reactions ran in triplicate. GAPDH was the endogenous reference. Data were acquired by Quantstudio Design&analysis System. Change fold in mRNA was calculated by the delta delta Ct methods. Primers used are listed in Table S2.

Adult Neural Stem Cell Culture

Briefly, DG tissue was dissected under a dissecting microscope. Minced DG tissue was then digested with a Papain (1 mg/ml, Worthington Biochemical Corporation) and DNase (0.01 mg/ml, Sigma-Aldrich) mixture for 30 min in a 37 ºC oven. Mechanical dissociation was then performed using a micropipette to yield a single-cell suspension. Cells were subjected to Percoll (Sigma; GE17-0891-01) density gradient centrifugation at 450g for 15 min without braketo remove debris. Dissociated cells were plated in a 24-well plate at a density of 2 DGs/well.

To induce active NSCs (aNSCs) or cell expansion, cells were cultured as neurospheres in proliferation medium DMEM/F12 (Hyclone) containing 1X B27 (Life Technologies), 1X N2 (Life Technologies), 1 µM N-acetyl-L cysteine (NAC, Amresco), 1 mM sodium pyruvate (Amresco), 20 ng/ml recombinant human epidermal growth factor (hEGFLife Technologies), and 20 ng/ml recombinant human fibroblast growth factor-basic (hFGF, Life Technologies). To induce quiescent NSCs (qNSCs), cells were cultured in quiescent medium (DMEM/F12 containing 1X B27, 1X N2, 1 µM NAC, 1 mM sodium pyruvate, 20 ng/ml hFGF, and 50 ng/ml bone morphogenetic protein 4 (BMP4, R&D Systems) as previously described (Knobloch et al., 2017).

To induce active or quiescent NSCs, the following process was carried out. In general, after NSCs were harvested from the adult mouse DG, they were cultured in proliferation medium for 2 days. On the third day, the proliferation medium was maintained to induce active NSCs or replaced with quiescent medium to induce quiescent NSCs. The cells were cultured in proliferation or quiescent medium for 1 week, and the number and diameter of neurospheres were evaluated for phenotype confirmation. Half of the medium was replaced every 2 days. For all in vitro experiments involving NSCs, cells were plated in 24-well cell culture dishes or on glass coverslips precoated with poly-D-lysine (PDL, Sigma-Aldrich). All experiments were performed with a minimum of three wells per condition and repeated at least 3 times.

Microscopic Image Quantification and Analysis

Immunofluorescent images were acquired using a laser scanning confocal microscope (Leica TCS SP8). Confocal Z-stack images were captured, and the X-Y area included the upper and lower blades of the DG. Images were viewed and counted using LAS AF Lite software (Leica) and analyzed using ImageJ software. Figures were produced with Photoshop CC 14.0 (Adobe).

For acute staining, cell number quantification was performed in triple-stained coverslips. Cells labeled with NSC markers (GFAP, NES, and SOX2) were analyzed for the co-expression of VCAM1. The number of marker-positive cells (at least 1000 cells) was counted for each group. Accord to the mouse brain atlas, brain sections located between -1.60 mm and -2.60 mm from bregma were considered the dorsal DG, while sections located between -2.60 mm and -3.60 mm from bregma were considered the ventral DG.
To analyze the numbers and percentages of cells in the brain sections, the number of marker-positive cells (at least 70 cells) was counted for each experimental group. To analyze the clones in the brain sections, the location of cells in relation to all the surrounding cells was assessed to determine lineage relationships. At least 30 clones were counted for each time point. To compare cell density between the dorsal and ventral DG, cell density was calculated by dividing the total number of counted marker positive cells by the area of interest (SGZ+GCL).

Western Blot

HEK293FT and bEND.3 cells growing in 6-well plates were collected, homogenized, and centrifuged according to standard protocols. Proteins were extracted for immunoblot analysis and probed with antibodies against VCAM1 (1:1000, Abcam, Cat#ab134047) and GAPDH (1:10000, Bioworld, Cat#AP0063) and fluorescent dye-conjugated secondary antibodies.

The Morris Water Maze Task

The task was carried out as described by Charles V Vorhees and Michael T Williams (Vorhees and Williams, 2006). The Morris Water Maze is an apparatus consist of an open circular pool and an overhead video camera. The pool filled with water at 20-22 °C, and was dyed by TiO2, a white non-toxic material. Around the outside of the pool 'extra maze cues' were displayed. 'North' was artificially determined and the pool was divided up equally into four quadrants: 'Northeast' (NE), 'Northwest' (NW), 'Southeast' (SE), and 'Southwest' (SW). A hidden platform submerged 1 cm below the water surface was placed in the middle of the quadrant of NE, which was named the target quadrant.
In this experiment, mice at 2 to 3 months old were examined, including WT, E;V and N;V which were treated by TAM at P21. After pre-training, mice were trained to learn the fixed location of the invisible platform during the acquiring period. The time and path from start location to the hidden platform was recorded by the video camera and specific software named water maze (Actimetrics, USA). Each mouse was given four trials each day with randomly order of start locations which were listed in table S3. The maximum trial length of swimming was 60 s, and on-platform length was 30 s. The acquiring trial lasted 5 days in same environment. Twenty-four hours after this acquiring trial, a probe trial was carried out. In this trial, the platform was removed from the pool, and the mice were allowed to swim for 90 s in the pool starting from a new location, which was opposite to the target quadrant, and the time spent in each quadrant and the number of crossing the hidden platform was recorded as parameter of spatial memory.

Table S1. Primers for genotyping, related to Figure 6 and Figure 7.

Name	**Sequence (5' to 3')**
Vcam1-loxp-F	ATG CCT GTG AAG ATG GTC GC
Vcam1-loxp-R	GAA GCC CAT TGC ACA AAG TT
Cre-F	GCG GTC TGG CAG TAA AAA CTA TC
Cre-R	GTG AAA CAG CAT TGC TGT CAC TT
EMX-F	CTA GGC CAC AGA ATT GAA AGA TCT
EMX-R	GTA GGT GGA AAT TCT AGC ATC ATC C
Nestin-F	AAG GTG TGG TTC CAG AAT CG
Nestin-R	CTC TCC ACC AGA AGG CTG AG

Statistical Analysis

Data are presented as mean ± SEM or mean. Statistical comparisons were conducted using two-tailed unpaired Student's t-tests, one-way ANOVA, two-way ANOVA, and the Bonferroni post-hoc test when appropriate (using Microsoft Excel and GraphPad Prism7). When an unpaired Student's t-test was performed, Levene's test was conducted to compare variances, and Welch's correction was applied in cases of unequal variance Significance was set at $p < 0.05$.

Table S2. Primers for qPCR, related to Figure 3.

Name	Sequence (5' to 3')
mKi67-F	CAT CCA TCA GCC GGA GTC A
mKi67-R	TGT TTC GCA ACT TTC GTT TGT G
mSox2-F	GCG GAG TGG AAA CTT TTG TCC
mSox2-R	CGG GAA GCG TGT ACT TAT CCT T
mNestin-F	GCT GGA ACA GAG ATT GGA AGG
mNestin-R	CCA GGA TCT GAG CGA TCT GAC
mVcam1-F	AAT CCA CGC TTG TGT TGA GCT CTG
mVcam1-R	GCA CAA GTG GCC CAC TCA TTT T
mGapdh-F	GTG GAG TCC ACT GGC GTC TTC A
mGapdh-R	AGC AGA GGG GGC AGA GAT GAT G

Table S3. Spatial Start Positions in the Morris Watermaze Task, related to Figure 6 and Figure 7.

Day	Trial 1	Trial 2	Trial 3	Trial 4
1	W	S	SE	NW
2	SE	W	NW	S
3	NW	SE	S	W
4	S	NW	W	SE
5	W	SE	S	NW
6	SW			

W: west, E: east, S: sourth, N: north.

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