Stem Cell Reports

Supplemental Information

Reduced Adult Hippocampal Neurogenesis and Cognitive Impairments following Prenatal Treatment of the Antiepileptic Drug Valproic Acid

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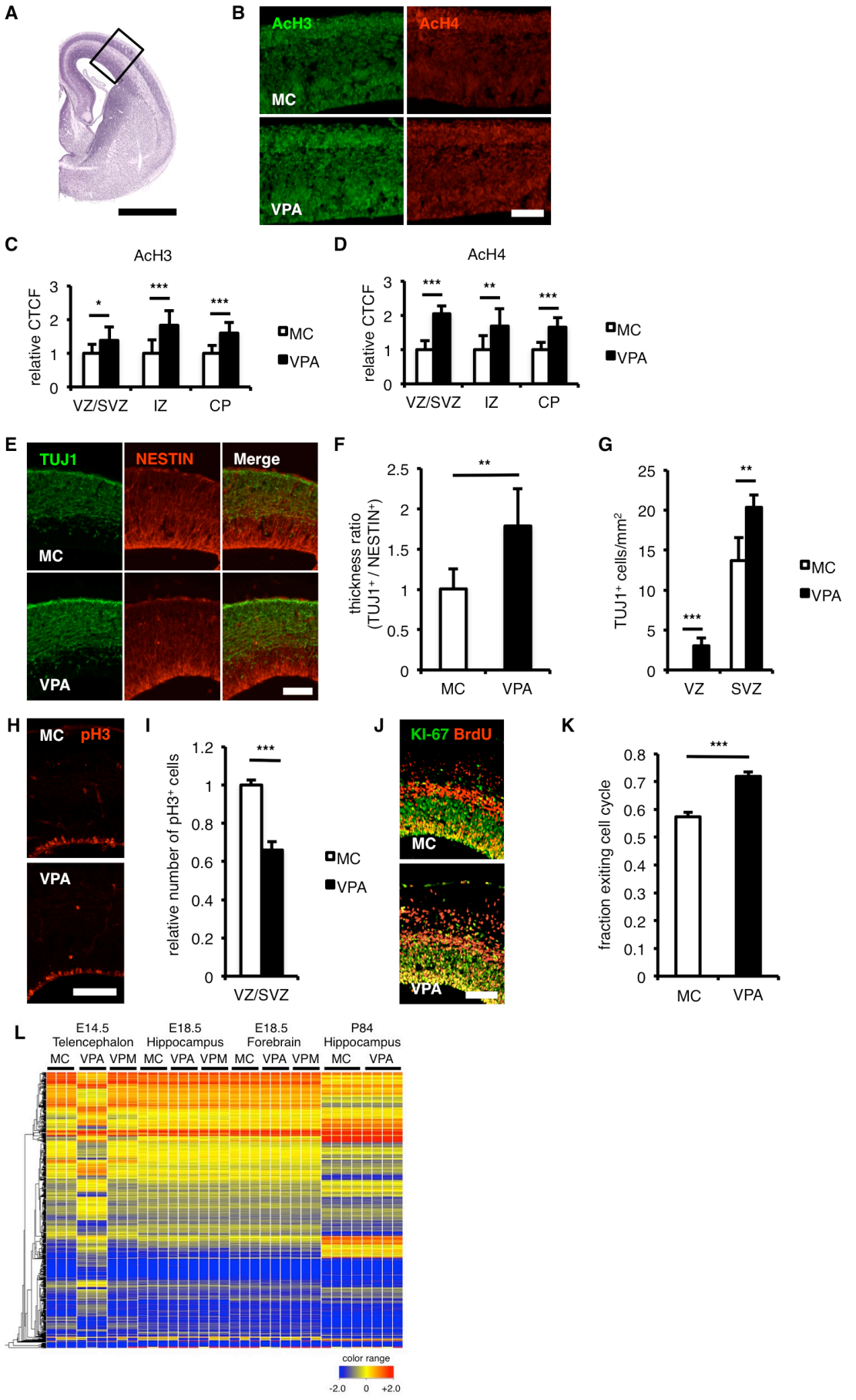
A
B
AcH3
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VZ/SVZ
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relative CTCF
VZ/SVZ
IZ
CP
MC
VPA
E
TUJ1
NESTIN
Merge
MC
VPA
F
thickness ratio (TUJ1+ / NESTIN+)
MC
VPA
G
TUJ1+ cells/mm2
VZ
SVZ
MC
VPA
H
MC
pH3
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I
relative number of pH3+ cells
VZ/SVZ
MC
VPA
J
KI-67 BrdU
MC
VPA
K
fraction exiting cell cycle
MC
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L
E14.5 Telencephalon
E18.5 Hippocampus
E18.5 Forebrain
P84 Hippocampus
MC VPA VPM MC VPA VPM MC VPA VPM MC VPA
color range
-2.0 0 +2.0

Figure S1, Related to Figure 1

Prenatal VPA treatment increases histone acetylation and enhances embryonic neurogenesis

(A) E15.5 forebrain sections from the region highlighted by the black rectangle were used for analysis. The image was modified from the Electronic Prenatal Mouse Brain Atlas. (B-D) VPA increases global histone acetylation of histones H3 (AcH3; green in B; quantification in C) and H4 (AcH4; red in B; quantification in D) in the embryonic forebrain. (E and F) The thickness ratio of regions positive for the immature neuron marker β-tubulin isotype III (TUJ1; green in E) and the NPC marker NESTIN (red in E) is increases in the cortex of VPA-treated mice (F), indicating that VPA enhances neurogenesis and depletes the NPC pool in the embryonic forebrain. (G) Quantification of $TUJ1^+$ cells in the VZ and SVZ in E. (H and I) VPA treatment reduces the number of mitotic NPCs ($pH3^+$; red in H) in the VZ and SVZ (I). (J) The fraction of cells exiting the cell cycle at E15.5 is higher in VPA-treated mice, as shown by an increased number of $BrdU^+$ cells (red) that were negative for KI-67 (green), 1 day after a single BrdU injection at E14.5. (K) Quantification of $BrdU^+$ cells that were negative for KI-67 compared to $BrdU^+$ cells that were still positive for KI-67 in J. (L) Hierarchical gene clustering of gene expression level shows that VPA transiently changed global gene expression through its HDAC-inhibiting activity. Red and blue bars indicate genes expressed at relatively high and low levels (more than 2.0-fold), respectively, in each stage and condition (n = 3 for each group at E14.5 and E18.5; n = 4 for each group at P84). See Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo), accession number GSE 42904, for complete data sets. MC, prenatal methylcellulose (vehicle); VPA, prenatal valproic acid; VPM, prenatal valpromide; VZ, ventricular zone; SVZ, subventricular zone; IZ,

intermediate zone; CP, cortical plate; CTCF, corrected total cell fluorescence. Data are represented as means. n = 3 for each group. Error bars are SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, two-tailed t-test. Scale bar is 1 mm in A, and 100 μm in B, E, H and J.

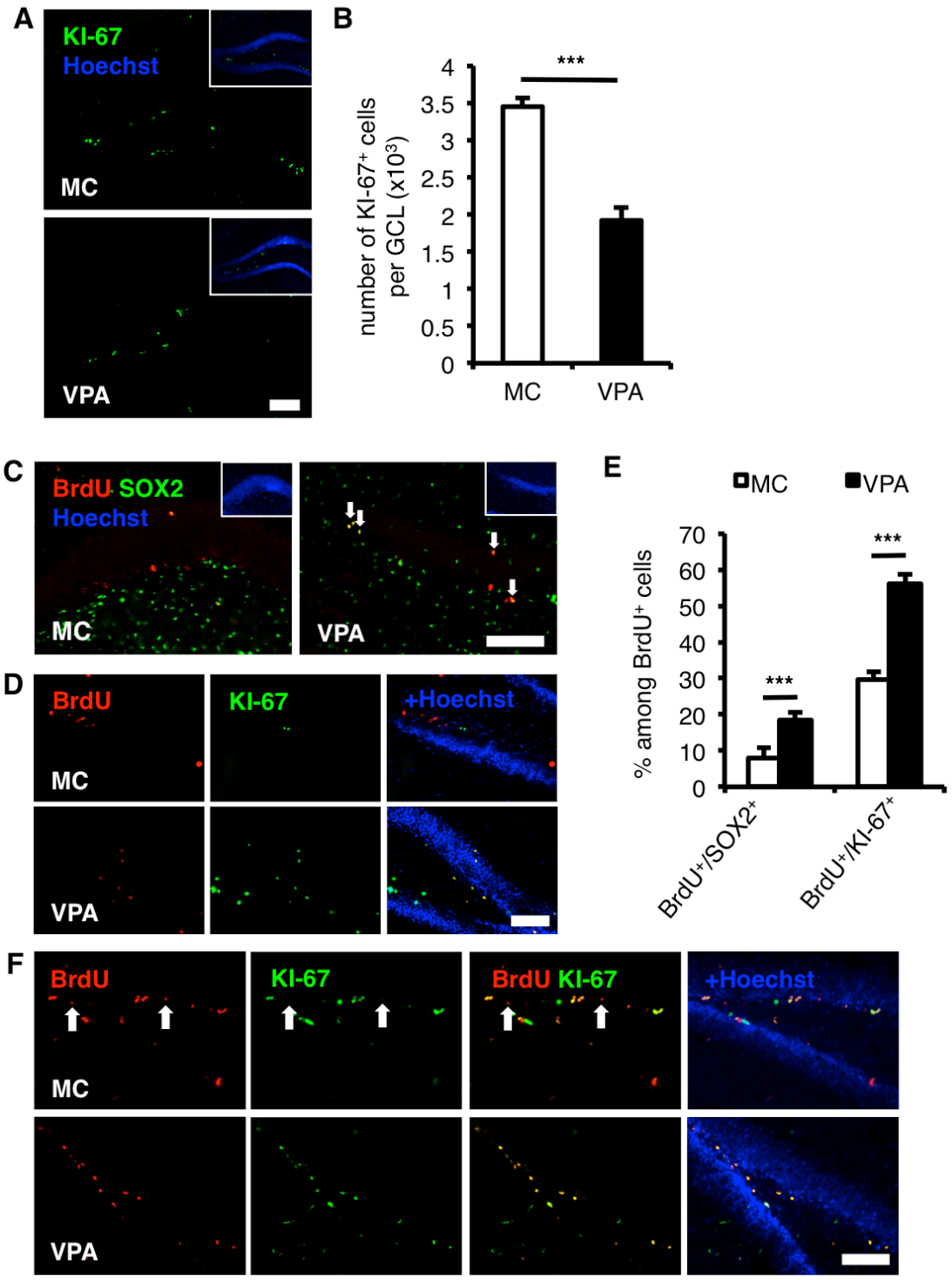

Figure S2, Related to Figure 3

VPA-treated mice have fewer proliferating cells in the DG of the hippocampus which differentiate more slowly than those in MC-treated mice

(A) Representative images of brain sections containing the DG and stained for KI-67

(green) and with Hoechst (blue) showed a reduction of proliferating cells in VPA-treated mice. (B) Quantification of KI-67^{+} cells in the granule cell layer (GCL). (C) Four weeks after the last BrdU injection, a higher proportion of BrdU-retaining cells (red) in VPA-treated mice (white arrows) still expressed the NPC marker SOX2 (green). (D) Four weeks after the last BrdU injection, a higher proportion of BrdU-retaining cells (red) in VPA-treated mice still expressed the proliferation marker KI-67 (green). (E) Quantification of BrdU^{+}SOX2^{+} and BrdU^{+}KI-67^{+} cells at 4 weeks after the last BrdU injection. (F) One day after the last BrdU injection, almost all BrdU^{+} cells (red) were still KI-67^{+} (green) in VPA-treated mice, while several BrdU^{+} but KI-67^{-} cells already existed in MC-treated mice (white arrows). MC, prenatal methylcellulose (vehicle); VPA, prenatal valproic acid. Data are represented as means. n = 3 for each group. Error bars are SD. ***$P < 0.001$, two-tailed t-test. Scale bars are 100 μm. See Figure 3A for experimental timeline.

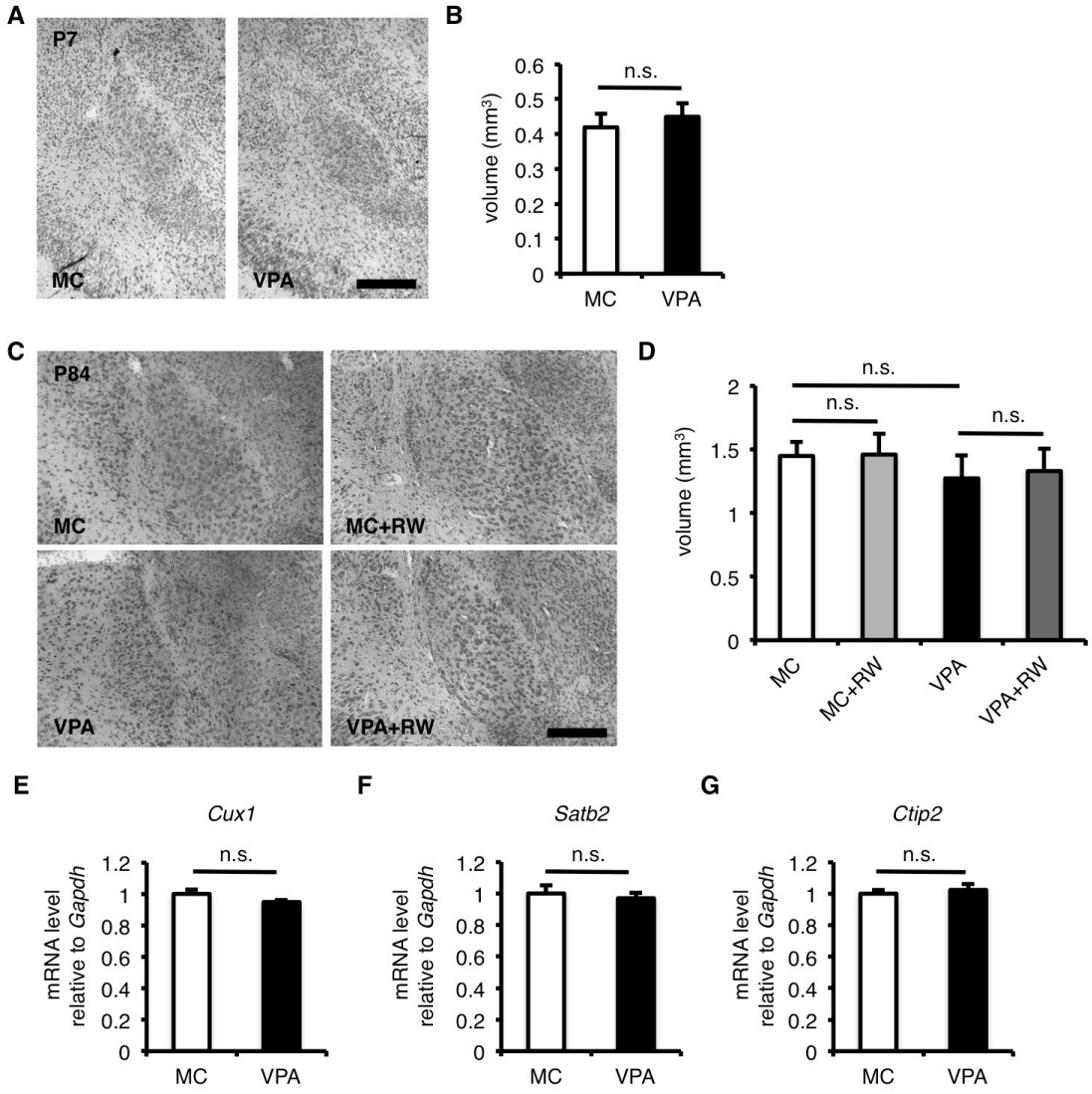

Figure S3, Related to Figure 3

The size of amygdala and expression levels of cortical-layer genes are not altered

(A-D) Representative images of brain sections containing amygdala stained with thionin (A and C), and the measurement of amygdala volume (B and D) showed no significant difference between MC- and VPA-treated mice. Postnatal voluntary running did not influence the amygdala size in both MC- and VPA-treated mice (D). (E-G) The expression levels of superficial-layer (*cut-like homeobox 1*, *Cux1*; E and *special AT-rich sequence binding protein 2*, *Satb2*; F), and deep-layer neuronal genes (*B-cell leukemia/lymphoma 11B*, *Bcl11b* also called *Ctip2*; G) are similar between MC- and VPA-treated mice in P84 cerebral cortex. MC, prenatal methylcellulose

(vehicle); MC+RW, prenatal methylcellulose and postnatal running; VPA, prenatal valproic acid; VPA+RW, prenatal valproic acid and postnatal running; *Gapdh, glyceraldehyde 3-phosphate dehydrogenase*. Data are represented as means. n = 3 for each group. Error bars are SD. n.s., not significantly different, two-tailed t-test. Scale bars are 250 μm.

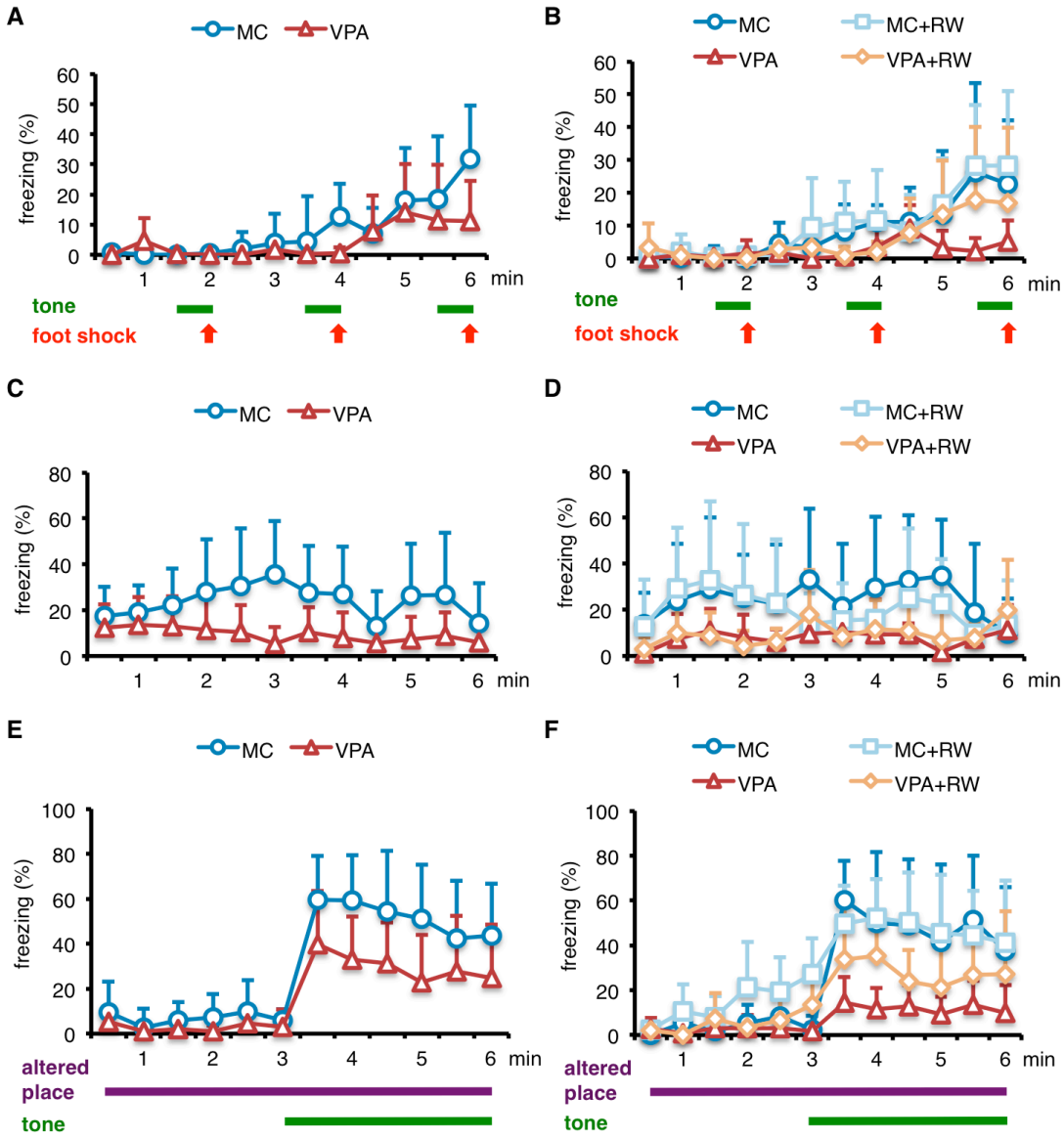

Figure S4, Related to Figure 2

Time course of freezing response in associative learning and memory tests

(A) VPA-treated mice (n = 12) displayed a lower freezing response than MC-treated mice (control; n = 12) in conditioned fear tests (day 1). (B) Voluntary running reinstates the freezing response in conditioned fear tests of VPA-treated mice (day 1; n = 7 for MC and VPA+RW; n = 8 for MC+RW and VPA). (C) VPA-treated mice (n = 12) displayed a lower freezing response than MC-treated mice (control; n = 12) in contextual fear tests (day 2). (D) Voluntary running does not reinstate the freezing

response in contextual fear tests of VPA-treated mice (day 2; n = 7 for MC and VPA+RW; n = 8 for MC+RW and VPA). (E) VPA-treated mice (n = 12) displayed a lower freezing response than MC-treated mice (control; n = 12) in cued fear tests (day 3). (F) Voluntary running reinstates the freezing response in cued fear tests of VPA-treated mice (day 3; n = 7 for MC and VPA+RW; n = 8 for MC+RW and VPA). MC, prenatal methylcellulose (vehicle); MC+RW, prenatal methylcellulose and postnatal running; VPA, prenatal valproic acid; VPA+RW, prenatal valproic acid and postnatal running. Data are represented as means. Error bars are SD.

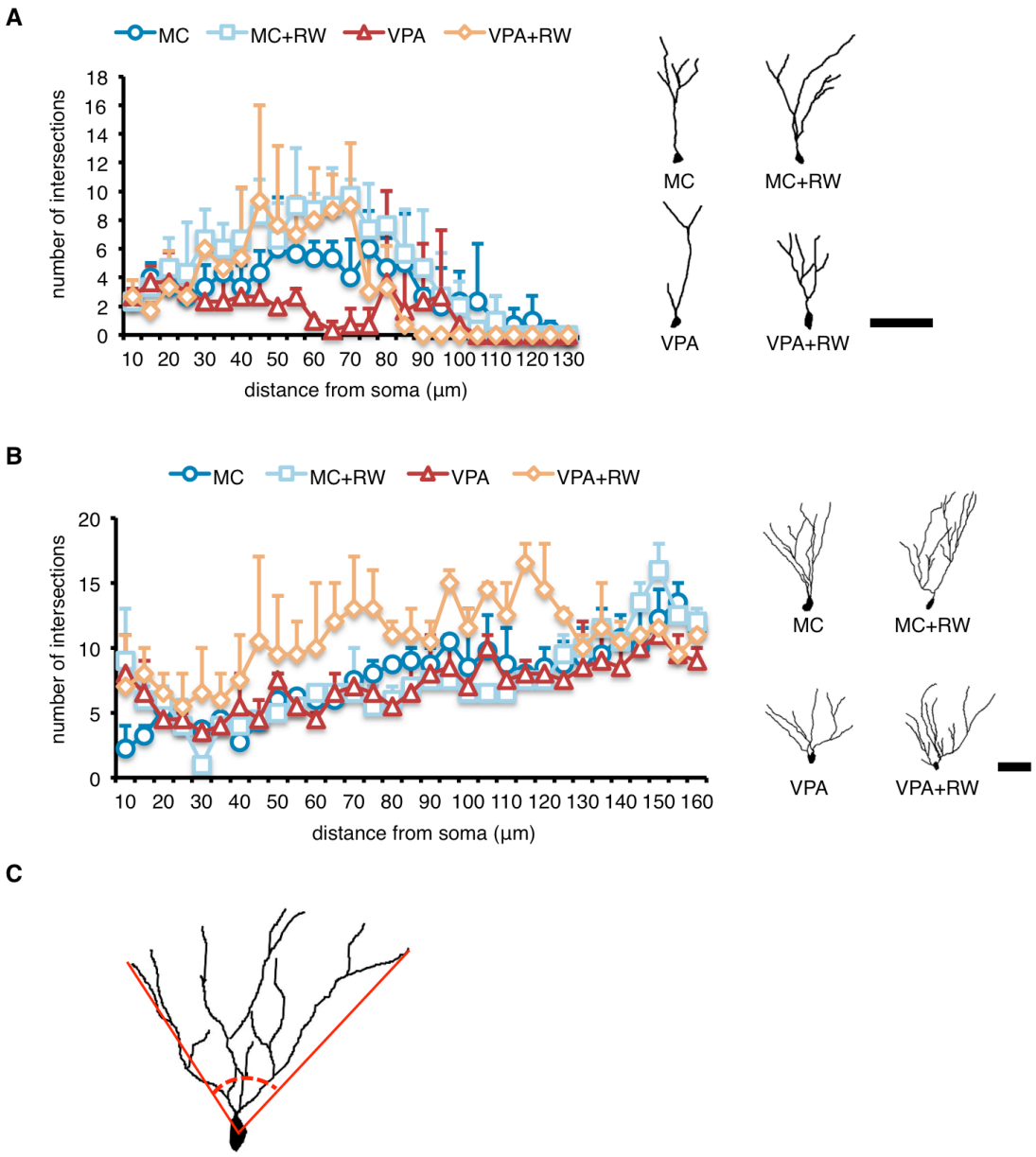

Figure S5, Related to Figure 5

Morphometry of DG neurons

(A) Sholl analysis showed that DCX^+ immature neurons in VPA-treated mice have a lower dendritic complexity than MC-treated mice, and voluntary running increases dendritic complexity in both MC- and VPA-treated mice. (B) Sholl analysis showed that Golgi-Cox stained neurons in VPA-treated mice have a similar dendritic complexity to MC-treated mice, and voluntary running increases dendritic complexity

in VPA-treated mice. The diagrams on the right in (A) and (B) shows representative neurons for each treatments. (C) Graphical example of maximal dendritic span measurement used for Figures 5G-5I. The solid red line represents the maximal dendritic span and dashed red line represents the angle that was used for analysis. MC, prenatal methylcellulose (vehicle); MC+RW, prenatal methylcellulose and postnatal running; VPA, prenatal valproic acid; VPA+RW, prenatal valproic acid and postnatal running. Data are represented as means. n = 3 for each group. Error bars are SD. Scale bar is 50 μm in A, and 100 μm in B.

Table S1, Related to Figure 2

Summary of behavior test results on 12-13-week-old male mice

Behavior Test	MC[a]	VPA[a]
Open Field		
- total distance (cm)	3648.30 ± 513.55	2436.49 ± 407.28*
- total center (sec)	94.33 ± 37.38	77.83 ± 48.24
- average speed (cm/sec)	4.79 ± 0.85	4.06 ± 0.67
- moving speed (cm/sec)	8.92 ± 0.66	8.10 ± 0.49
- move episode (times)	187.17 ± 11.37	184.17 ± 11.77
- total movement (times)	320.04 ± 40.39	299.71 ± 39.02
- distance per movement (cm)	15.33 ± 2.63	13.21 ± 1.93*
- duration per movement (sec)	1.72 ± 0.20	1.62 ± 0.17
Light/Dark Transition		
- dark distance (cm)	830.88 ± 122.36	639.25 ± 66.66***
- light distance (cm)	312.03 ± 127.66	237.76 ± 120.83
- dark time (sec)	227.92 ± 29.47	224.33 ± 31.14
- light time (sec)	85.21 ± 31.02	84.75 ± 30.13
- number of transitions (times)	14.00 ± 5.70	8.42 ± 4.58*
- latency to enter light (sec)	83.33 ± 68.79	98.00 ± 48.26
Elevated Plus Maze		
- total distance (cm)	1591.45 ± 263.67	1372.97 ± 235.90*
- open arms time (sec)	181.46 ± 56.73	196.00 ± 46.52
- closed arms time (sec)	418.54 ± 56.73	404.00 ± 46.52
- open arms entry (times)	4.75 ± 3.47	5.42 ± 3.37
- closed arms entry (times)	28.33 ± 7.28	21.83 ± 4.28*
- total entry (times)	33.08 ± 8.27	27.25 ± 6.40
Y-Maze		
- total distance (cm)	1365.93 ± 228.01	1095.63 ± 154.07**
- total entry (times)	16.92 ± 3.15	12.66 ± 2.42***
- alternation rate (% correct)	66.15 ± 13.01	50.51 ± 21.18*
Fear Conditioning		
- conditioning (% freezing)	8.31 ± 5.81	4.40 ± 4.10*
- contextual (% freezing)	23.97 ± 12.79	9.32 ± 5.39**
- cued (% freezing)	51.81 ± 18.46	29.91 ± 18.03**
- moving speed without foot shock (cm/sec)	0.90 ± 0.40	1.00 ± 0.40
- moving speed with foot shock (cm/sec)	11.40 ± 3.10	8.10 ± 2.60
Prepulse Inhibition		
- 80/120 dB (% startle)	31.55 ± 23.59	12.21 ± 26.83
- 85/120 dB (% startle)	28.82 ± 22.73	24.15 ± 22.03
- 90/120 dB (% startle)	38.82 ± 20.95	34.35 ± 21.94
- 95/120 dB (% startle)	57.35 ± 17.32	41.02 ± 19.23*
- 100/120 dB (% startle)	74.71 ± 12.16	65.66 ± 18.38
- 105/120 dB (% startle)	90.88 ± 3.90	83.24 ± 11.66

Tail Suspension (% immobility)	53.61 ± 26.07	54.53 ± 26.82
Thermal Hyperalgesia		
- left paw withdrawal latency (sec)	9.37 ± 1.24	9.37 ± 2.04
- right paw withdrawal latency (sec)	8.70 ± 1.71	9.37 ± 2.02
Tactile Allodynia		
- left paw, 0.02g (allodynia score)	0.08 ± 0.31	0.25 ± 0.26
- right paw, 0.02g (allodynia score)	0.17 ± 0.33	0.08 ± 0.19
- left paw, 0.16g (allodynia score)	0.33 ± 0.33	0.08 ± 0.23
- right paw, 0.16g (allodynia score)	0.08 ± 0.23	0.08 ± 0.23

[a] n = 12 mice.

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, two-tailed t-test.

MC, prenatal methylcellulose (vehicle); VPA, prenatal valproic acid.

Table S2, Related to Figure 4

Summary of behavior test results and two-way ANOVA analysis with or without postnatal running on 12-13-week-old male mice

Behavior Test	MC[a]	MC+RW[b]	VPA[b]	VPA+RW[a]	Two-way ANOVA factors		
					Prenatal treatment	Postnatal activity	Interaction
Open Field							
- total distance (cm)	2910.14 ± 195.27	2843.67 ± 674.96	2634.54 ± 447.83	2884.70 ± 519.33	$F(1, 26) = 0.46$, $p = 0.50$	$F(1, 26) = 0.25$, $p = 0.62$	$F(1, 26) = 0.75$, $p = 0.39$
- total center (sec)	162.57 ± 77.93	223.31 ± 87.23	151.00 ± 97.77	183.36 ± 65.68	$F(1, 26) = 0.89$, $p = 0.35$	$F(1, 26) = 2.30$, $p = 0.14$	$F(1, 26) = 0.21$, $p = 0.65$
- average speed (cm/sec)	4.84 ± 0.32	4.74 ± 1.12	4.39 ± 0.75	4.80 ± 0.86	$F(1, 26) = 0.47$, $p = 0.50$	$F(1, 26) = 0.26$, $p = 0.62$	$F(1, 26) = 0.73$, $p = 0.40$
- moving speed (cm/sec)	8.96 ± 0.41	9.73 ± 0.91	8.12 ± 0.57	9.43 ± 0.80	$F(1, 26) = 6.03$, $p < 0.05$	$F(1, 26) = 16.07$, $p < 0.001$	$F(1, 26) = 1.07$, $p = 0.31$
- move episode (times)	196.71 ± 12.22	173.50 ± 21.23	180.75 ± 13.36	178.43 ± 10.33	$F(1, 26) = 0.71$, $p = 0.41$	$F(1, 26) = 5.33$, $p < 0.05$	$F(1, 26) = 3.56$, $p = 0.07$
- total movement (times)	324.36 ± 16.72	291.94 ± 55.80	323.62 ± 38.70	305.07 ± 41.72	$F(1, 26) = 0.27$, $p = 0.61$	$F(1, 26) = 2.84$, $p = 0.10$	$F(1, 26) = 0.21$, $p = 0.65$
- distance per movement (cm)	14.81 ± 1.16	16.41 ± 3.30	14.56 ± 2.05	16.10 ± 2.20	$F(1, 26) = 0.20$, $p = 0.66$	$F(1, 26) = 3.34$, $p = 0.08$	$F(1, 26) = 0.001$, $p = 0.97$
- duration per movement (sec)	1.65 ± 0.08	1.69 ± 0.21	1.79 ± 0.15	1.71 ± 0.17	$F(1, 26) = 1.83$, $p = 0.19$	$F(1, 26) = 0.13$, $p = 0.72$	$F(1, 26) = 0.76$, $p = 0.39$
Light/Dark Transition							
- dark distance (cm)	857.43 ± 53.37	820.00 ± 216.94	672.85 ± 122.91**	682.67 ± 148.20**	$F(1, 26) = 8.55$, $p < 0.01$	$F(1, 26) = 0.06$, $p = 0.80$	$F(1, 26) = 0.19$, $p = 0.67$
- light distance (cm)	353.29 ±	294.43 ±	339.70 ±	288.81 ±	$F(1, 26) = 0.01$,	$F(1, 26) = 1.21$,	$F(1, 26) = 0.01$,

	117.37	117.09	173.78	124.93	$p = 0.91$	$p = 0.28$	$p = 0.91$
- dark time (sec)	211.29 ± 24.58	235.31 ± 27.17	203.75 ± 51.36	224.07 ± 41.45	$F(1, 26) = 0.61$, $p = 0.44$	$F(1, 26) = 2.54$, $p = 0.12$	$F(1, 26) = 0.02$, $p = 0.89$
- light time (sec)	101.36 ± 26.82	76.12 ± 27.86	111.06 ± 53.21	86.00 ± 44.39	$F(1, 26) = 0.62$, $p = 0.44$	$F(1, 26) = 2.96$, $p = 0.10$	$F(1, 26) = 0.001$, $p = 0.99$
- number of transitions (times)	13.29 ± 4.39	14.62 ± 7.03	11.37 ± 5.63	12.14 ± 5.90	$F(1, 26) = 1.12$, $p = 0.30$	$F(1, 26) = 0.24$, $p = 0.63$	$F(1, 26) = 0.02$, $p = 0.89$
- latency to enter light (sec)	37.14 ± 14.46	77.75 ± 58.32	119.00 ± 197.46	76.71 ± 96.32	$F(1, 26) = 0.90$, $p = 0.35$	$F(1, 26) = 0.001$, $p = 0.99$	$F(1, 26) = 0.94$, $p = 0.34$
Y-Maze							
- total distance (cm)	733.94 ± 217.46	681.06 ± 298.66	595.24 ± 350.61	578.36 ± 176.34	$F(1, 26) = 1.40$, $p = 0.25$	$F(1, 26) = 0.12$, $p = 0.73$	$F(1, 26) = 0.03$, $p = 0.86$
- total entry (times)	9.29 ± 2.98	8.50 ± 3.46	7.12 ± 4.05	7.14 ± 1.57	$F(1, 26) = 2.19$, $p = 0.15$	$F(1, 26) = 0.11$, $p = 0.75$	$F(1, 26) = 0.12$, $p = 0.73$
- alternation rate (% correct)	59.66 ± 20.13	69.96 ± 16.78	26.37 ± 18.94**	66.86 ± 12.82	$F(1, 26) = 9.76$, $p < 0.01$	$F(1, 26) = 15.85$, $p < 0.001$	$F(1, 26) = 5.59$, $p < 0.05$
Fear Conditioning							
- conditioning (% freezing)	8.51 ± 6.83	9.84 ± 7.33	2.38 ± 1.73*	5.80 ± 5.23	$F(1, 26) = 6.38$, $p < 0.05$	$F(1, 26) = 1.30$, $p = 0.26$	$F(1, 26) = 0.25$, $p = 0.62$
- contextual (% freezing)	24.54 ± 20.61	20.00 ± 13.96	7.71 ± 4.39**	9.62 ± 6.37**	$F(1, 26) = 8.30$, $p < 0.01$	$F(1, 26) = 0.08$, $p = 0.78$	$F(1, 26) = 0.47$, $p = 0.50$
- cued (% freezing)	48.25 ± 26.13	47.22 ± 16.02	11.95 ± 5.06**	28.09 ± 16.74	$F(1, 26) = 20.03$, $p < 0.001$	$F(1, 26) = 1.43$, $p = 0.24$	$F(1, 26) = 1.85$, $p = 0.19$

[a] n = 7 mice.

[b] n = 8 mice.

*$P < 0.05$, **$P < 0.01$, two-tailed t-test to MC.

MC, prenatal methylcellulose (vehicle); MC+RW, prenatal methylcellulose and postnatal running; VPA, prenatal valproic acid; VPA+RW, prenatal valproic acid and postnatal running.

Supplemental Experimental Procedures

Animal Group and Treatment. Mice were randomly assigned to each group on embryonic day (E)6.5. The VPA and VPM groups received an oral administration of 300 mg/kg valproic acid (VPA; Sigma) and valpromide (VPM; Wako), respectively, once a day on E12.5, E13.5, and E14.5. The MC group received an oral administration of identical volumes of 0.5% (w/v) MC (Wako) once a day on the same days. Pups from the MC and VPA groups were weaned at postnatal day (P)28 and 4 male mice per litter were randomly selected and housed in one cage. Four groups were designated as MC (pups from an MC-treated mother), MC+RW (pups from an MC-treated mother, with running wheel), VPA (pups from a VPA-treated mother), and VPA+RW (pups from a VPA-treated mother, with RW). MC+RW and VPA+RW mice were housed with a RW (Fast-Trac, BioServ) and were allowed to run freely in their cage immediately after weaning. To analyze adult neurogenesis, 100 mg/kg BrdU was injected intraperitoneally for 7 consecutive days starting on P84. Mice were sacrificed one day (proliferation group) or 4 weeks (differentiation group) after the last BrdU injection. For behavioral and electrophysiological analysis, separate sets of 12-13-week-old male mice with the same group designations were used.

Immunohistochemistry. Mice were anesthesized and perfused with PBS followed by 4% PFA in PBS on the indicated days. The brain was dissected, and postfixed overnight in the same fixative at 4 °C. For cryosectioning, fixed tissues were cryoproctected in 10% sucrose in PBS overnight at 4 °C, then in 20% sucrose in PBS overnight at 4 °C, and embedded in OCT compound (Tissue Tek). Cryostat sections

(15 μm) of embryonic and P7 brains were cut and affixed to MAS-coated glass slides (Matsunami Glass), while other postnatal brain sections (40 μm) were cut and suspended in PBS in 6- or 12-well plates (Nunc, Greiner). For some antibodies, antigen retrieval was conducted either by incubation in L.A.B. Solution (Polysciences) for 15 min at room temperature (RT) or by autoclaving in Target Retrieval Solution (Dako) for 15 min at 105 °C. For BrdU immunostaining, sections were incubated with 2 N HCl for 30 min at 37 °C. After 3 washes with PBS, the sections were incubated for 1 h at RT in blocking solution (PBS containing 3% FBS and 0.1% Triton X-100). They were then incubated overnight at 4 °C with the appropriate primary antibodies. The following primary antibodies were used: rabbit anti-acetyl-histone H3 (1:1000, Millipore 06-599), rabbit anti-acetyl-histone H4 (1:1000, Millipore 06-866), rabbit anti-β-tubulin isotype III (TUJ1; 1:1000, Covance PRB-435P), mouse anti-NESTIN (1:250, Millipore MAB353), goat anti-doublecortin (DCX; 1:500, Santa Cruz sc-8066), rabbit anti-doublecortin (DCX; 1:1000, Abcam ab18723), mouse anti-human KI-67 (1:500, BD Pharmingen 550609), rat anti-BrdU (1:1000, AbD Serotec OBT0030), mouse anti-phospho-histone H3 (pH3; 1:1000, Cell Signaling 9706), mouse anti-NEUN (1:100, Millipore MAB377), rabbit anti-S100β (1:500, Swant 37A), rabbit anti-SOX2 (1:500, Millipore AB5603), rabbit anti-TBR2 (1:500, Abcam ab23345), rabbit anti-IBA1 (1:500, Wako 019-19741), and rat anti-mouse CD68 (1:500, AbD Serotec MCA1957GA). After 3 washes with PBS, the sections were incubated for 2 h at RT with the appropriate secondary antibodies. The following secondary antibodies were used: Cy3-conjugated donkey anti-rabbit, Cy3-conjugated donkey anti-mouse, Cy3-conjugated donkey anti-goat, Cy3-conjugated donkey anti-rat, Cy5-conjugated donkey anti-rabbit, Cy5-conjugated donkey anti-mouse (all 1:500, Jackson ImmunoResearch), Alexa Fluor 488-conjugated donkey

anti-mouse, and Alexa Fluor 488-conjugated donkey anti-rabbit (both 1:500, Invitrogen). After 3 washes with PBS, nuclei were stained for 15 min at RT with Hoechst 33258 (Nacalai Tesque). For floating postnatal sections, after being washed with PBS, sections were affixed to MAS-coated glass slides (Matsunami Glass). Sections were mounted on cover slips with Immu-Mount (Thermo Scientific), and examined and photographed using an Axiovert 200M fluorescence microscope (Zeiss), an AF6000 fluorescence microscope (Leica), or an LSM 710 confocal microscope (Zeiss), equipped with a camera and appropriate epifluorescence filters. Cell fluorescence was measured using ImageJ (http://rsbweb.nih.gov/ij), and corrected total cell fluorescence (CTCF) was calculated as described previously (Burgess et al., 2010; Gavet and Pines, 2010). Ten cells per area were picked randomly in a section and 3 mice from each group were used for analysis.

Cell counts and volume measurements. Cell counting within the indicated areas was conducted manually using an Axiovert 200M fluorescence microscope (Zeiss), an AF6000 fluorescence microscope (Leica), or an LSM 710 confocal microscope (Zeiss) equipped with a camera and appropriate epifluorescence filters. For BrdU counting in the adult neurogenesis experiment, the total number of BrdU-positive cells was counted in every sixth section (240 μm apart). BrdU-positive cells were counted throughout the rostro-caudal extent of the granule cell layer (GCL), and the derived numbers were multiplied by 6 (slice series) to obtain total cell number per GCL. For BrdU-double (or -triple) immunostaining with other markers, every twelfth section (480 μm apart) from the proliferation and differentiation groups was used and double (or triple) labeling was confirmed in three-dimensional reconstructions of z-series. From each mouse, 50 BrdU-positive cells dispersed throughout the GCL were

picked randomly and analyzed for double (or triple) immunoreactivity. Volume measurements of amygdala were done on basolateral amygdala (BLA) complex (the portion between the external capsule and the amygdalar capsule), which includes the lateral and basolateral nuclei. Area of BLA complex was manually traced by ImageJ (http://rsbweb.nih.gov/ij) in every twelfth section. Volume was estimated by multiplying the derived number with the interval thickness between serial sections (180 μm for P7, 480 μm for P84).

Gene expression analysis. These procedures were conducted according to the Percellome method (Kanno et al., 2006) to normalize mRNA expression values to sample cell numbers by adding external spike mRNAs to the sample in proportion to the genomic DNA concentration and utilizing the spike RNA quantity data as a dose-response standard curve for each sample. Brain samples were prepared from E14.5 (3 h after the final oral administration of the drugs), E18.5, and 12-week-old (P84) mice. Samples were homogenized and lysed in 500 ml of RLT buffer (QIAGEN). Two separate 10-μl aliquots were treated with DNAse-free RNase A (Nippon Gene) in a 1.5-ml tube for 30 min at 37 °C, followed by proteinase K (Roche) for 3 h at 55 °C, and then transferred to a 96-well black plate. PicoGreen fluorescent dye (Molecular Probes) was added to each well, and the plates were incubated for 2 min at 30 °C. The DNA concentration was measured using a 96-well fluorescence plate reader with excitation at 485 nm and emission at 538 nm. Lambda phage DNA (PicoGreen kit, Molecular Probes) was used as standard. The appropriate amount of spike RNA cocktail was added to the sample homogenates in proportion to their DNA concentration. Five independent *Bacillus subtilis* poly-A RNAs were included in the grade-dosed spike cocktail. Total RNAs were purified using an RNeasy Mini kit

(QIAGEN), according to the manufacturer's instructions. First-strand cDNAs were synthesized by incubating 5 μg of total RNA with 200 U SuperScript II reverse transcriptase (Invitrogen) and 100 pmol T7-$(dT)_{24}$ primer [5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-$(dT)_{24}$-3']. After second-strand synthesis, the double-stranded cDNAs were purified using a GeneChip Sample Cleanup Module (Affymetrix), according to the manufacturer's instructions, and labeled by *in vitro* transcription using a BioArray HighYield RNA transcript labeling kit (Enzo Life Sciences). The labeled cRNA was again purified using a GeneChip Sample Cleanup Module and treated with fragmentation buffer at 94 °C for 35 min. For hybridization to a GeneChip Mouse Genome 430 2.0 Array (Affymetrix), 15 μg of fragmented cRNA probe was incubated with 50 pM control oligonucleotide B2, 1x eukaryotic hybridization control (1.5 pM BioB, 5 pM BioC, 25 pM BioD and 100 pM Cre), 0.1 mg/ml herring sperm DNA, 0.5 mg/ml acetylated BSA and 1x manufacturer-recommended hybridization buffer in a 45 °C rotisserie oven for 16 h. Washing and staining were performed in a GeneChip Fluidics Station (Affymetrix) using the appropriate antibody amplification, washing and staining protocols. The phycoerythrin-stained arrays were scanned as digital image files, which were analyzed with GeneChip Operating Software (Affymetrix). The expression data were converted to copy numbers of mRNA per cell by the Percellome method, quality controlled, and analyzed using Percellome software (Kanno et al., 2006) and GeneSpring (Agilent Technologies). The GeneChip data have been deposited in the NCBI Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO series accession number GSE 42904.

For real-time qPCR, total RNAs were isolated from P84 cortex and hippocampus using Sepasol-RNA I Super G (Nacalai Tesque), and treated with DNase I (Promega).

cDNAs were synthesized from 1 μg total RNA with SuperScript VILO cDNA Synthesis Kit (Invitrogen) as recommended by the manufacturer. qRT-PCR was performed by MX3000p (Agilent Technologies) or StepOnePlus (Applied Biosystems) using KAPA SYBR FAST qPCR Master Mix Universal kit with ROX as a reference dye (KAPA Biosystems). The expression of target genes was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*). The gene-specific primers were as follows (5′-3′): *Cux1*: Cux1-S, TGATGGATATGAAGCGGATG; Cux1-AS, TCAGCTGGTGTTGTGGCTG; *Satb2*: Satb2-S, GGAGAATCTCTGCACCATC; Satb2-AS, GAGCTCTCCTTAGTTGGCTG; *Ctip2*: Ctip2-S, CCCGACCCTGATCTACTCAC; Ctip2-AS, TTCTCCTGCTTGGGACAGATG; *Bdnf*: Bdnf-S, CAGGAGTACATAACGGCCACC; Bdnf-AS, GTAGGCCAAGTTGCCTTGTCCGT; and *Gapdh*: Gapdh-S, ACCACAGTCCATGCC ATCAC; Gapdh-AS, TCCACCACCCTGTTGCTGTA.

Behavioral tests. Experimental apparatus and image analyzing software (Image OF4, Image LD2, Image EP2, Image FZ2, SR-9040, Image TS2 and Time YM2) were obtained from O'Hara & Co. Image OF4, Image LD2, Image EP2, Image FZ2, and Image TS2 were developed from the public domain software ImageJ. All experiments were performed sequentially using the same set of male mice and were conducted between 13:30 and 16:30. The level of background noise during behavioral testing was about 50 dB. After each trial, the apparatus was wiped clean.

Open field test. Locomotor activity was measured for 10 min using an open field apparatus made of white plastic (50 x 50 x 40 (H) cm). An LED light system was positioned 50 cm above the center of the field (50 lux at the center of the field). Total

distance travelled (cm), time spent in the central area (30% of the field) (sec), and frequency of movement were measured by Image OF4 (Tanemura et al., 2002).

Light/dark transition test. The apparatus used for the light/dark transition test consisted of a cage (21 x 42 x 25 (H) cm) divided into two chambers by a partition with an opening. One chamber was brightly illuminated (250 lux), whereas the other chamber was dark (2 lux). A mouse was placed in the dark area and allowed to move freely between the two chambers through the opening for 5 min. The latency for the first move into the light area, the total number of transitions and the time spent on each side were measured by Image LD2.

Elevated plus maze test. The plus-shaped apparatus consisted of four arms (25 x 5 cm) connected to a central square area (5 x 5 cm). Two opposed arms were enclosed with 20-cm high transparent walls and the other two were left open. The floor of the maze was made of white plastic and was elevated 60 cm above the room floor (200 lux at the center of the apparatus). A mouse was placed in the central square area of the maze, facing one of the arms, and its behavior was recorded for 10 min: the total distance traveled (cm), total time spent in open arms and the central square area (sec), and total number of entries into any of the arms were measured by Image EP2 (Tanemura et al., 2002).

Contextual/cued fear conditioning test. The apparatus consisted of a conditioning chamber (or a test chamber) (17 x 10 x 10 (H) cm) made of clear plastic with a ceiling and placed in a soundproof box. The chamber floor had stainless steel rods (2-mm diameter) spaced 5 mm apart for electric foot shock to the mouse. The soundproof box was made of white-colored wood, and was equipped with an audio speaker and light source (35 lux at the center of the floor). A CCD camera was positioned 20 cm above the ceiling of the chamber. During the conditioning trial (Day 1), mice were

placed individually in the conditioning chamber in the soundproof box and, after 90 sec, they were given three tone-shock pairings (30 sec of tone, 75 dB, 10 KHz followed by 3 sec of 0.1-mA electric shock at the end of the tone) separated by 90 sec. They were then returned to their home cage. The next day (Day 2), as a contextual fear test, they were returned to the conditioning chamber for 6 min without tone or shock. On the third day (Day 3), they were placed in a novel chamber, of different make and lacking stainless steel rods, which was situated in the soundproof box and, after 3 min, the conditioning tone only (no shock) was presented for 3 min (35 lux at the center of the floor). The freezing response of the mice was measured by Image FZ2 as a consecutive 2-sec period of immobility. Freezing rate (%) was calculated as [time freezing/session time] x 100 as described previously (Tatebayashi et al., 2002).

Prepulse inhibition test. Startle responses and prepulse inhibition (PPI) of startle responses were measured using an automatic startle reflex measurement system (SR-9040; O'Hara & Co.). A test session was started by placing a mouse in a Plexiglas cylinder, where it was left undisturbed for 10 min. The duration of white noise that was used as the startle stimulus was 40 msec for all trial types. The startle response was recorded by accelerometer for 140 msec (measuring the response every 1 msec) starting with the onset of the prepulse stimulus. The background noise level in each chamber was 70 dB. The peak startle amplitude recorded during the 140-msec sampling window was used as the dependent variable. A test session consisted of 6 trial types (two types for startle stimulus-only trials, and four types for prepulse inhibition trials). The intensity of the startle stimulus was 120 dB. The prepulse sound was presented 100 msec before the startle stimulus, and its intensity was either 80, 85, 90, 95, 100 or 105 dB. Six combinations of prepulse and startle stimuli were employed (80-120, 85-120, 90-120, 95-120, 100-120 and 105-120 dB). Six blocks of

the 6 trial types were presented in pseudorandom order such that each trial type was presented once within a block. The average inter-trial interval was 15 sec (range: 10-20 sec).

Tail suspension test. Mice were suspended 60 cm above the floor for 3 min by holding their tail. The immobility time during this period was scored. Immobility was defined as the absence of all movement except for those required for respiration, and was measured by Image TS2.

Y-maze alternation test. Exploratory activity was measured using a Y-maze apparatus made of clear plastic. The maze had 3 identical arms (40 x 9 x 16 (H) cm) at 120° to each other. The center platform was a triangle with 9-cm side-length. Each mouse was placed at the end of one arm, with its head directed to the walls, and allowed to freely explore the apparatus for 5 min. Sequential alternations in entering the arms were recorded by Time YM2.

Measurement of thermal hyperalgesia. A measurement of thermal hyperalgesia was performed as described previously (Imai et al., 2013) with minor modifications. To assess the sensitivity to thermal stimulation, each of the hind paws of the mice was tested individually using a thermal stimulus apparatus (model 33 Analgesia Meter; IITC/Life Science Instruments, Woodland Hills, CA, USA). The intensity of the thermal stimulus was adjusted to achieve an average baseline paw withdrawal latency of approximately 7 to 10 s in naive mice. Only quick hind paw movements (with or without licking of hind paws) away from the stimulus were considered to be a withdrawal response. The latency of paw withdrawal after the thermal stimulus was determined as the average of 3 trials per paw on each side.

Measurement of tactile allodynia. A measurement of tactile allodynia was performed as described previously (Imai et al., 2013) with minor modifications. To quantify the

sensitivity to a tactile stimulus, paw withdrawal in response to a tactile stimulus was measured using von Frey filaments (North Coast Medical, Inc., Morgan Hill, CA, USA) with a bending force of 0.02 g or 0.16 g. The von Frey filament was applied to the plantar surface of the hind paw for 3 sec, and this was repeated 3 times at intervals of at least 5 sec. Each of the hind paws was tested individually. Paw withdrawal in response to a tactile stimulus was evaluated by scoring as follows: 0, no response; 1, a slow and slight withdrawal response; 2, a slow and prolonged flexion withdrawal response (sustained lifting of the paw) to the stimulus; 3, a quick withdrawal response away from the stimulus without flinching or licking; 4, an intense withdrawal response away from the stimulus with brisk flinching and/or licking. The latency of paw withdrawal in response to a tactile stimulus was determined as the average of 2 trials per paw on each side.

Golgi-Cox staining. Golgi-Cox staining was done using a Rapid GolgiStain Kit (FD NeuroTechnologies) according to the protocol provided by the manufacturer, with minor modifications. The brain was removed from the skull without any perfusion, and then sectioned (100 μm) on a cryostat. After sections were mounted on cover slips, they were examined and photographed using an Axiovert 40 CFL microscope (Zeiss) equipped with a camera.

Cell and tissue morphometrics. Analysis of DCX-positive immature neurons was conducted from the 3D reconstruction of Z stack confocal images. The 2D projection images were used for further analysis. Twelve cells in total (3 cells / section x 4 sections) were picked per mouse and 4 mice from each group were used for the analysis. Golgi-stained neurons were analyzed from 2D images. Twelve sparse and

well-impregnated cells (3 cells / section x 4 sections) were selected per mouse and 3 mice from each group were used. The thickness of the immunostained area was measured at 6 different locations per section; 3 mice from each group were used. Images were traced and analyzed manually using GIMP (http://www.gimp.org) and ImageJ (http://rsbweb.nih.gov/ij). Maximum dendritic span of DG neurons and of the basal portion of CA1 neurons was measured as the angle between the two fartherst ends of the dendritic arborization and the center of the soma (see Figure S5C in Supplemental Information). For the apical portion of CA1 neurons, the same dendritic span measurement was conducted but stratum radiatum-oriented dendrites were excluded from the analysis. Dendritic complexity was measured using the Sholl analysis plugin for ImageJ (http://labs.biology.ucsd.edu/ghosh/software/index.html).

Voltage-sensitive dye imaging. *Slice preparation.* Hippocampal slices (400 μm) were prepared from 12-13-week-old male mice, decapitated under deep ether or isoflurane anesthesia. The brains were removed and cooled in ice-cold artificial cerebrospinal fluid (ACSF; 124 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgSO_4$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, and 10 mM glucose, pH 7.4) bubbled with 95% O_2:5% CO_2 gas. After cooling for 5 min, the hippocampus was dissected out along with the surrounding cortex and sliced into 400-μm transverse sections with a vibratome (Leica VT-1200). Following incubation in gassed ACSF for 3–5 min, each slice was transferred onto a fine-mesh membrane filter (Omni Pore membrane filter, JHWP01300; Millipore), held in place by a thin Plexiglas ring (inner diameter, 11 mm; outer diameter, 15 mm; thickness, 1–2 mm). These slices were transferred to a moist chamber continuously supplied with a humidified O_2/CO_2 gas mixture. The temperature was held at 32 °C for 1 h, and after a further 1-h incubation at room

temperature the slices were stained for 25 min with 100 µl of VSD solution (0.2 mM di-4-ANEPPS (Molecular Probes) in 2.5% ethanol, 0.13% Cremaphor EL (Sigma), 1.17% distilled water, 48.1% FBS, and 48.1% ACSF). The slices were subjected to experiments after at least a 1-h incubation at room temperature following VSD washout.

Optical recording. The Plexiglas ring supporting each slice (see above) was placed in an immersion-type recording chamber. Slices were continuously perfused with prewarmed (31 °C) and oxygenated ACSF (bubbled with a 95% O_2:5% CO_2 gas mixture) at a rate of 1 ml/min. Custom laboratory-designed epifluorescence optics, used to view the slices during experiments, consisted of a custom-made objective lens (Olympus MYCAM 5x/0.6 WI; the final magnification of the system was x 5) and a Leica Microsystems MZ-APO (f = 55 mm ×1.0) projection lens. Excitation light was provided by a halogen lamp source (150 W; MHW-G150LR; Moritex) projected through an excitation filter ($\lambda = 530 \pm 10$ nm) and reflected onto the hippocampal slice by a dichroic mirror ($\lambda = 575$ nm). Emission fluorescence from the slice passed through an emission filter ($\lambda > 590$ nm) and was projected onto a C-MOS imager (MiCAM Ultima; BrainVision). The intensity of fluorescence emitted by the slice prior to stimulation was averaged and used as reference intensity (F_0). The fractional change in fluorescence [$\Delta F_{(t)} = F_{(t)} - F_0$] was normalized to the F_0 ($\Delta F/F_0$), and this value was used as the optical signal. Optical signals referred to below represent signals filtered in spatial and temporal dimensions with a digital Gaussian kernel of 5x5x3 (horizontal x vertical x temporal; $\sigma \approx 1$). We analyzed optical signals offline using a procedure developed for Igor Pro (WaveMetrics). At a wavelength of 610 nm, VSD fluorescence decreases in response to the depolarization of the cell membrane. To fit the polarity of the response to conventional membrane potential changes, we

expressed the optical signal in a polarity that matched the membrane potential change. For example, decreased fluorescence, which corresponds to depolarization, was represented as a positive deflection. Electrical stimulations (200 μA, bipolar 200 μsec) were applied with constant current pulses (A395, WPI) through a glass microcapillary tube (5 μm inner diameter; filled with ACSF) placed either on Schaffer collateral afferents in the CA3/CA1 border of CA1, on the granule cell layer to stimulate the mossy fiber pathway, or in the molecular layer of the upper blade in the dentate gyrus. The stimulation was applied with at least 20-sec intervals.

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