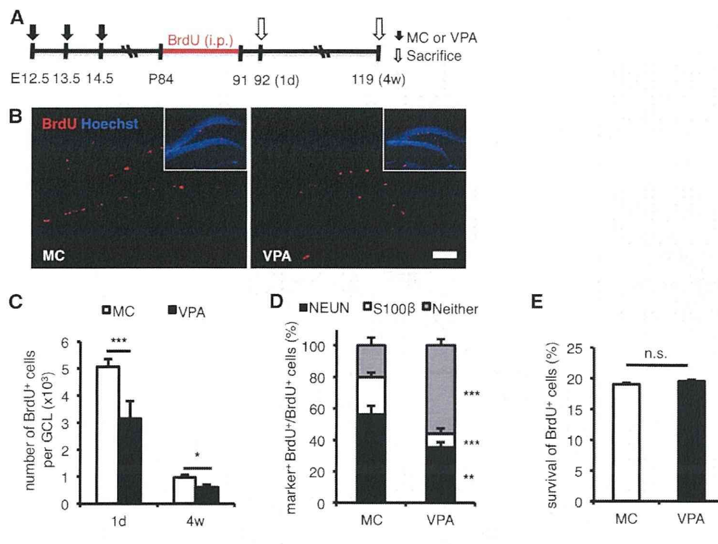

Figure 3. Prenatal VPA Treatment Has the Long-Term Effect on the Adult Neurogenesis

(A) Experimental timeline of prenatal VPA treatment and adult neurogenesis analysis. E, embryonic day; P, postnatal day; 1d, 1 day after the last intraperitoneal (i.p.) BrdU injection; 4w, 4 weeks after the last i.p. BrdU injection.

(B) Representative images of brain sections including the hippocampal DG stained for BrdU (red) and with Hoechst 33258 (blue),1 day after the last BrdU injection. See also Figure S2A for KI-67 staining.

(C) Quantification of $BrdU^+$ cells in the granule cell layer (GCL), 1 day (1d; n = 8 for each group) and 4 weeks (4w; n = 8 for each group) after the last BrdU injection, shows a reduction of $BrdU^+$ cells in the hippocampus. See also Figure S2B for quantification of $KI\text{-}67^+$ cells in the GCL.

(D) NPC differentiation into $NEUN^+$ neurons and $S100\beta^+$ astrocytes is impaired in VPA-treated mice, as shown by a reduced proportion of marker-positive and $BrdU^+$ cells among total $BrdU^+$ cells at 4 weeks after the last BrdU injection (n = 4 for each group).

(E) $BrdU^+$ cell survival is similar in VPA- and MC-treated mice. Quantification of $BrdU^+$ cell survival in each group as a percentage of $BrdU^+$ cells at 4w relative to $BrdU^+$ cells at 1d (n = 8 for each group).

MC, prenatal methylcellulose (vehicle); VPA, prenatal valproic acid. Data are represented as means. Error bars indicate the SD. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, n.s., not significantly different, two-tailed t test. Scale bar, 100 μm. See also Figures S2 and S3.

prenatal treatment and postnatal activity as factors, the results did not show a significant effect of postnatal activity on the performance of mice in hippocampus-dependent learning and memory tests (Table S2). This is mainly because voluntary running in MC-treated mice made no significant contribution to the performance in hippocampus-dependent learning and memory tests (conditioning: $F(1, 13) = 0.13$, $p = 0.72$; contextual: $F(1, 13) = 0.26$, $p = 0.62$; cued: $F(1, 13) = 0.01$, $p = 0.93$; Y-maze: $F(1, 13) = 1.17$, $p = 0.30$; one-way ANOVA). In addition, we found no significant interaction between prenatal drug treatment and postnatal activity in relation to the behavior of mice, except for the correct alternation in Y-maze ($F(1, 26) = 5.59$, $p < 0.05$, two-way ANOVA; Table S2). Therefore, it seems likely that only a certain level of adult neurogenesis is required for mice to perform normally in these hippocampus-dependent learning and memory tests, so that MC-treated mice with or without a RW showed no significant differences in these tests even though MC-treated mice with a RW displayed higher levels of neurogenesis than those without one. However, because the level of adult neurogenesis in the VPA-treated mice was below this threshold, the mice performed poorly in these tests; voluntary running improved their performance, probably by increasing adult neurogenesis in the hippocampus to above the threshold level.

We next sought to determine whether adult neurogenesis in the DG of VPA-treated mice generated normal or abnormal neurons. We found that DCX-positive immature neurons (Figures 5A and S5A) and Golgi-stained mature neurons (Figures 5B and S5B) in the DG of VPA-treated mice displayed an abnormal morphology with shorter total dendritic length (Figures 5D and 5E) and fewer dendrite processes toward the molecular layer, as shown by a wider maximum dendritic span compared to MC-treated mice (Figure 5G). The dendritic complexity of Golgi-stained neurons in VPA-treated mice, however, was similar to that of MC-treated mice (Figures 5F and S5B). Surprisingly, this abnormal morphology could also be overcome by voluntary running (Figures 5A, 5B, 5D, 5E, 5G, S5A, and S5B). Taken together, these observations suggest that the restoration of learning and memory deficiencies in VPA-treated mice through running is involved in increased neurogenesis, which gives rise to neurons with normal morphology.

Several mechanisms have been proposed to be responsible for the voluntary running-induced increase in adult hippocampal neurogenesis. Voluntary running can induce expression of neurotrophic factors such as *brain-derived neurotrophic factor* (*Bdnf*) in the hippocampus (van Praag,

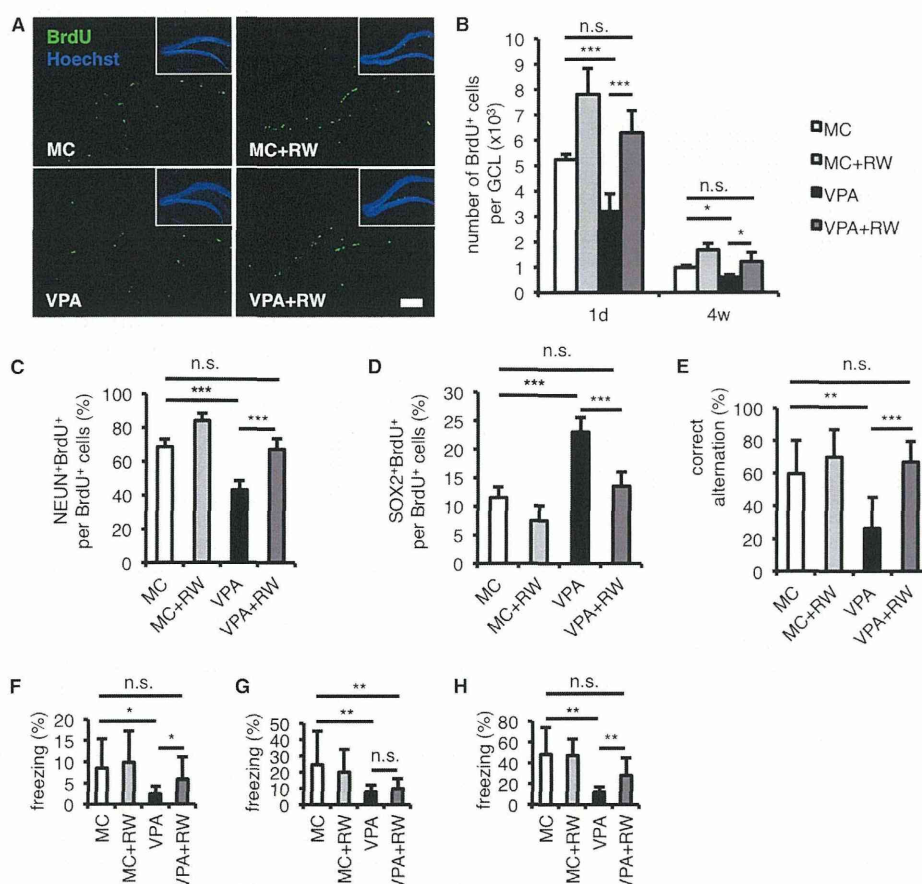

Figure 4. Voluntary Running Restores Adult Neurogenesis and Cognitive Deficiencies of VPA-Treated Mice

(A) Representative images of brain sections including the hippocampal DG stained for BrdU (green) and with Hoechst 33258 (blue), 1 day after the last BrdU injection. See Figure 2A for the experimental timeline.

(B) Quantification of $BrdU^+$ in the granule cell layer (GCL), 1 day (1d; n = 8 for each group) and 4 weeks (4w; n = 8 for each group) after the last BrdU injection, shows an increased number of $BrdU^+$ cells in the hippocampus after voluntary running.

(C and D) Voluntary running recovers the proportion of $BrdU^+$ cells that had differentiated into $NEUN^+$ neurons (C) and the ones that still expressed SOX2 (D) among total $BrdU^+$ cells at 4 weeks after the last BrdU injection (n = 4 for each group).

(E) Reduction of correct-arm alternation in Y-maze tests of VPA-treated mice is recovered by voluntary running (n = 7 for MC and VPA + RW; n = 8 for MC + RW and VPA).

(F–H) Voluntary running recovers the freezing response in conditioning (F; day 1) and in cued fear associative tests (H; day 3), but not in contextual fear associative tests (G; day 2; n = 7 for MC and VPA + RW; n = 8 for MC + RW and VPA). See also Figures S4B, S4D, and S4F for the time course of the freezing response and Table S2 for a summary of behavior data.

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 indicate the SD. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, n.s., not significantly different, two-tailed t test. Scale bar, 100 μm. See also Figure S4 and Table S2.

2009; Farmer et al., 2004), and *Bdnf* has been suggested to have an important role in adult neurogenesis, neuronal maturation, and dendrite arborization (Tolwani et al., 2002; Bekinschtein et al., 2011; Stranahan, 2011). Indeed, we found an increased level of *Bdnf* expression after running in both MC- and VPA-treated mice hippocampus

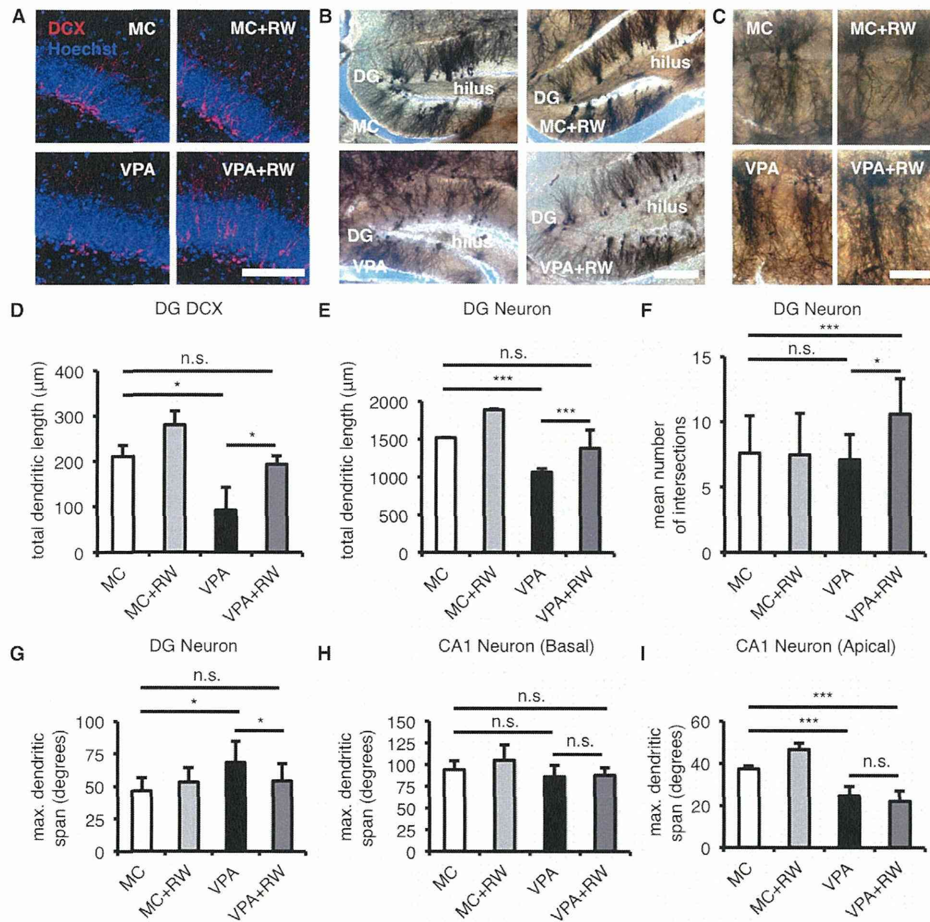

Figure 5. Voluntary Running Restores Neuronal Morphology in VPA-Treated Mice

(A) Impaired morphology of DCX^{+} young neurons in the DG of VPA-treated mice is recovered by voluntary running (n = 4 for each group). Scale bar, 100 μm.

(B) Impaired morphology of Golgi-Cox stained neurons in the DG of VPA-treated mice is recovered by voluntary running (n = 3 for each group). Note that voluntary running recovered non-molecular layer-oriented dendrites in VPA-treated mice to molecular layer-oriented ones. Scale bar, 100 μm.

(C) Impaired morphology of Golgi-Cox stained neurons in the CA1 of VPA-treated mice is not recovered by voluntary running (n = 3 for each group). Note that the less-ramified and straighter apical dendrites in VPA-treated mice could not be recovered by voluntary running. Scale bar, 50 μm.

(D–F) Voluntary running recovers total dendritic length of DCX^{+} young neurons (D) and Golgi-Cox stained neurons (E) and increases dendritic complexity of Golgi-Cox stained neurons (F) in the DG of VPA-treated mice. See also Figures S5A and S5B for Sholl analysis.

(G–I) Abnormal dendritic span of DG neurons (G), but not of apical dendritic span of CA1 neurons (I), is recovered by voluntary running in VPA-treated mice, while basal dendrites of CA1 neurons show similar dendritic span across groups (H).

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 indicate the SD. *$p < 0.05$, ***$p < 0.001$, n.s., not significantly different, two-tailed t test. See also Figure S5.

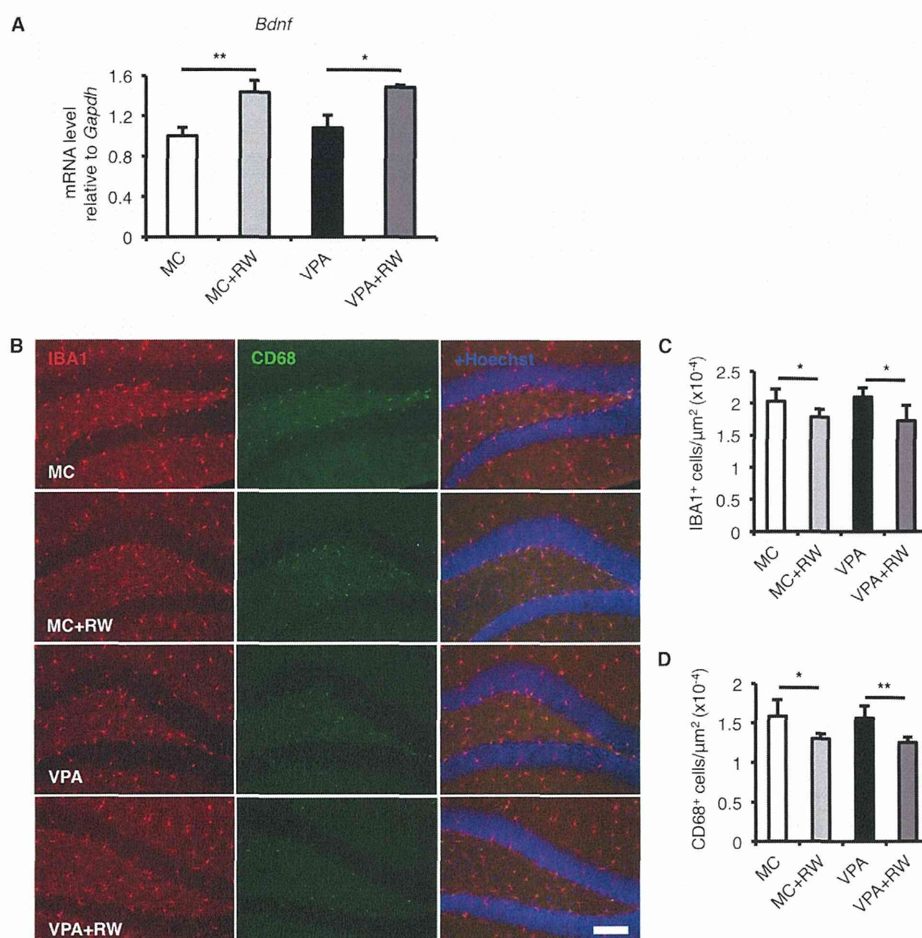

Figure 6. Voluntary Running Increases the *Bdnf* Expression Level and Reduces Microglia and Activated Microglia in the Hippocampus

(A) The expression level of *brain-derived neurotrophic factor* (*Bdnf*) was increased by voluntary running in both MC- and VPA-treated mice.
(B–D) Voluntary running reduced the number of $IBA1^+$ microglia (red; B and C) and $CD68^+$-activated microglia (green; B and D) in both MC- and VPA-treated mice.
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 indicate the SD. $*p < 0.05$, $**p < 0.01$, two-tailed t test. Scale bar, 100 μm.

(Figure 6A). Voluntary running has also been shown to reduce the number of microglia and its activation (Gebara et al., 2013; Kohman et al., 2013), and previous reports showed that microglia can suppress the adult hippocampal neurogenesis (Sierra et al., 2010; Vukovic et al., 2012; Matsuda et al., 2015). Interestingly, we found that the number of IBA1-positive microglia and CD68-positive-activated microglia was also decreased by voluntary running in both MC- and VPA-treated mice (Figures 6B–6D). Therefore, it is plausible that voluntary running helped VPA-treated mice through the increased adult hippocampal neurogenesis that was caused by the increase level of *Bdnf* expression and the reduction of microglia and its activated form in the hippocampus.

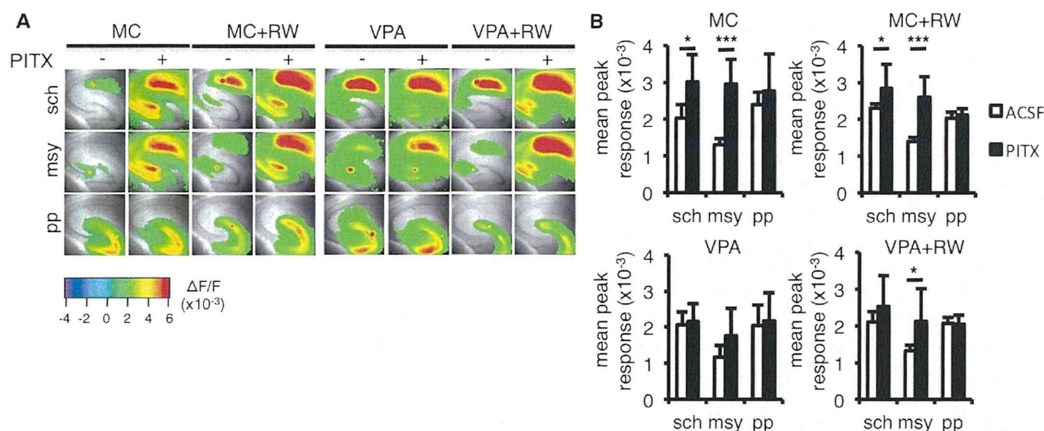

Figure 7. Voluntary Running Restores Neuronal Activity in VPA-Treated Mice

(A) Representative pseudocolor activity map images of brain slices including the hippocampus show that voluntary running can only recover the impairment of $GABA_A$ receptor-mediated inhibition in the mossy fiber pathway (msy) of VPA-treated mice, after treatment with the $GABA_A$ receptor channel antagonist picrotoxin (PITX) (n = 6 for MC, n = 9 for MC + RW, n = 7 for VPA, n = 8 for VPA + RW). Electrical stimulation was applied to Schaffer collateral afferents at the CA3/CA1 border of CA1 (sch); to the granule cell layer to stimulate the mossy fiber pathway (msy); and to the molecular layer of the upper blade in the DG (pp).

(B) Quantification of the neural response in artificial cerebrospinal fluid (ACSF), with (black bars) or without PITX (white bars; n = 6 for MC, n = 9 for MC + RW, n = 7 for VPA, n = 8 for VPA + RW). Note that although the augmentation of the neural response caused by $GABA_A$ receptor-mediated inhibition with PITX application seen in sch and msy was abolished in VPA-treated mice, voluntary running could restore the augmentation only in the msy.

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 indicate the SD. *$p < 0.05$, ***$p < 0.001$, two-tailed t test.

Voluntary Running Cannot Mitigate Abnormal Neuronal Morphology or Function of the Hippocampal CA1 Region in VPA-Treated Mice

The freezing response in the contextual fear test after voluntary running by VPA-treated mice did not recover to the level displayed by MC-treated mice (Figures 4G and S4D; Table S2). It has been proposed that recall of contextual memories relies more on CA1 than other regions in the hippocampus (Hall et al., 2001). Indeed, we found that apical dendrite morphology was abnormal in CA1 neurons of VPA-treated mice, and this defect was not repaired by voluntary running (Figures 5C, 5H, and 5I). Moreover, when we examined the region-specific restoration by voluntary running of neuronal activity in the hippocampus, the basal neuronal responses upon electrical stimulation were not affected by VPA nor voluntary running in the three major synaptic connections in the hippocampus (CA3-CA1, Schaffer collateral afferent [sch]; DG-CA3, mossy fiber [msy]; EC-DG, perforant pathway [pp]) possibly due to some homeostatic balancing mechanism (Turrigiano and Nelson, 2004; Turrigiano, 2011). However, when the excitatory activity was measured, with the increase in activity caused by an inhibitor for $GABA_A$ receptor picrotoxin (PITX) application, the effect of VPA became apparent. That is, the PITX induced an increase in sch and msy in MC-treated mice, which was not seen in VPA-treated mice, suggesting the impairment of inhibitory action in VPA-mice. The voluntary running could restore the characteristics only in msy (Figure 7). These results may explain why the recovery of neurogenesis and neuronal morphology of the DG in VPA-treated mice could not ameliorate their poor performance in the contextual associative test. Previous studies have indicated that ablation of adult neurogenesis in the DG leads to defective performance in a contextual associative test (Saxe et al., 2006; Wojtowicz et al., 2008) (but see Shors et al., 2002), but enhanced neurogenesis or running was weakly related to the performance in the test (Wojtowicz et al., 2008).

DISCUSSION

The precise pathology underlying postnatal impairment of cognitive function in children of epileptic expectant mothers treated with VPA, commonly used AED, is

unknown. Based on our findings in mice, we propose that the impairment is attributable to the untimely enhancement of embryonic neurogenesis, which leads to depletion of the NPC pool and consequently to a decreased level of postnatal neurogenesis in the hippocampus. Children of epileptic expectant mothers treated with VPA may also have hippocampal neurons with abnormal morphology and activity, as were observed in this study. Although prenatal exposure to AEDs such as VPA may have detrimental effects that persist until adulthood, we suggest that these effects could be mitigated by a simple physical activity such as running. Our results thus offer a straightforward strategy to help children born to epileptic mothers.

The cognitive deficits associated with prenatal VPA exposure might not due solely to the reduced neurogenesis with the abnormal neuronal morphology in the hippocampus, and there is a possibility that the low freezing responses in fear associative tests were contributed by the deficiency in amygdala, nociception, and/or motoric functions. Nevertheless, our data suggested that the reduced neurogenesis associated with the abnormal neuronal morphology in the hippocampus were very likely to be correlated with the observed cognitive deficits for several reasons. First, voluntary running is well known for its effect on enhancing both adult neurogenesis in the DG of the hippocampus and hippocampus-dependent learning and memory (Zhao et al., 2008), and this voluntary running could recover the cognitive deficit, if not all, in VPA-treated mice with reduced neurogenesis in the DG. Second, to the best of our knowledge, there are no reports to date that show a direct contribution of voluntary running to the enhancement of amygdala function that subsequently leads to an improvement in the cued fear response. Third, based on experiments that we have conducted, we could not find any significant differences in amygdala size and in the expression levels of cortical layer-specific genes of MC- and VPA-treated mice, with or without voluntary running. Fourth, total traveled distance in the open field, elevated plus and the Y-maze tests, and the number of light/dark transitions were not significantly different between MC- and VPA-treated mice, although in our earlier experiment some of these parameters showed modest differences. Moreover, in fear associative test, VPA-treated mice move similarly to MC-treated mice before the start of the tone (pre-tone), which indicate that motor deficiency is unlikely to be the main cause of low freezing responses in VPA-treated mice. Fifth, MC- and VPA-treated mice have similar basal nociceptive response and startle response to electric footshock during the conditioning for fear-associative test, thus it seems unlikely that VPA-treated mice have abnormal nociception and cannot sense the foot shock. Taking these facts into consideration, we therefore suggested that the reduced neurogenesis associated with the abnormal neuronal morphology in the hippocampus were very likely to be a critical cause of the observed cognitive deficits. However, we still cannot completely exclude the possibility that changes in other brain areas may also contribute to the deficits, warranting further future investigation.

We and others have shown previously that VPA treatment induces neuronal differentiation but suppresses glial differentiation of cultured multipotent NPCs (Hsieh et al., 2004; Balasubramaniyan et al., 2006; Murabe et al., 2007; Abematsu et al., 2010; Juliandi et al., 2012). We have now demonstrated that VPA also increases histone acetylation in the embryonic forebrain and induces neuronal differentiation of embryonic NPCs. Previous study have shown that VPA promotes neuronal differentiation by increasing histone H4 acetylation at proneural gene promoters (Yu et al., 2009). However, several studies have suggested that the activation of GSK-3β/β-catenin and/or ERK pathway is the main cause for the increase neurogenesis of NPCs by VPA (Yuan et al., 2001; Jung et al., 2008; Hao et al., 2004; Go et al., 2012). It has been suggested that VPA might have various cellular effects that will depend on the context of VPA usage and/or cell type and experimental design used in the study, which warrant further research to reveal the connection between these effects (Kostroucheová et al., 2007; Rosenberg, 2007).

We suggest that gene expression change caused by VPA is attributable mainly to its HDAC-inhibiting activity. To date, more than a dozen HDACs have been characterized and they are classified into at least three major groups. In particular, HDAC1 and HDAC2, belonging to the class I group, have been reported to regulate NPC differentiation (Sun et al., 2011). NPCs express high levels of HDAC1 and some of them also express low levels of HDAC2 (MacDonald and Roskams, 2008). Interestingly, as NPCs are committed to the neuronal lineage, expression of HDAC2 is upregulated while that of HDAC1 is downregulated and becomes undetectable in most post-mitotic neurons (MacDonald et al., 2005; MacDonald and Roskams, 2008); on the other hand, HDAC1 expression is sustained in the majority of cells in glial lineages (astrocytes and oligodendrocytes), in which HDAC2 is not detected (Shen et al., 2005; MacDonald and Roskams, 2008). Moreover, HDAC2, but not HDAC1, was found to inhibit astrocytic differentiation (Humphrey et al., 2008). Therefore, although VPA is capable of inhibiting both HDAC1 and HDAC2 (Kazantsev and Thompson, 2008), it is tempting to speculate that the main target of VPA in HDAC inhibition-mediated neuronal differentiation of NPCs is HDAC1. It will be of interest to explore this possibility in a future study.

Neurogenesis in the adult mammalian brain occurs throughout life and has been clearly demonstrated at two

locations under physiological conditions: the SVZ of the lateral ventricle and the subgranular zone (SGZ) of the DG in the hippocampus (Alvarez-Buylla and Lim, 2004). Several studies have shown that hippocampal neurogenesis is regulated by both physiological and pathological activities at different stages, including (1) proliferation of NPCs, (2) fate determination and differentiation of NPCs, and (3) survival, maturation, and integration of newborn neurons (Zhao et al., 2008). Furthermore, each of these stages is subject to regulation by numerous intrinsic and extrinsic factors (Suh et al., 2009). Genetic and environmental factors that affect adult hippocampal neurogenesis also cause alteration in cognitive performance, suggesting roles for adult hippocampal neurogenesis in learning and memory (Zhao et al., 2008). Our results showed that VPA-treated mice have a decreased level of postnatal neurogenesis in the hippocampus, which correlates with their poor performance in learning and memory tests. We have shown here and elsewhere (Hsieh et al., 2004; Jessberger et al., 2007) that VPA can reduce the proliferation of NPCs, and this reduction, together with the enhancement of neurogenesis, probably led to the depletion of the NPC pool in VPA-treated mice. It is possible that this depletion caused a slower differentiation of the residual NPCs in order to maintain required number of NPC pool during life. This possibility is an interesting avenue to be explored in the future.

In accordance with previous studies (van Praag et al., 1999a, 1999b), we found that voluntary running augments hippocampal neurogenesis of both MC- and VPA-treated mice, and it restores learning and memory deficiencies in VPA-treated mice. A previous report has shown the same restoration of decreased hippocampal neurogenesis and learning deficits in aged rodents by voluntary running (van Praag et al., 2005), although the precise molecular mechanisms responsible for voluntary running-induced neurogenesis remain undetermined (Deng et al., 2010). Here, we propose that at least the increase expression level of *Bdnf*, and the reduction of activated microglia may contribute to the restoration of impaired hippocampal neurogenesis and neuronal morphology in the DG of VPA-treated mice after voluntary running. However, future exploration is necessary to reveal the direct connection between the increase expression level of *Bdnf* and the reduction of microglia and its activated form in the hippocampus after voluntary running.

EXPERIMENTAL PROCEDURES

Animal Treatment

All experiments were carried out according to institutional animal experimentation guidelines, which comply with the NIH Guide for Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and their suffering. Pregnant C57BL/6 mice were individually housed in plastic breeding cages with free access to water and pellet diet in a 12-hr light-dark cycle. For a detailed description of groups and treatments, see the Supplemental Experimental Procedures.

Immunohistochemistry, Nissl, and Golgi Staining

Mice were anesthetized and perfused with PBS followed by 4% PFA in PBS. The brain was dissected, postfixed, and processed for immunohistochemistry. For Nissl staining, brain sections were defatted with xylene, hydrated through a graded ethanol series (100%, 95%, and 70%), and washed with water before stained with 0.2% thionin solution (pH 4.0). Sections were then dehydrated in water and a graded ethanol series (70%, 95%, and 100%), clear in xylene, and mounted with Entellan (Merck). For Golgi staining, the brain was removed from the skull without any perfusion and then sectioned (100 μm) on a cryostat. For a more detailed description and list of antibodies, see the Supplemental Experimental Procedures.

Measurement and Morphometrics

For a detailed description of cell count, volume measurement and cell/tissue morphometrics, see the Supplemental Experimental Procedures.

Gene Expression Analysis

For a detailed description of GeneChip and real-time qPCR procedures, see the Supplemental Experimental Procedures.

Behavioral Tests

Behavioral experiments were performed sequentially using male mice. For a more detailed description, see the Supplemental Experimental Procedures.

Voltage-Sensitive Dye Imaging

Experiment was done using hippocampal slices. For a more detailed description, see the Supplemental Experimental Procedures.

Statistics

Statistical analyses were performed by Student's two-tailed t test (unpaired) and one-way or two-way ANOVA using R software (http://www.r-project.org) (n indicates individual mice).

ACCESSION NUMBERS

The accession number for the GeneChip data reported in this paper is GEO: GSE42904.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2015.10.012.

AUTHOR CONTRIBUTIONS

B.J. and K.N. conceived and designed the study. B.J., K. Tanemura, K.I., T.T., Y.F., M.O.I., N.M., and D.I. carried out the experiments. B.J., K. Tanemura, K.I., T.T., M.A., T.S., K. Tsujimura, M.N., and K.N. analyzed the data. J.K. supported the experiments. B.J. and K.N. wrote the manuscript.

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