Inhibition of GSK3β improves hippocampus-dependent learning and rescues neurogenesis in a mouse model of fragile X syndrome

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Fragile X syndrome (FXS), a common inherited form of intellectual disability with learning deficits, results from a loss of fragile X mental retardation protein (FMRP). Despite extensive research, treatment options for FXS remain limited. Since FMRP is known to play an important role in adult hippocampal neurogenesis and hippocampus-dependent learning and FMRP regulates the adult neural stem cell fate through the translational regulation of glycogen synthase kinase 3β (GSK3β), we investigated the effects of a GSK3β inhibitor, SB216763, on Fmr1 knockout mice (Fmr1 KO). We found that the inhibition of GSK3β could reverse the hippocampus-dependent learning deficits and rescue adult hippocampal neurogenesis at multiple stages in Fmr1 KO mice. Our results point to GSK3β inhibition as a potential treatment for the learning deficits seen in FXS.

INTRODUCTION

Fragile X syndrome (FXS), one of the most common forms of inherited intellectual disability, is caused by the functional loss of fragile X mental retardation protein (FMRP) (1). Both human FXS patients and FMRP-deficient animal models suffer from an array of deficits, including impaired cognition, learning and memory (2–7). There have been extensive efforts devoted to developing treatments for FXS; unfortunately, however, therapeutic options remain limited (8).

We and others have shown that Fmr1 knockout (Fmr1 KO) mice have elevated the levels of glycogen synthase kinase 3β (GSK3β); GSK3 inhibition has been considered as a therapy for FXS (9,10). In fact, lithium can reverse the anxiety and social behavioral deficits of Fmr1 KO mice (10–13). However, since lithium is a non-specific inhibitor for GSK3β, the mechanism of action of the drug is unclear (14). In contrast, SB216763 (SB), a small-molecule competitive inhibitor, specifically blocks GSK3β kinase activity, with minimal effects on other kinases (15,16). Whether the inhibition of GSK3β using a specific inhibitor, such as SB, could have therapeutic effects in FXS has yet to be explored.

Neurogenesis persists throughout life in restricted germinal zones of mammalian brains. Neurons produced in the dentate gyrus (DG) of the adult hippocampus can integrate into existing neural circuitry and are therefore ideal for integrating new memory (17). We previously showed that the deletion of FMRP specifically from adult neural stem/progenitor cells (aNSCs) results in hippocampus-dependent learning deficits in mice (3,9). At a molecular level, FMRP regulates the expression of GSK3β, a negative regulator of β-catenin and the canonical Wnt signaling pathway, which modulates hippocampal neurogenesis (9,18), but whether the inhibition of GSK3β could rescue impaired hippocampal neurogenesis and hippocampus-dependent learning in Fmr1 KO mice has not been evaluated.

In this study, we set out to determine whether a specific inhibitor for GSK3β, SB, could be used to treat Fmr1 KO mice. Indeed, we found that SB treatment improved hippocampus-dependent learning and rescued hippocampal neurogenesis in adult Fmr1 KO mice. These findings suggest that GSK3β inhibition might make a good potential therapy for treating the learning deficits seen in FXS.

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SB treatment improves hippocampus-dependent learning in Fmr1 KO mice

To determine whether SB could ameliorate hippocampus-dependent learning deficits in Fmr1 KO mice, we gave adult male Fmr1 KO mice and wild-type (WT) littermates either SB or vehicle via intraperitoneal (i.p.) injection for 2 weeks. At 2 weeks after the last injection, we subjected these mice to two hippocampus-dependent learning tests in which Fmr1 KO mice are known to show deficits: the trace conditioning learning test and delayed non-matching-to-place radial arm maze (DNMP-RAM) (3). We first tested mice on the trace conditioning test to assess hippocampus-dependent associative learning. Consistent with our previous findings (3), vehicle-treated Fmr1 KO mice performed worse (less freezing) in both contextual and tone trace conditioning tests compared with vehicle-treated WT mice (Fig. 1C and D, WT + Veh versus KO + Veh). On the other hand, SB-treated Fmr1 KO mice performed significantly better (longer duration of freezing) than vehicle-treated Fmr1 KO mice in both the contextual test (Fig. 1C; n = 7, KO; n = 6, WT) and the tone test (Fig. 1D; n = 7, KO; n = 6, WT), indicating that SB treatment had no effect on trace learning in WT mice.

Next, we tested mice in a DNMP-RAM task designed to assess their hippocampus-dependent spatial learning and memory (Fig. 2A) (3,19). Consistent with previous findings (3), vehicle-treated Fmr1 KO mice performed significantly worse in both test settings than vehicle-treated WT mice (Fig. 2B, WT + Veh versus KO + Veh, separation 2, n = 7; KO, n = 6, post hoc t-test, P<sub>WT+ Veh–KO + Veh</sub> = 0.05; separation 4, n = 7; KO, n = 6, post hoc t-test, P<sub>WT+ Veh–KO + Veh</sub> = 0.029). Although SB administration had no effect on the DNMP-RAM task in WT mice, SB-treated Fmr1 KO mice performed significantly better than vehicle-treated Fmr1 KO mice in both separation 2 [Fig. 2B; n = 7,
KO; n = 6, WT; two-way ANOVA, significant effect of genotype (P = 0.038), marginal significant effect of genotype (P = 0.064) and no effect of interaction between genotype and treatment (P = 0.227), post hoc t-test, P_{KO+ SB− KO + Veh} = 0.018 and separation 4 [Fig. 2B; n = 7, KO; n = 6, WT; two-way ANOVA, significant effect of genotype (P = 0.012), marginal significant effect of genotype (P = 0.05) and no effect of interaction between genotype and treatment (P = 0.124), post hoc t-test, P_{KO+ SB− KO + Veh} = 0.02]. Taken together, these data suggest that SB treatment has a rescue effect on the hippocampus-dependent learning deficits in Fmr1 KO mice.

**SB treatment enhances GSK3β phosphorylation in Fmr1 KO mice**

GSK3β phosphorylation at Ser-9 leads to reduced kinase activity, and SB is known to inhibit GSK3β activity by enhancing Ser-9 phosphorylation (14). We therefore examined the status of GSK3β Ser-9 phosphorylation in the hippocampus of SB-treated mice. Consistent with our previous report (9), the levels of total GSK3β were significantly higher in the hippocampus of Fmr1 KO mice compared with WT mice, and SB treatment had no effect on total GSK3β levels [Fig. 3A and B; n = 4, two-way ANOVA, significant effect of genotype (P < 0.001), with no significant effect of treatment (P = 0.722) and interaction between genotype and treatment (P = 0.042), post hoc t-test, P_{KO+ Veh− KO + SB} = 0.31]. Consistent with published literature (10,12,13), we found that the phosphorylation of GSK3β at Ser-9 was lower in vehicle-treated KO mice compared with vehicle-treated WT mice (Fig. 3C). Interestingly, SB at the dosage we used showed no effect on GSK3β Ser-9 phosphorylation in WT mice; however, GSK3β phosphorylation levels were significantly higher in SB-treated KO mice compared with vehicle-treated KO mice [Fig. 3C; two-way ANOVA, significant effect of genotype (P = 0.01), treatment (P < 0.001) and interaction between genotype and treatment (P = 0.004), post hoc t-test, P_{KO+ Veh− KO + SB} = 0.006]. Consequently, the SB treatment rescued the levels of Ser-9-phosphorylated GSK3β in KO mice to the WT level while having no effect on WT mice at this dosage [Fig. 3D; two-way ANOVA, significant effect of genotype (P < 0.001), treatment (P < 0.007) and interaction between genotype and treatment (P = 0.004), post hoc t-test, P_{KO+ Veh− KO + SB} = 0.039, P_{WT+ Veh− WT + SB} = 0.22]. GSK3β is a negative regulator of β-catenin and the canonical Wnt signaling pathway, which modulates hippocampal neurogenesis (18). Fmr1 KO mice exhibit reduced β-catenin levels (9). We therefore investigated whether SB treatment could rescue β-catenin levels in the hippocampus of Fmr1 KO mice. Reminiscent of what we found with GSK3β, we saw no changes in the levels of β-catenin in WT mice after SB treatment, but β-catenin levels were significantly increased in SB-treated Fmr1 KO mice compared with vehicle-treated Fmr1 KO mice [Fig. 3A and E; two-way ANOVA, significant effect of genotype (P < 0.001), treatment (P < 0.001) and interaction between genotype and treatment (P < 0.001), post hoc t-test, P_{KO+ Veh− KO + SB} = 0.005, P_{WT+ Veh− WT + SB} = 0.22]. These findings indicate that the inhibition of GSK3β, at the appropriate dosage, can be used to elevate the level of β-catenin in pathological conditions, without affecting normal physiological conditions, which may explain why SB could rescue learning in Fmr1 KO mice without affecting WT mice.

**SB treatment rescues adult hippocampal neurogenesis deficits in Fmr1 KO mice**

Adult hippocampal neurogenesis has important roles in hippocampus-dependent learning and memory (17). Deletion or restoration of FMRP in adult NSCs directly affects adult hippocampal neurogenesis and hippocampus-dependent learning (3). The rescue effects of SB on learning in Fmr1 KO mice prompted us to test whether SB could rescue adult hippocampal neurogenesis. Using BrdU to label newborn cells, we evaluated neurogenesis in SB-treated mice (Fig. 4A). We found...
that the number of BrdU+ cells at 7 weeks post-BrdU injections (6 weeks post-SB injections) was no different between vehicle-treated Fmr1 KO and WT mice and SB treatment did not affect the number of BrdU+ cells in either WT or Fmr1 KO mice [Fig. 4B and C; two-way ANOVA, no significant effect of genotype (P = 0.085), treatment (P = 0.77) and interaction between genotype and treatment (P = 0.73)], consistent with our previous report (9). Therefore, SB treatment does not affect the number of new cells in the adult DG.

Since FMRP deficiency affects the cell fate specification of adult NSCs (3,9), we next determined the phenotypes of these newborn cells using antibodies for mature neurons (Fig. 4D, NeuN) and astrocytes (Fig. 4E, S100ß). Consistent with our previous observations (3,9), we found that vehicle-treated Fmr1 KO mice had significantly reduced neuronal differentiation (Fig. 4F, BrdU+NeuN+/BrdU+, WT, n = 5; KO, n = 4; post hoc t-test, P<0.001) and significantly increased astrocyte differentiation (Fig. 4G, BrdU+S100ß+/BrdU+, WT, n = 5; KO, n = 4; post hoc t-test, P<0.001). Although SB treatment did not change the NSC fate specification in WT mice, it indeed rescued deficits of neuronal and astrocyte differentiation in Fmr1 KO mice [Fig. 4F, neuron: two-way ANOVA, marginal significant effect of genotype (P = 0.052) and interaction between genotype and treatment (P = 0.074) and with no significant effect of treatment (P = 0.1276), post hoc t-test, P<0.001; Fig. 4G, astrocyte: two-way ANOVA, marginal significant effect of genotype (P = 0.006) and interaction between genotype and treatment (P = 0.022) and no significant effect of treatment (P = 0.172); post hoc t-test, P<0.001). Thus, these results imply that SB treatment can rescue adult neurogenesis deficits in Fmr1 KO mice by altering NSC fate determination.

**SB treatment rescues neuronal maturation deficits of new neurons in Fmr1 KO mice**

FMRP is known to regulate the maturation and synaptic plasticity of neurons (20). Previously, we demonstrated that a lack of FMRP in newborn adult hippocampal neurons significantly impaired neuronal dendrite development (3). To determine whether SB treatment could rescue this deficit in adult Fmr1 KO mice, we analyzed the dendritic arborization of newborn DG neurons using a retrovirus-based single-cell tracking method. Since green fluorescent protein (GFP)-expressing recombinant retrovirus could only infect dividing cells, stereotoxic grafting of this virus into the postnatal DG allowed us to
track the fate of the labeled NSCs and analyze the morphology of differentiated new neurons (21–24). Using this method, we analyzed GFP-expressing newborn neurons in the DG of mice treated with either SB or vehicle (Fig. 5A). Quantitative assessment indicated that vehicle-treated Fmr1 KO mice had less complexity (Fig. 5B) and shorter dendrites (Fig. 5C) compared with vehicle-treated WT mice, consistent with our previous report (3). Although SB treatment had no effect on dendritic complexity or dendritic length of new neurons in WT mice, SB-treated Fmr1 KO mice exhibited significantly increased dendritic complexity (Fig. 5B, multivariate ANOVA, $P_{KO + Veh - KO + SB} = 0.029$) and dendritic length [Fig. 5C, two-way ANOVA, significant effect of genotype ($P = 0.013$) and marginal significant interaction between genotype and treatment ($P = 0.069$) and no significant effect of treatment ($P = 0.159$), post hoc t-test, $P_{KO + Veh - KO + SB} = 0.037$] compared with vehicle-treated Fmr1 KO mice.

Another parameter for assessing neuronal maturation is the density of spines on neuronal dendrites (25). FMRP deficiency is known to lead to an increased density of spines and a larger proportion of immature thin spines (26). We therefore quantified the number of spines on secondary dendrites of these GFP-expressing new neurons imaged by high-resolution confocal microscopy. Quantitative analyses indicated that the spine density of new neurons in the DG of Fmr1 KO mice was significantly higher than in WT mice (Fig. 5D and E, post hoc t-test, $P_{WT + Veh - KO + Veh} < 0.0001$), consistent with the literature (27,28). However, SB treatment had no effect on the spine density of new neurons in either Fmr1 KO mice or WT mice [Fig. 5D and E, two-way ANOVA, significant effect of genotype ($P < 0.001$) and no significant effect of treatment ($P = 0.11$) and interaction between genotype and treatment ($P = 1$), post hoc t-test, $P_{KO + Veh - KO + SB} = 0.21$]. Therefore, SB treatment could
partially rescue the maturation of new neurons in the DG of Fmr1 KO mice by enhancing neuronal dendritic complexity.

**SB treatment promotes integration of new neurons into neural networks in Fmr1 KO mice**

Newly generated neurons in the DG are progressively integrated into neuronal circuits after 4–6 weeks of age (17). Neuronal integration can be assessed by the expression of activity-dependent immediate early genes, such as c-Fos (29). It has been shown that Fmr1 KO mice have a decreased proportion of c-Fos+ neurons (30). We therefore investigated whether SB treatment could rescue the integration of newborn DG neurons in Fmr1 KO mice by analyzing the proportion of newly generated neurons (BrdU+NeuN+) that were c-Fos positive (Fig. 6A). Quantitative data revealed that the percentage of c-Fos-expressing newborn neurons (c-Fos+BrdU+NeuN+) was lower in vehicle-treated Fmr1 KO mice than in vehicle-treated WT mice (Fig. 6B, WT+Veh versus KO+Veh). SB treatment did not affect the percentage of c-Fos-expressing newborn neurons in WT mice but significantly increased the percentage of c-Fos-expressing newborn neurons in the KO mice, rescuing it to similar levels as seen in the WT mice [Fig. 6B, n = 4, KO, n = 3, two-way ANOVA, marginal significant effect of genotype (P = 0.0613) and treatment (P = 0.025) and with marginal significant interaction between genotype and treatment (P = 0.076), post hoc t-test, P_{KO+ Veh−KO + SB} = 0.029]. Thus, these results suggest that SB treatment could rescue the decreased integration of new DG neurons in Fmr1 KO mice.

**DISCUSSION**

In this study, we show that treatment with a specific GSK3β inhibitor, SB, rescues hippocampus-dependent learning deficits in Fmr1 KO mice. We demonstrate that, at the molecular level, SB restores β-catenin levels by enhancing GSK3β phosphorylation at Ser-9. At the cellular level, SB rescues the cell fate differentiation of NSCs, dendritic morphogenesis and integration of newborn DG neurons in the hippocampus of Fmr1 KO mice. Our findings suggest that the inhibition of GSK3β could make a good potential treatment for the learning deficits in FXS.

FXS is one of the most prevalent genetic mental disorders and is associated with an array of neurological and neuropsychiatric deficits, particularly impaired cognition, learning and memory (2–7). Despite extensive efforts, today there are still are only limited treatments for FXS. It has been reported that metabotropic glutamate receptor
Not all findings are consistent with this theory (8). For example, blocking mGluR using (32, 33). These findings led to the ‘mGluR theory’ of FXS (31), and the treatment of Fmr1 KO mice with mGluR antagonists [e.g. 2-methyl-6-(phenylethynyl)pyridine (MPEP)] can correct heightened audiogenic seizure susceptibility, abnormal center-field behavior and impaired pre-pulse inhibition (32, 33). These findings led to the ‘mGluR theory’ of FXS pathophysiology (34). However, not all findings are consistent with this theory (8). For example, blocking mGluR using MPEP in Fmr1 KO mice results in a greater degree of spine immaturity, including an increased proportion of immature dendritic spines (35). Therefore, other FMRP-regulated pathways may underlie functional phenotypes seen in FXS. Recently, we and others have found that GSK3β, a kinase critical for cellular signal transduction and a negative regulator of the canonical Wnt signaling pathway, exhibits elevated activity in the brains of Fmr1 KO mice (9, 10). In fact, lithium can reverse the anxiety and aberrant social behavior of Fmr1 KO mice (10–13). However, whether these effects are the result of specific inhibition of the GSK3β pathway is unclear. We previously found that the inhibition of GSK3β using SB, a more specific GSK3β inhibitor than lithium, could rescue NSC deficits that result from FMRP deficiency in vitro (9). Data shown here have confirmed that the inhibition of GSK3β could rescue adult hippocampal neurogenesis in vivo, as well as learning deficits in Fmr1 KO mice. Therefore, our data, together with other published literature, suggest that the inhibition of GSK3β could make a promising therapy for treating the learning abnormalities seen in FXS. Whether SB treatment can rescue neurogenesis and learning after the drug treatment remains a question. The half-life of SB is unknown (36). Two other selective GSK inhibitors, CHIR98023 and CHIR99021, have a reported half-life of 90 min, suggesting that the plasma half-life of SB might be in a similar range (37). However, the therapeutic effect of GSK3β inhibitor is reversible after treatment stops (10), suggesting that GSK3β inhibitor delivery might only be sufficient to affect ongoing neurogenesis. We have shown that FMRP deficiency leads to impaired NSC differentiation, morphological maturation, as well as survival of young neurons (3, 9). It has been shown that the young neurons are at the most vulnerable stage during adult neurogenesis (38). The 2-week SB treatment may have neural protective effect on young neurons in addition to promoting neuronal maturation. In fact, it has been shown that Gsk3β inhibitor can protect neuronal death in stroke and neurodegenerative disease (10, 39, 40).

Loss of FMRP leads to an increased density of dendritic spines and a larger proportion of immature spines in neurons (20, 26). Our data here suggest that the inhibition of GSK3β at the dosage we used in this study rescued the dendritic arborization deficit in the newborn DG neurons of Fmr1 KO mice. Since the neuronal maturation of DG new neurons do not differ significantly between young, middle-age and aged rats (41), it is possible that SB has similar effect on neuronal maturation in older Fmr1 KO mice. Interestingly, the inhibition of GSK3β was not effective at correcting the dendritic spine density pathology in the newborn DG neurons of Fmr1 KO mice. The differential effect of SB on dendritic arborization and spine density suggests that GSK3β might play different roles in regulating these two aspects of neuronal maturation. While GSK3β/β-catenin/Wnt signaling is an important mediator of dendritic development (42), FMRP may regulate spine density through mechanisms independent of Wnt signaling. For example, we know that FMRP regulates the spine morphology through the FMRP-dependent translational regulation of cytoskeletal or scaffold proteins needed for dendritic spine stability, such as MAP1B, PSD-95 or Shank (43). In addition, dendritic spine density can be regulated by FMRP via the Rac/p21-activated kinase signaling pathway (44). Thus, the inhibition of GSK3β alone may be insufficient to correct the dendritic spine density deficit in the newborn DG neurons of Fmr1 KO mice.

Interestingly, a study by Ring et al. (45) suggests that the infusion of GSK3β inhibitors into WT rodents has little effect in the expression levels of β-catenin. Here, we showed that SB treatment did not affect the expression level of β-catenin in WT mice, but did have significant effects on Fmr1 KO mice. A similar example is that lithium treatment does not significantly improve the cognitive performance of WT mice, but has significant therapeutic effects on a mouse model of spinocerebellar ataxia type 1 disease (46). Our data are also consistent with literature, showing that the inhibition of GSK3β has no significant effect on the behavioral test performance of WT mice (10–13). The lack of effect of SB treatment on β-catenin levels could be due to relatively the low dosage of SB we used in our study. It has been shown that the effect of GSK3 inhibitors on β-catenin is dosage-dependent in cultured cells (47, 48). In addition, Dash et al. (49) have shown that the administration of GSK3 inhibitors, lithium and SB in WT mice leads to increased GSK3β phosphorylation and mild decrease in β-catenin levels at 5 mg/kg dosage but has no significant effect at 2.5 mg/kg dosage. We decided to use 2 mg/kg SB for our study mainly because that this dosage has been shown to exert significant effects on hippocampal neurogenesis and hippocampal-dependent learning in genetic mutant (DISC1 or HIF1 knockout) mice.
with elevated Gsk3β activity but not in WT mice (50, 51). Indeed, we observed that 2 mg/kg SB could inhibit GSK3β phosphorylation and rescue β-catenin expression in Fmr1 KO mice. Since this dosage of SB is lower than the dosage that affects the β-catenin level in WT animals (49), it is not surprising that we did not see any changes in β-catenin levels in our SB-treated WT animals. Together, these findings suggest that the inhibition of GSK3β, at the appropriate dosage, might be used to treat pathological conditions that involve increased expression of GSK3β, without affecting normal physiological conditions.

In summary, the therapeutic potential shown by GSK3β inhibitors is encouraging in an FXS mouse model. However, translating these results into patient treatment is still highly challenging. Many components of the Wnt signaling pathway are overexpressed or mutated in several types of cancer (52). GSK3 inhibitors, by enhancing Wnt signaling, might be potentially oncogenic (14). In order to translate GSK3 inhibitor treatment into human trials, long-term evaluation is required to fully evaluate the oncogenic effect and toxicity of these agents in humans because these compounds may have to be administered for a long period of time for treating. Therefore, a new generation of selective, safe and blood-brain barrier penetrable inhibitor of GSK3β need to be developed.

MATERIALS AND METHODS

Animal treatment

Mice were housed in an ALAAC-approved facility at UNM on a 12-h reverse light/dark cycle, with lights on at 08:00 h. Behavioral assessments were performed between 1000 and 1500 h on adult mice (8–10 weeks old). All procedures and husbandry were in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were performed according to protocols approved by the University of New Mexico Animal Care and Use Committee. The Fmr1 KO mice bred onto the C57B/L6 genetic background were as described previously (3,9). Briefly, Fmr1 heterozygous female mice were bred with C57B/L6 WT male mice and Fmr1 WT and KO male littermate mice were used for all experiments.

GSK3β inhibitor (SB, Sigma-Aldrich, S3442) was made in a stock concentration of 20 mg/ml in dimethyl sulfoxide and then diluted to 0.3 mg/ml in sunflower oil. Adult Fmr1 KO and WT C57BL/6 mice (8–10 weeks old) were given SB (2 mg/kg) injections i.p. every other day for 14 days based on the literature (10,50). For neurogenesis analysis, the mice also received a total of four BrdU injections (50 mg/kg, Sigma-Aldrich, B5002) on days 7 and 8. At 4 weeks post-last SB injection, some mice were subjected to behavioral tests followed by perfusion, and their brains were saved for subsequent neurogenesis analysis. Other mice were sacrificed for fresh brain tissue collection for protein expression analyses.

Immunohistology and neurogenesis analyses

In vivo neurogenesis analyses were performed essentially as described in our publications (9,21–24,53). Mice were then euthanized at 6 weeks after the last BrdU injection by i.p. injection of sodium pentobarbital, followed by transcardiac perfusion with saline, followed by 4% paraformaldehyde (PFA). The brains were dissected out, post-fixed overnight in 4% PFA, and then equilibrated in 30% sucrose. The 40-µm brain sections were generated using a sliding microtome and stored in a −20° freezer as floating sections in 96-well plates filled with cryoprotectant solution (glycerol, ethylene glycol and 0.1 M phosphate buffer, pH 7.4, 1:1:2 by volume).

We performed immunohistological analysis on 1-in-6 serial floating brain sections (240 µm apart) based on the published method (9,21,23,24,53). The primary antibodies used were: rat-anti-BrdU (1:3000, Abcam, ab-6326), mouse anti-NeuN (1:5000; Millipore, MAB377), rabbit anti-S100β (1:500; Dako, Z0311) and rabbit anti-c-Fos (1:500; Millipore, AB1584). Fluorescent secondary antibodies were used at 1:250 dilutions (donkey from Jackson ImmunoResearch or goat from Invitrogen). After staining, sections were mounted, coverslipped and maintained at 4°C in the dark until analysis. BrdU-positive cells in the granule layer were counted using unbiased stereology (Stereoinvestigator, MBF Biosciences, Inc.) with a 5-µm guard zone, and the total number of BrdU+ cells in the DG was then calculated by Stereoinvestigator, as described in our publications. The results were presented as the number of BrdU+ cells in a cubic millimeter of DG. Phenotype analysis of BrdU+ cells was performed as described previously (9,21,22,53). Briefly, 50 BrdU+ cells in the DG were randomly selected, and their phenotypes (double labeling with either NeuN or S100β) were determined using either a Zeiss LSM510 laser scanning confocal microscope or a Nikon TE2000 microscope equipped with spin disc confocal microscope and MetaMorph quantification software. The data were analyzed using two-way ANOVA (GraphPad software, www.graphpad.com).

In vivo retrovirus grafting and neuronal maturation analyses

Retroviral vector CAG-EGFP and retrovirus production were performed as described previously (21–24,54). Briefly, CAG-EGFP retroviral vector DNA and packaging plasmid DNA were transfected into cultured 293T cells using calcium phosphate methods. The medium containing lentivirus was collected at 40, 64 and 88 h post-transfection, pooled, filtered through a 0.2-μm filter and concentrated using an ultracentrifuge at 65 000g for 2 h at 20°C using a SW27 rotor (Beckman). The virus was washed once with phosphate-buffered saline (PBS) and then resuspended in 50 µl of PBS. We routinely obtained 1 × 109 infectious viral particles/ml.

Retroviral grating was performed as described (21–24,54). Briefly, 7–8-week-old C57BL/6 male mice were anesthetized with isofluorane, and virus (1.5 µl with titer greater than 5 × 10⁹/µl) was injected stereotaxically into the dentate gyrus (DG) using the following coordinates relative to bregma: anteroposterior, −1(1/2) × d mm; lateral, +/−1.8 mm. At 4 weeks post-viral grafting, mice were perfused with 4% PFA for neuronal maturation analysis.

For dendritic branching analysis on 300-µm-thick floating brain sections, eGFP+ neurons were imaged on an LSM
510 confocal with a 20× per oil objective. Z-stacks of eGFP+ dendrites were captured at 8-μm intervals, and the dendrites and the cell body of single eGFP+ neurons were analyzed by Neurolucida software (MicroBrightField, Inc.). Data were extracted for Sholl analysis and the total dendritic length from each eGFP+ neuron.

For dendritic spine density analyses, 1-in-6 40-μm floating brain sections containing eGFP+ cells were used for analysis. eGFP+ neurons were imaged on an LSM 510 confocal with a 100× per oil objective. Protrusions were counted along 10 μm long dendrite segments measured using Image-J software (NIH Image). The ‘dendritic spine density’ result was calculated as the number of spines per 10 μm length of dendrite. A minimum of 40 dendritic fragments (10-μm each) from a minimum of 4 eGFP+ neurons were quantified from each animal.

Western blot
Frozen hippocampal tissue was homogenized in RIPA buffer and protein concentrations obtained using Protein Assay Dye (Bio-Rad, 500-0006). Twenty micrograms of protein lysate from each sample was separated on 8–16% sodium dodecyl sulfate–polyacrylamide electrophoresis gels (Bio-Rad, 161-1222) and then transferred to nitrocellulose membranes (Bio-Rad, 162-0115). Membranes were processed following the ECL western blotting substrate (Pierce, 32106). Anti-FMRP (Millipore, MAB2160), anti-Phospho-Ser-9-GSK3β (Cell Signaling, 9322), anti-GSK3β (BD Transduction Laboratories, 610201) and anti-β-catenin (Millipore, 05-613) were used as primary antibodies at the concentrations recommended by the manufacturers. Horseradish peroxidase-conjugated secondary antibodies were obtained from Pierce. For loading controls, membranes were stripped and reprobed with the antibody against β-actin (Sigma, A3853).

Trace conditioning tests
These tests were performed based on a published method (3). Apparatus: animals were placed into a Coulbourn Habitest™ fear conditioning system equipped with a stainless-steel grid floor for administration of a foot shock. Training: after 90 s of habitation in the conditioning apparatus, a 15-s tone of 80 dB was presented. After a 30-s delay, a 1-s electric foot shock of 0.7 mA was presented. This cycle was presented a total of seven times with a 210-s inter-trial interval. Mice were then returned to their home cages. Testing: at 24 h following training, mice were tested for both tone and context freezing. For the tone freezing test, mice were placed into a clean, rat-sized housing cage. After 3 min of acclimation, mice were exposed to a series of three 15-s tones of 80 dB, without subsequent shock, separated by a 165-s inter-trial interval. Freezing behavior during tone, including the first 90 s immediately following the tone, was measured. For the context freezing, mice were placed into the conditioning context for 180 s and were observed for freezing behavior throughout the context testing period. Movement, other than that associated with respiration, was recorded. Behavior was analyzed in a blind fashion with regard to genotype and drug treatment. All training was videotaped for subsequent viewing, rescoring and documentation. The trace conditioning apparatus was cleaned with 70% isopropyl alcohol between each mouse.

Delayed bon-match to place-8-way radial arm maze (DNMP-RAM) test
This test was performed based on a published method (3). The radial arm maze was purchased from Coulbourn Instruments and consisted of an octagonal-shaped center arena (28 cm diameter × 25.5 cm height) with eight arms (68.5 × 9.5 × 12.1 cm, 1 × w × h) radiating out from the center arena. The arms are made of clear Plexiglas with a wire grid floor and a clear Plexiglas top. Mice were maintained on a calorie-restricted diet (1.5 g of food per day) beginning 2 days prior to testing, continuing throughout the testing period. Testing was conducted over 5 consecutive days with two sessions on each of the arm separations in the morning and two sessions in the afternoon. On the first 2 days of testing, mice were permitted to self-correct, or if they failed to self-correct in 90 s, they were placed in the baited arm for 60 s prior to being returned to the home cage.

In a delayed non-match to position or place paradigm, mice were presented with a choice between a familiar and a novel arm, where the correct choice is the novel arm. Mice received eight trials per day for 5 consecutive days (20 trials of each spatial separation). Mice received one trial (consisting of a sample phase + choice phase) and were returned to a holding cage, and all other mice were tested before the second round of testing began.

In the sample phase, only the start and sample arms were accessible to the mouse, and the sample arm was baited with mouse chow and Honey Nut Cheerios™. Mice were removed from the maze and placed in a holding cage if they had either spent 30 s in the sample arm or had left the sample arm. During the choice phase, a new arm is open and baited with food, while the start and sample arms are open but not baited. The correct choice arms varied in distance from the sample arm by either two (separation 2) or four arms (separation 4). Mice that entered the correct choice (food-baited) arm were considered to have scored as correct, whereas entries into the sample arm or the start arm were scored as incorrect. Failure to make a selection in 3 min was also scored as incorrect, although this happened rarely. Mice were allowed to self-correct choices and retrieve the Cheerio before being removed from the maze. Each day, mice received two trials (sample + choice phase) for each of the two separations (separations 2 and 4) per day for 5 consecutive days.

The radial arm maze was rotated between sample and choice presentations, such that the exact spatial locations within the room for the start and sample arms were held constant during the trials, but the arms themselves changed to prevent the use of odor cues. The rotation took ~15 s. Repeated-measures ANOVA between group and separations were carried out using SPSS (v.18) for each experiment; post hoc Student’s t-tests with Bonferroni corrections were used as needed.
Statistical analysis

Statistical analysis was performed using the ANOVA and Student’s t-test, unless specified, with the aid of SPSS version 18. All percentages were arcsine-transformed before statistical analysis. All data were shown as the mean with the standard error of the mean (mean ± SEM). Probabilities of < 0.05 were considered as significant. The Bonferroni correction was used to control the type I error (55).

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Conflict of Interest statement. None declared.

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