Supplementary information

Liver X receptor β regulates the development of the dentate gyrus and autistic-like behavior in the mouse

SI Methods and Materials.

Immunohistochemical analyses and immunofluorescence

The brains were dissected and fixed in 4% paraformaldehyde (PFA) for 24 h at 4ºC. For paraffin sections, tissues were processed for paraffin embedding, and coronal sections (5 μm thick) were collected. For cryostat sections, the brains were post-fixed in 30% sucrose solution with 4% PFA at 4ºC, and coronal cryosections (20 μm or 40 μm) were collected. Paraffin sections were deparaffinized in xylene, rehydrated through graded alcohol, and processed for antigen retrieval by boiling in 10 mM citrate buffer (pH 6.0) for 2 min. The sections were incubated in 0.5% H_2O_2 in PBS for 30 min at room temperature to quench endogenous peroxidase and then incubated in 0.5% Triton X-100 in PBS for 30 min. To block nonspecific binding, sections were incubated in 3% bovine serum albumin (BSA) for 1 h at 4°C. For LXRβ staining, retrieval was improved by incubating the sections with 0.15 units/ml of β-galactosidase for 2 h. For BrdU staining, sections were incubated in 2 M HCl for 10 min at room temperature before Triton X-100 incubation. Sections were then incubated with anti-LXRβ (1:200; made in Jan-Ake Gustafsson's laboratory, Karolinska Institute, Novum, Sweden), anti-Tbr2 (1:400, Abcam, Cambridge, UK), anti-BLBP (1:400, Millipore, Bedford, MA), anti-GFAP (1:1000, Millipore), anti-Sox2 (1:500, Millipore), anti-Calretinin (1:1,000, Millipore), anti-Calbindin

(1:1000, Millipore), anti-Prox1(1: 500, Covance, Madison, WI), anti-BrdU (1:200, BD Pharmingen, San Diego, CA), and anti-Nestin (1:400, BD Pharmingen) in 1% BSA and 0.1% Triton 100 overnight at room temperature. BSA was used instead of primary antibodies in negative controls. After washing, sections were incubated with the corresponding secondary antibodies in 1:200 dilutions for 2 h at room temperature, followed by incubation with the avidin-biotin-peroxidase complex for 2 h and 3,3-diaminobenzidine tetrahydrochloride as the chromogen. For immunofluorescence, sections were incubated with corresponding secondary antibodies, Cy3-or 488-conjugated (both at 1:500, Jackson ImmunoResearch, West Grove, PA) (3 h, at room temperature), respectively. Sections were then mounted in Vectashield (Vector Laboratories, Burlingame, CA), and counterstained with 4',6-diamidino-2-phenylindole DAPI (Sigma-Aldrich, St. Louis, MO).

BrdU Labeling

For analysis of S-phase progenitor cells, mice received a single injection of BrdU (50 mg/kg, i.p.) at P2, P7, P10, and P14, and were sacrificed 2 h later. Adult mice received a total of four injections at a dose of 100 mg/kg BrdU, and were then sacrificed 2 h after the last BrdU injection. For the migration study, BrdU was given to pregnant females at E15.5 and embryos were removed at E18.5, or pups were injected with BrdU at P2 and sacrificed at P7. For the differentiation study, pups were injected with BrdU at P5 and P6, then sacrificed at P14.

Golgi staining

We used the FD Rapid Golgi Stain kit (FD NeuroTechnologies, Baltimore, MD)

to perform Golgi staining following the manufacturer's protocol (1). Briefly, the freshly dissected brains from P14 were immersed in impregnation solution (made by mixing equal volumes of solutions A and B) and stored at room temperature for two weeks in the dark. The brains were then transferred into solution C and kept for 48 h at 4°C in the dark. Afterward, they were sliced to a thickness of 100 μm and stained using standard staining procedures. Granule neurons in the identical area of the hippocampal SGZ of each group were randomly selected, and one to two equivalent-length dendritic segments from each neuron were chosen for quantification. The density of the dendrite spines was manually quantified according to the criteria described.

Western blot analysis

The hippocampi were harvested from WT and LXRβ KO mice at P7 and P14. The protein content was measured using a BCA protein assay, with BSA as a standard. The protein was mixed with 5×loading buffer and then subjected to electrophoresis in a 12% sodium dodecyl sulfate polyacrylamide gel and electrically transferred onto a polyvinylidene difluoride transfer membrane. Membranes were blocked in 5% fat-free milk and then incubated overnight at 4°C with the primary antibodies followed by peroxidase-conjugated secondary antibody labeling for 1 h at room temperature. The following primary antibodies were used: anti-β-actin (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), anti-Notch1 (1:1000, Santa Cruz Biotechnology), anti-Jagged1 (1:1000, Santa Cruz Biotechnology), anti-NICD (1:1000, Abcam), anti-Smad4 (1:1000, Santa Cruz Biotechnology), anti-BLBP (1:1000, Millipore),

anti-Hes1 (1:1000, Millipore), anti-PSD95 (1:1000, Millipore), and anti-synaptophysin (1:1000, Sigma-Aldrich). All western blot analyses data were representative of at least three independent experiments. Specific protein bands on the membranes were visualized by the enhanced chemiluminescence method (Amersham, Piscataway, NJ) according to the manufacturer's instructions.

Culture and differentiation of L2.3 cells

According to previous method (2), the rat RGC line L2.3 (a gift from Professor HD Li) was cultured in standard DMEM/F12 (Invitrogen, USA) supplemented with glucose (25 mM; Sigma-Aldrich, USA), heparin (2 μg/ml; Sigma-Aldrich), B27 (50 ×; Gibco, USA), basic fibroblast growth factor (10 ng/ml; BD Bioscience, USA) and penicillin and streptomycin (100 U/ml; Gibco) at 37 °C in a humidified atmosphere containing 5 % CO_2.To induce differentiation, the cells were cultured on polylysine- and laminin-coated coverslips in 50× B27 containing serum-free medium (DMEM/F12 with 25 mM glucose and 100 U/ml penicillin and streptomycin) for 7 days. On the first day of differentiation, the cells were labeled with 10 μM BrdU. After 7-day differentiation and fixation in 4% paraformaldehyde, the cells were incubated in 2M HCl for 30 min at 37°C and processed for BrdU immunostaining after washing with 0.1 M borate buffer for 10 min.

Cell proliferation assay of L2.3

The cell proliferation assay of L2.3 was performed on day 1-3 after different doses of T0901317 treatment. In brief, L2.3 neurosphere cells were exposed to T0901317 for 1-3 days, then dissociated into single cells and cultured on polylysine-

and laminin- coated coverslips. After 6h attachment, the cells were fixed with 4% paraformaldehyde.

Fluorescence-activated cell sorting (FACS) analysis of the cell cycle

The cells were fixed in 70% ethanol solution for 12 h at 4 °C after being harvested and were then stained using the Cycletest™ Plus DNA Reagent Kit (BD Bioscience). The result was determined using a FACS Calibur Flow Cytometer (BD Bioscience), and analyzed applying the ModFit 2.0 software (BD Bioscience). At least 20,000 cells in each sample were harvested and analyzed.

Immunocytochemistry and cell counting

For immunostaining, cells were fixed with 4% PFA for 30 min at 4°C, permeabilized with 0.25% Triton X-100 for 10 min at room temperature, and nonspecific staining blocked with 3% BSA and 10% normal goat serum for 1 h at room temperature. The cells were incubated with primary antibodies overnight at 4°C, followed by incubation with secondary antibodies for 1 h at 37°C.Nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI, Beyotime, China). The cells were washed three times with PBS between procedures. Primary antibodies and their dilutions were as follows: rabbit anti-Ki67 antibody, mouse anti-BrdU (1:300,Abcam, UK), rabbit anti-BrdU (1:300, Bioss, China), mouse anti-Tuj1 (1:400; Beyotime), and rabbit anti-GFAP (1:400; Abcam, UK). Secondary antibodies and their dilutions were as follows: Alexa Fluor® 488 goat anti-mouse IgG (1:500, Life Technologies, USA), Alexa Fluor® 568 goat anti-rabbit IgG (1:500, Life Technologies), Alexa Fluor® 488 goat anti-rabbit IgG (1:400, Beyotime) and Cy3 goat anti-mouse IgG (1:500,

Beyotime). Negative control experiments were performed in parallel using normal goat serum instead of the primary antibodies. After immunostaining, the cells were observed and analyzed using a fluorescence microscopy system (Leica, Germany). The number of total cells in each field was measured by counting DAPI-positive nuclei. The expression level of each antigen was determined by calculating the ratio of positive cells to the total number of cells. Each result represents the mean of three independent experiments.

Behavioral tests

The mice used for all behavioral analyses were 3-month-old adult male mice. Behavioral experiments were performed between 10:00 a.m. and 6:00 p.m. Mice were allowed to rest for 24 h in between behavioral tests. The following assays were performed: the novel object and novel location recognition, Morris water maze, nest building, and three-chambered social interaction tests, and spontaneous repetitive behaviors. For all behavioral experiments, experimenters were blinded to genotype. After each experiment, all the apparatuses were wiped clean to remove traces of the previous assay.

Novel object and novel location recognition tasks

Novel object recognition task

Testing was performed in an open field arena constructed with opaque gray Plexiglas as described (3). The stimulus objects varied in shape, color, and texture, but were similar in size. The day prior to testing, mice were habituated to the empty testing apparatus for 5 min. During the training session, two identical copies of the

objects were positioned in the open field arena and the mouse was allowed to explore both objects for 5 min. After the completion of the training session, mice were tested for object memory. During this test trial, one object was replaced with another novel object, which was of similar size but different shape and color than the previous one. Then, the same test mouse was placed in the center, and allowed to explore the arena and the two objects. The movement of the mice was recorded for 5 min. The discrimination index (DI) was calculated as follows: DI = Tn/ (Tf + Tn); Tn and Tf were the times taken for the mouse to explore the novel and familiar object, respectively.

Novel location recognition task

This task assesses the ability of the mice to recognize a novel spatial arrangement of familiar objects. Procedures were similar to those described in the novel object recognition task, except that identical copies of objects were used in the recognition phase, with one of them moved to a new location in the open field. Mice were allowed to explore two familiar objects, with one in a new location.

Morris water maze

The Morris water maze test was performed as described elsewhere (4). In brief, mice were trained to locate a hidden platform based on distal visual cues to escape from the pool. Mice received four training trials per day (with different starting points) for 5 consecutive days. On day 6, the platform was removed and a probe test was performed. The next day, the platform was moved to the opposite quadrant and the reversal task of the test was started. Mice again received four training trials per day to

locate the new platform. A probe test was performed on day 10.

Social interaction

Mice were tested for social interaction in a three-chambered apparatus as previously described (5). The test mouse was first placed in the middle chamber and allowed to explore for 5 min. After the habituation period, an unfamiliar C57BL/6J male (stranger 1), that had no prior contact with the subject mice, was placed in one of the side chambers, and a novel object was placed in the opposite side. Both doors to the side chambers were then unblocked and the subject was allowed to explore the entire social test box for a 10-min session. Measures were taken of the amount of time spent in each chamber.

At the end of the first 10-min session, each mouse was tested in a second 10-min session to quantify social preference for a second stranger. A second, unfamiliar mouse was placed in the chamber where an object was placed during the first 10-min session. The test mouse had a choice between the first, already-investigated unfamiliar mouse (stranger 1), and the novel unfamiliar mouse (stranger 2). Measures were taken of the amount of time spent in each chamber of the apparatus during the second 10-min session.

Self-grooming test

To measure self-grooming, mice were individually placed into a standard mouse cage. After a 5-min habituation period, mice were video recorded for 10 min with a horizontally mounted camera (5). The total time spent self-grooming was scored by a trained observer blind to the treatment group.

Nest building

Mice were individually housed overnight, with food, water and sawdust bedding. On the first day of testing, one compressed cotton square (Nestlet, 5 × 5 cm, Ancare, Bellmore, NY, USA) was placed in the cage to facilitate nest building. The presence and the quality of each nest was evaluated on the following day according to five-point scale using the method of Deacon(6).

Imaging and quantification

RGCs were identified according to their GFAP immunoreactivity colabeled with BLBP or Sox2 and the presence of a radial glia-like apical process traversing the GCL. These cells divide slowly to produce IPCs, expressing Tbr2, and divide rapidly to produce neuronal committed IPCs, expressing DCX. Upon DCX expression, progenitors exit the cell cycle, gradually mature, and express Calretinin, Prox1, and CB.

Quantification of BrdU and DCX-positive cells in adult mice was carried out using an unbiased design-based stereology protocol, with a microscope (AxioSkop; Zeiss, Jena, Germany) at a magnification of × 40. Tissue for this purpose was derived from free-floating sections. Every tenth section, spanning the rostrocaudal extent of the hippocampus, was taken, and the total number of positive cells per DG was estimated by multiplying the total number of positive cells counted by section periodicity.

To determine the number of cells in the postnatal DG (within two weeks after birth) that were positive for CB, Prox1, Tbr2, BrdU, Sox2, or double-positive for

GFAP/BLBP and GFAP/Sox2 on the paraffin sections, the cells in the DG were quantified, and results were expressed as the number of cells per section. Twelve sections for markers in each brain were assessed. For progenitor migration analysis, the distribution of BrdU-positive cells was analyzed in the different compartments of the hippocampus (ventricular zone [VZ], fimbrio-dentate junction [FDJ], and DG at E18.5; marginal zone [MZ], GCL, and hilus at P7), the percentage of BrdU-labeled cells in each area was determined, and results were plotted as histograms. To examine the differentiation of BrdU-positive cells into neurons positive for the neuronal marker Prox1, or into glia immune-positive for the glial marker GFAP, the percentages of BrdU positive cells that colocalized with Prox1 or GFAP were assessed.

Statistical analysis

Data were analyzed by the Student's t-test, one or two-way analysis of variance (ANOVA) followed by Fisher's protected least-significant difference post hoc tests or at least significant difference multiple-comparison t-test. Significance was reached at values of $P < 0.05$. Statistical analysis was performed using the Statistical Product and Service Solutions software V13.0 (SPSS, IBM, Chicago, IL).

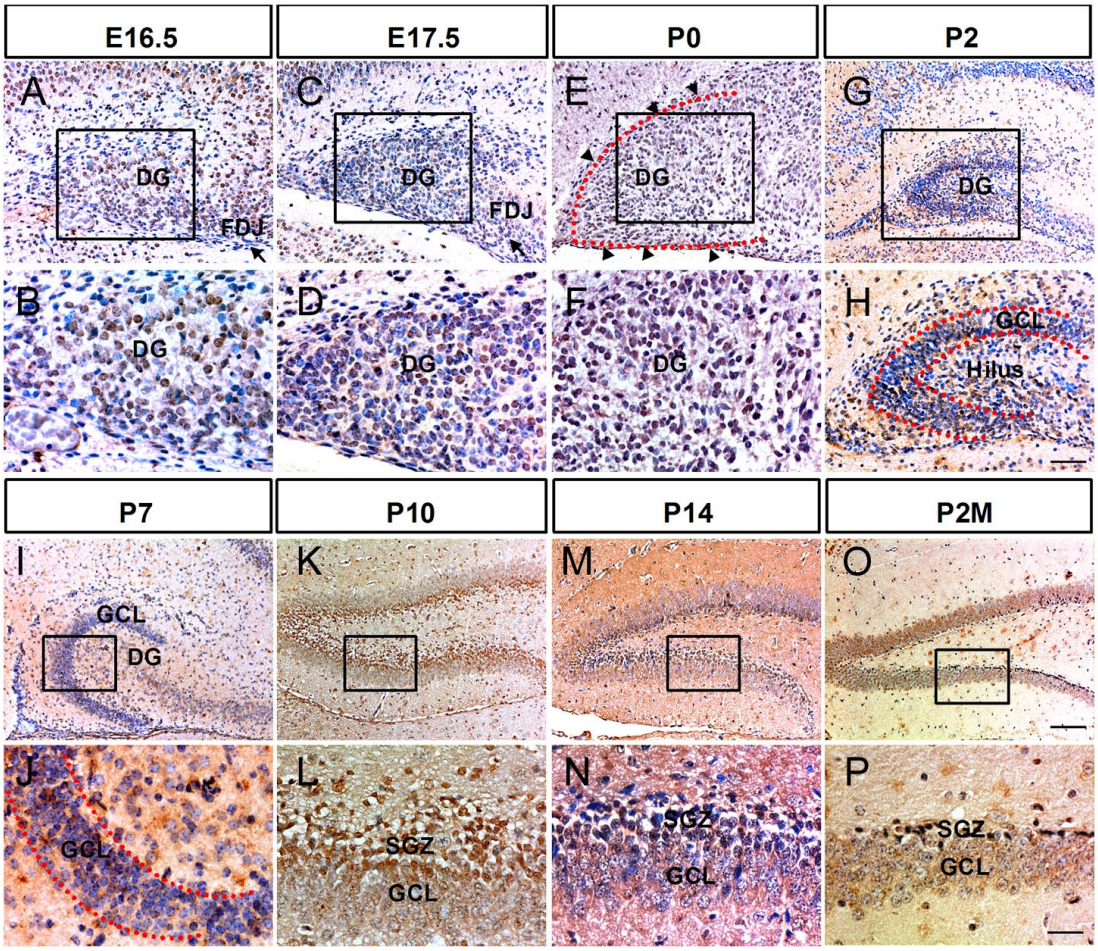

Fig.S1. LXRβ expression in the developing DG of mice from E16.5 to P 2M.

LXRβ expression was detected in both DG primordium and FDJ (arrows in A and C) at E16.5 (A and B) and E17.5 (C and D). At P0 (E and F), LXRβ was expressed in the developing dentate in the transient proliferative zone including the hilus and DG blades (arrowheads in E). LXRβ expression was higher in the GCL at P2 (G and H) and P7 (I and J). LXRβ expression was mainly localized in the SGZ at P10 (K and L), P14 (M and N) and P2M (O and P). (B, D, F, H, J, L, N and P) Images are higher-power views of the boxed areas in A, C, E, G, I, K, M and O.Scale bar: A, C, E, H, 50 μm; G, I, K, M, O, 100μm; B, D, F, J, L, N, P, 25μm. DG, dentate gyrus; FDJ, fimbriodentate junction; HF, hippocampal fissure; GCL, granular cellular layer; SGZ, subgranular zone.

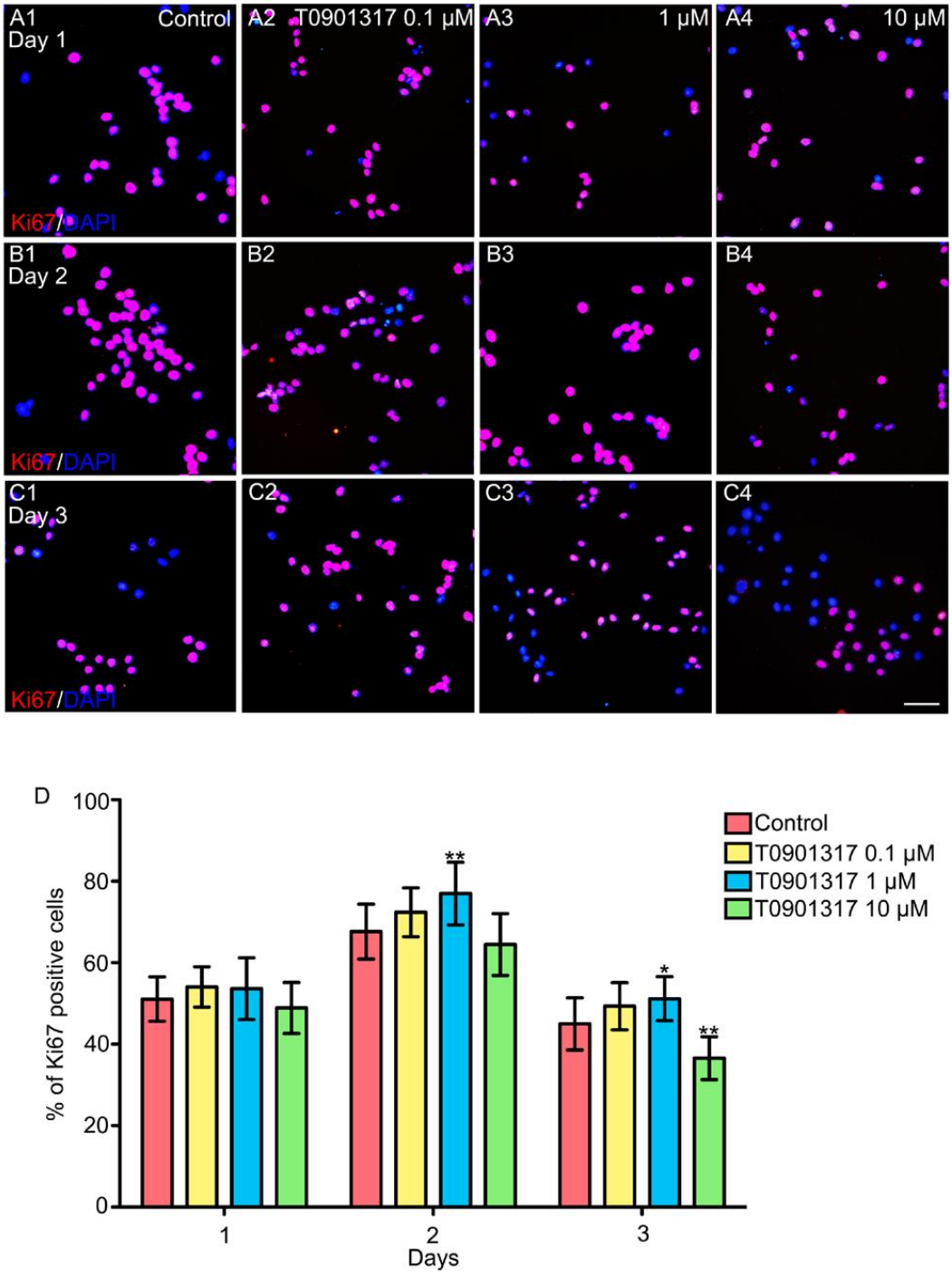

Fig.S2.Analysis of Ki67 expression after treatment with T0901317.

(A–C) Representative images of L2.3 cells double-stained with Ki67 (red) and DAPI (blue). (D)Statistical analysis of Ki67 expression. The data represent the mean ± s.d. of three separate experiments. *$P < 0.05$, **$P < 0.01$, versus control cells. Scale bar in C4=50 μm and applies to A1-C4.

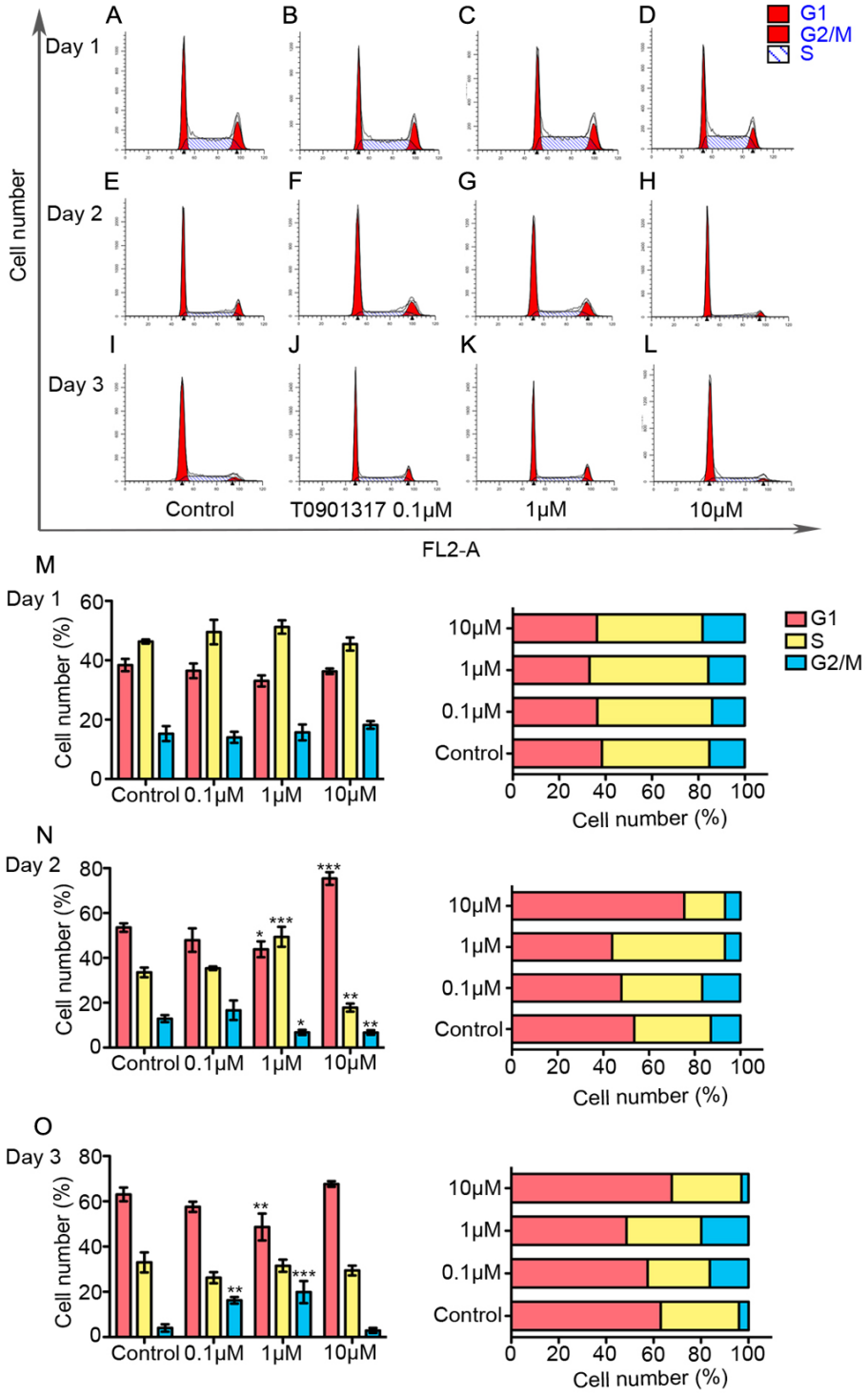

Fig.S3. FACS analysis of the cell cycle of L2.3 cells exposed to T0901317 for 3 days.

(A-L) Representative cell-cycle data.(M-O) Statistical analysis. Data represent the mean ± s.d. of three independent determinations. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, versus control cells.

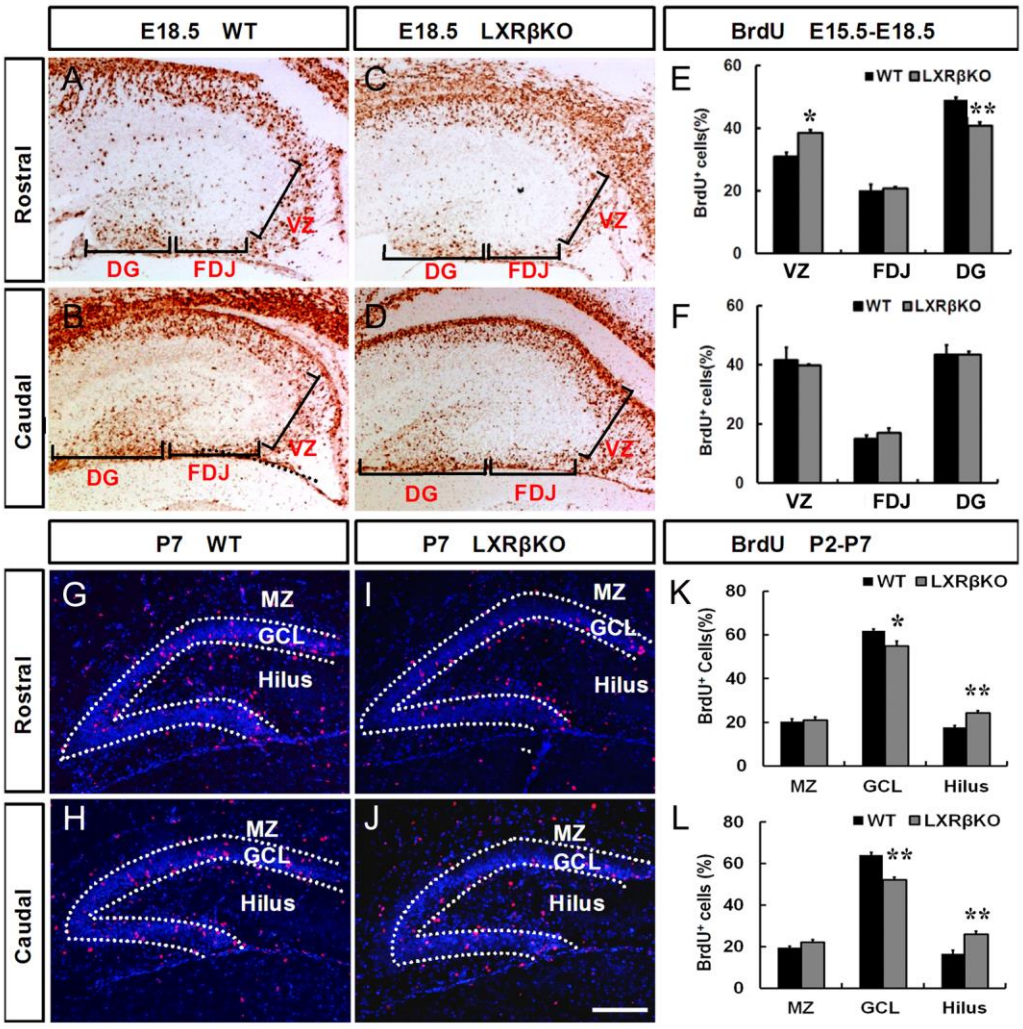

Fig.S4. LXRβKO mice show neuronal migration abnormalities.

(A-F) Representative immunohistological images of the LXRβKO and control E18.5 hippocampus for BrdU at rostral(A, C and E) and caudal levels (B, D and F). BrdU was administered at E15.5 and the mice were killed at E18.5. The distribution of BrdU- positive cells is altered in the LXRβKO hippocampus at rostral level. (G-L) Representative immunohistological images of the LXRβKO and control P7 hippocampus for BrdU at rostral(G, I and K) and caudal levels (H, J and L). BrdU was administered at P2 and the mice were killed at P7. The distribution of BrdU- positive cells is altered in the LXRβKO hippocampus at both rostral and caudal level. Data are presented as mean ±s.e.m (n=3).*P< 0.05; **P< 0.01,Student's t-test. Scale bar: A-D, G-J, 100μm. DG, dentate gyrus; FDJ, fimbriodentate junction; VZ, ventricular zone; SGZ, subgranular zone; MZ, marginal zone ; GCL, granule cell layer.

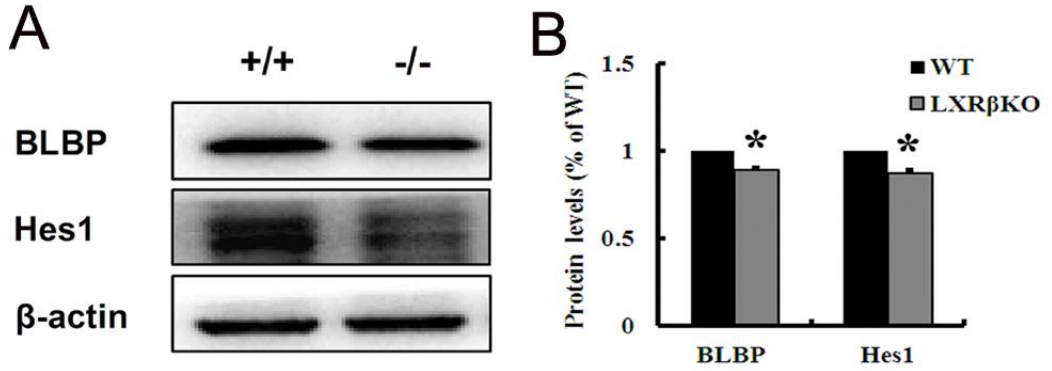

Fig.S5.Western blot analysis of BLBP and Hes1 in P7 WT and LXRβ KO hippocampus.

(A)Representative immunoblots of BLBP and Hes1.(B)Graph representing the densitometric analysis of BLBP and Hes1.Data are presented as mean ± s.e.m. (n = 3); **P*< 0.05, Student's t-test.

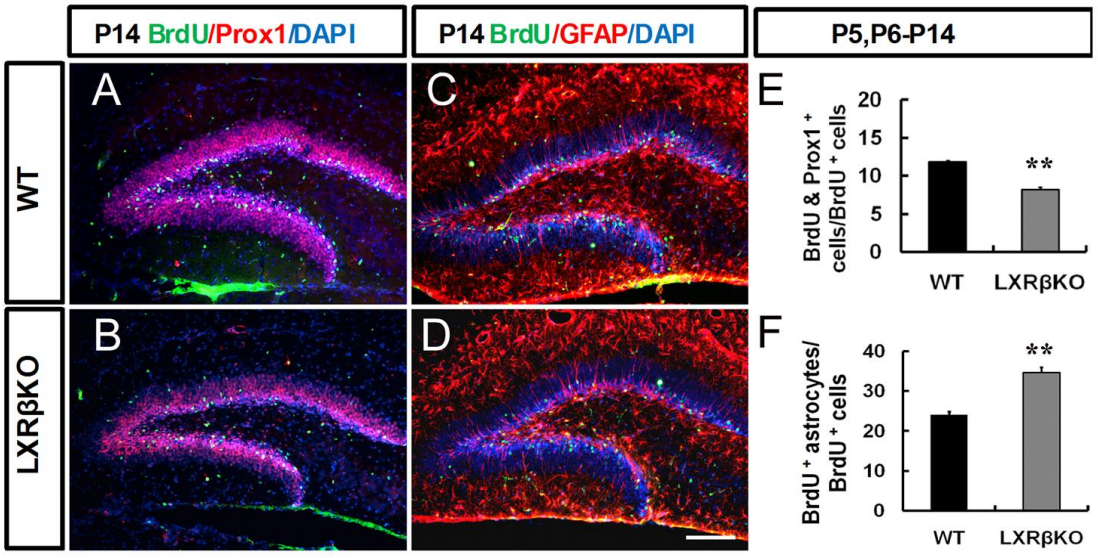

Fig.S6.Loss of LXRβ altered neuronal differentiation in developing DG in vivo.

Representative images of $BrdU^+$ /$Prox1^+$ (A and B) and $BrdU^+$ /$GFAP^+$ (C and D) double positive cells in the DG of WT (A and C) and mutant animals (B and D) at P14. Percentages of $BrdU^+$ /$Prox1^+$ (E) and $BrdU^+$ /$GFAP^+$ (F) double positive cells among the total number of BrdU cells. Data are expressed as mean ± s.e.m. n=3 ;***P* < 0.01, versus controls. Scale bar in D=100 μm and applies to A-D.

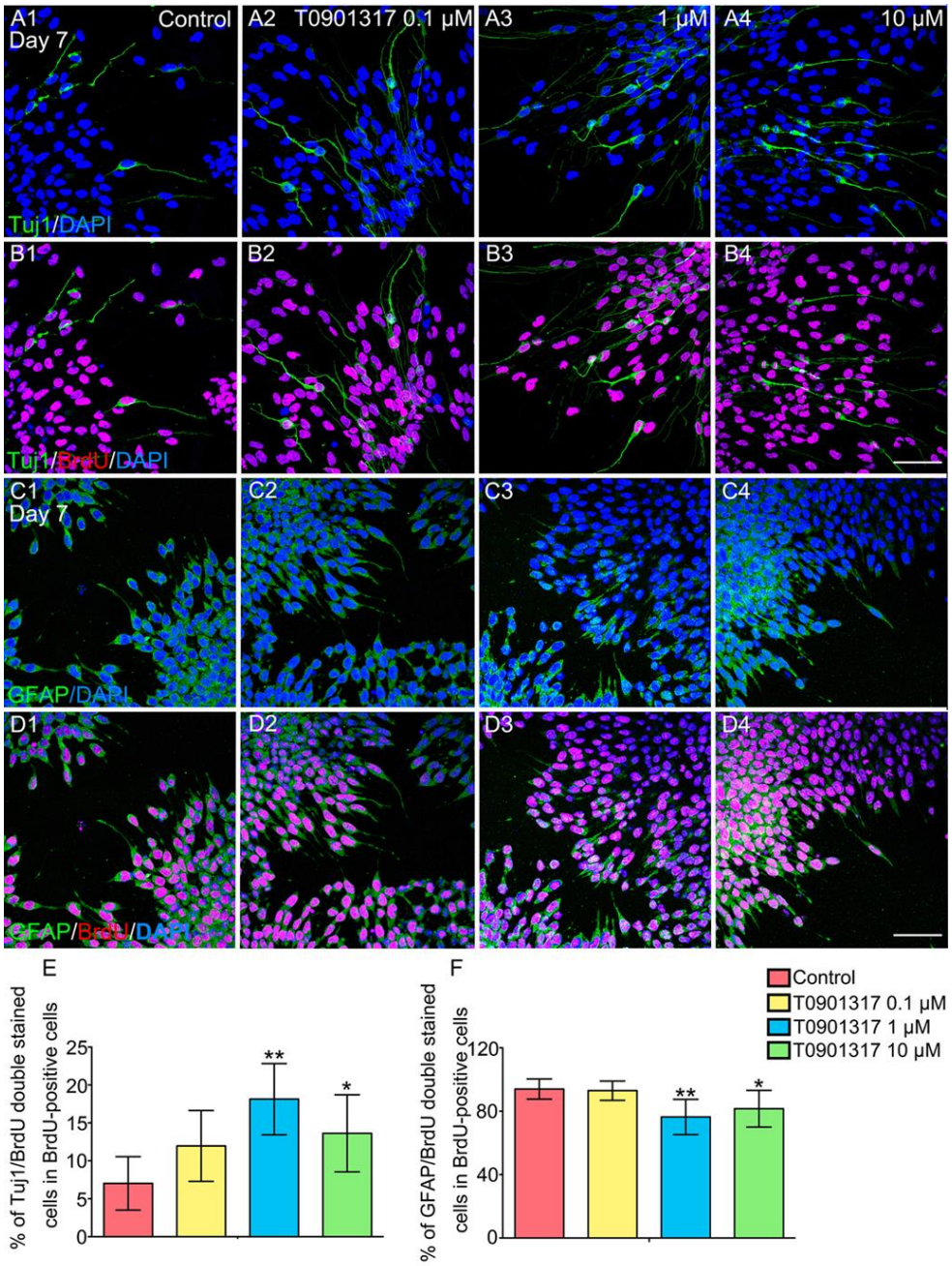

Fig.S7. Immunocytochemical analysis of Tuj1/BrdU and GFAP/BrdU double positive cells derived from L2.3 cells on day 7.

(A-B) Representative images of Tuj1/BrdU expression. (C-D) Representative images of GFAP/BrdU expression. (E) Percentage of Tuj1/BrdU double positive cells in BrdU-positive cells. (F) Percentage of GFAP/BrdU double positive cells in BrdU-positive cells. Data from three separate determinations are expressed as the mean ± s.d. **P* < 0.05, ***P* < 0.01, versus control cells. Scale bars in B4 and D4 = 50 μm, and applies to A1-D4.

References

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