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Supplemental Information

Hippocampal 7α-Hydroxylated Neurosteroids Are Raised by Training and Bolster Remote Spatial Memory with Increase of the Spine Densities

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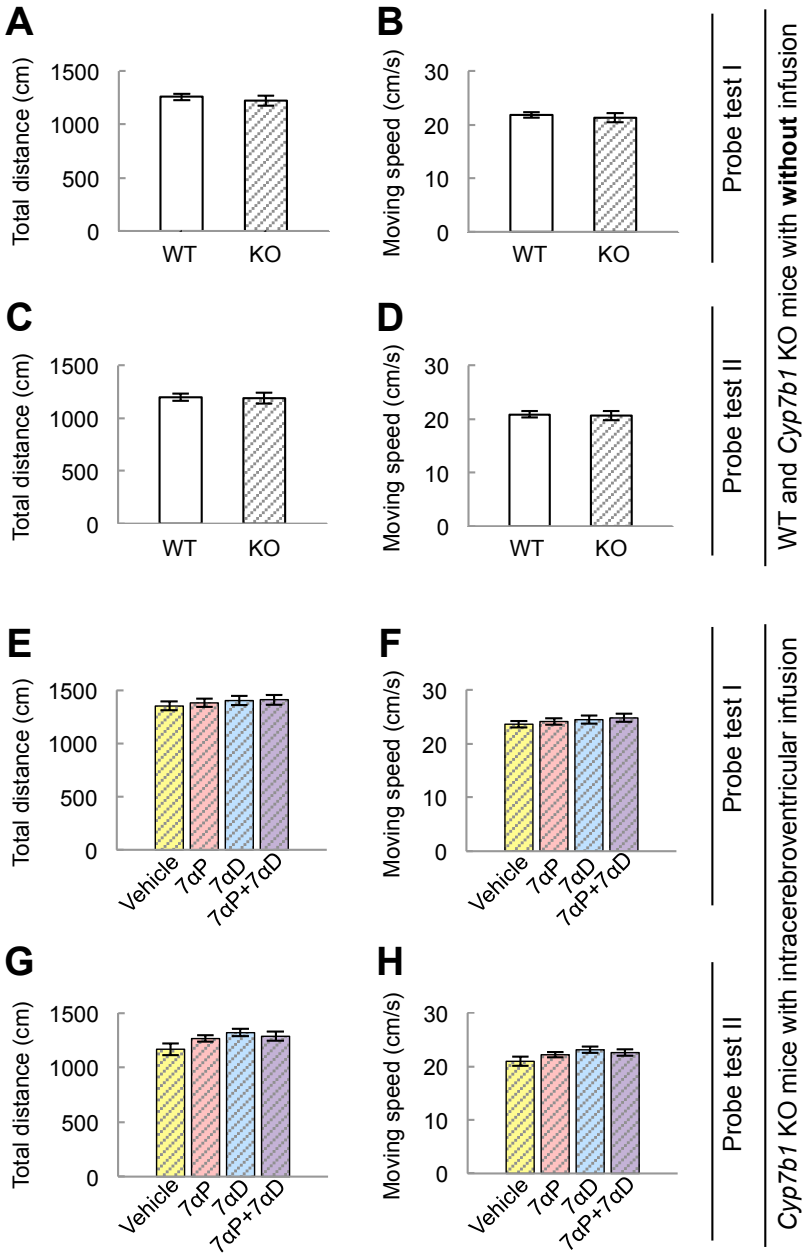

Figure S1. Activity parameters in probe tests of the Morris water maze test. Related to Figure 2 and Figure 6.

Total distance (**A**, **C**, **E**, **G**) and moving speed (**B**, **D**, **F**, **H**) of mice in the probe tests of the Morris water maze test (Fig 2, 6). Activities of (**A**, **B**) probe test I and (**C**, **D**) probe test II for *Cyp7b1* KO and the littermate WT mice in Fig 2. Not significant (P>0.05) by Student's t test. Activities of (**E**, **F**) probe test I and (**G**, **H**) probe test II for *Cyp7b1* KO mice with intracerebroventricular infusion of steroids in Fig 6. Not significant (P>0.05) by Dunnett's test *versus* vehicle. Values are shown as the mean ± SEM.

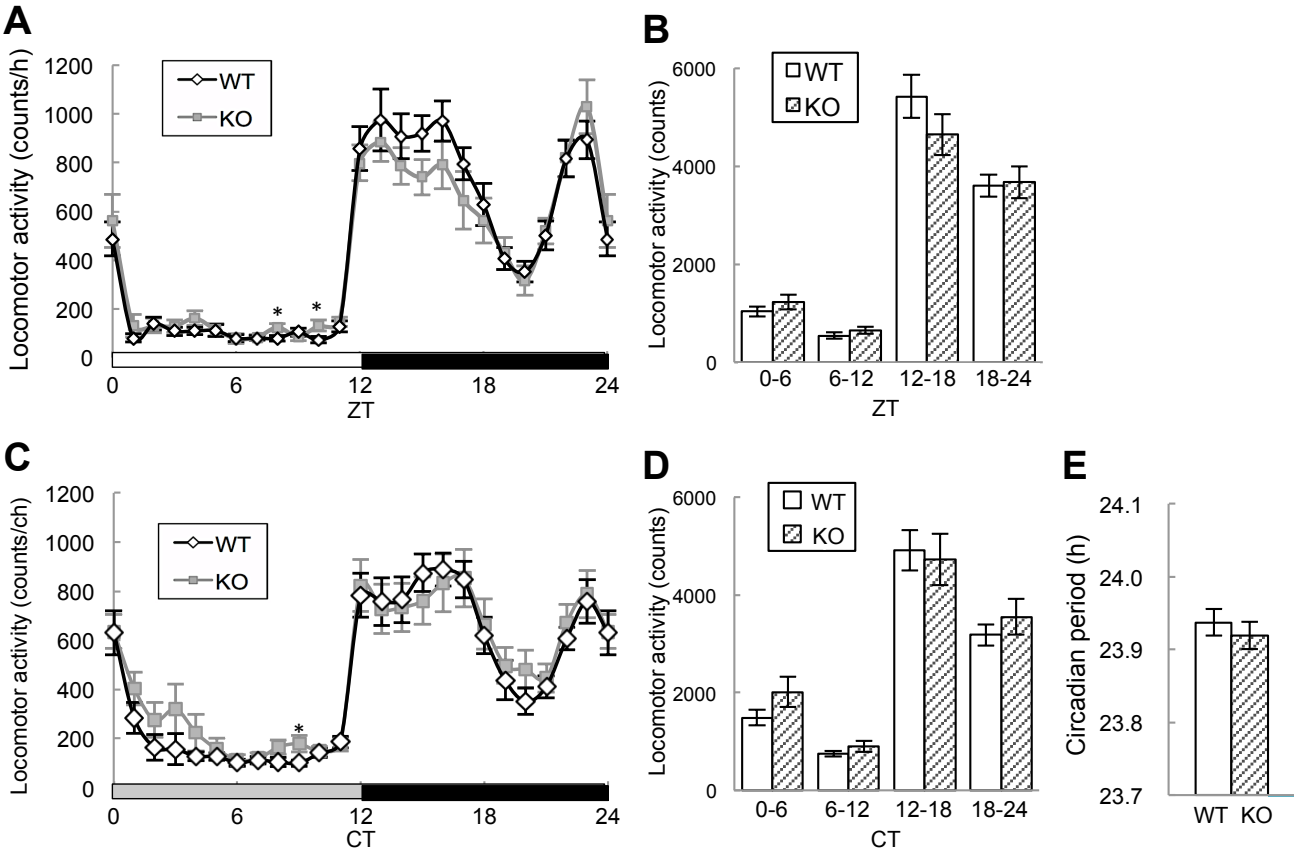

Figure S2. Locomotor activities and circadian periods of *Cyp7b1* KO mice and the littermate WT mice. Related to Figure 2.

Cyp7b1 KO and the littermate WT mice were entrained to a 12 hr/12 hr light/dark cycle (LD) and transferred to constant darkness (DD). Their free moving locomotor activities were monitored using infrared area sensors. (**A**) Averaged locomotor activities in LD (counts in one hour) were plotted against zeitgeber time (ZT). (**B**) Averaged locomotor activities in LD (counts in six hours) during the four time zones (ZT0-6, 6-12, 12-18, 18-24). (**C**) Averaged locomotor activities in DD (counts in one circadian hour) were plotted against circadian time (CT). (**D**) Averaged locomotor activities in DD (counts in six circadian hours) during the four time zones (CT0-6, 6-12, 12-18, 18-24). (**E**) Circadian periods in DD. In A-E, values are calculated from the activities in 14 days for each mouse and shown as the mean ± SEM (WT, n = 15; KO, n = 14). $^{*}P<0.05$ by Student's t test.

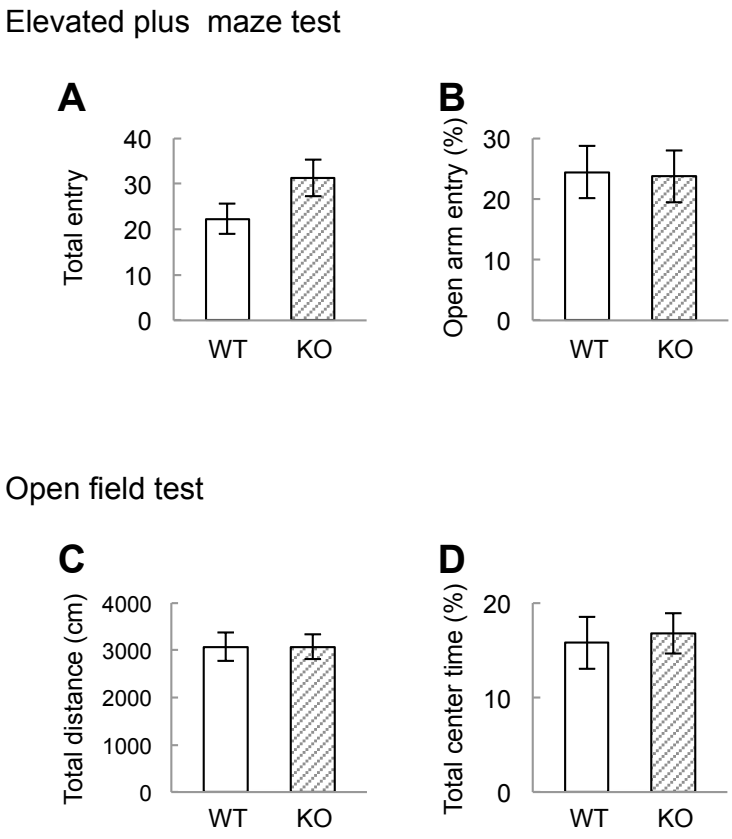

Figure S3. Anxiety-like behavior of *Cyp7b1* KO mice. Related to Figure 2.

Cyp7b1 KO and the littermate WT mice were subjected to elevated plus maze (EPM) test and open field (OF) test. Each mouse was subjected to both or one of these behavioral tests in the following order at least every second day. EPM test: (**A**) number of total arm entries and (**B**) ratio of number of open arm entries (WT, $n = 16$; KO, $n = 10$). OF test: (**C**) total distance explored for OF and (**D**) time ratio spent in center area (WT, $n = 12$; KO, $n = 11$). Values are shown as the mean ± SEM. Not significant ($P>0.05$) by Student's t test.

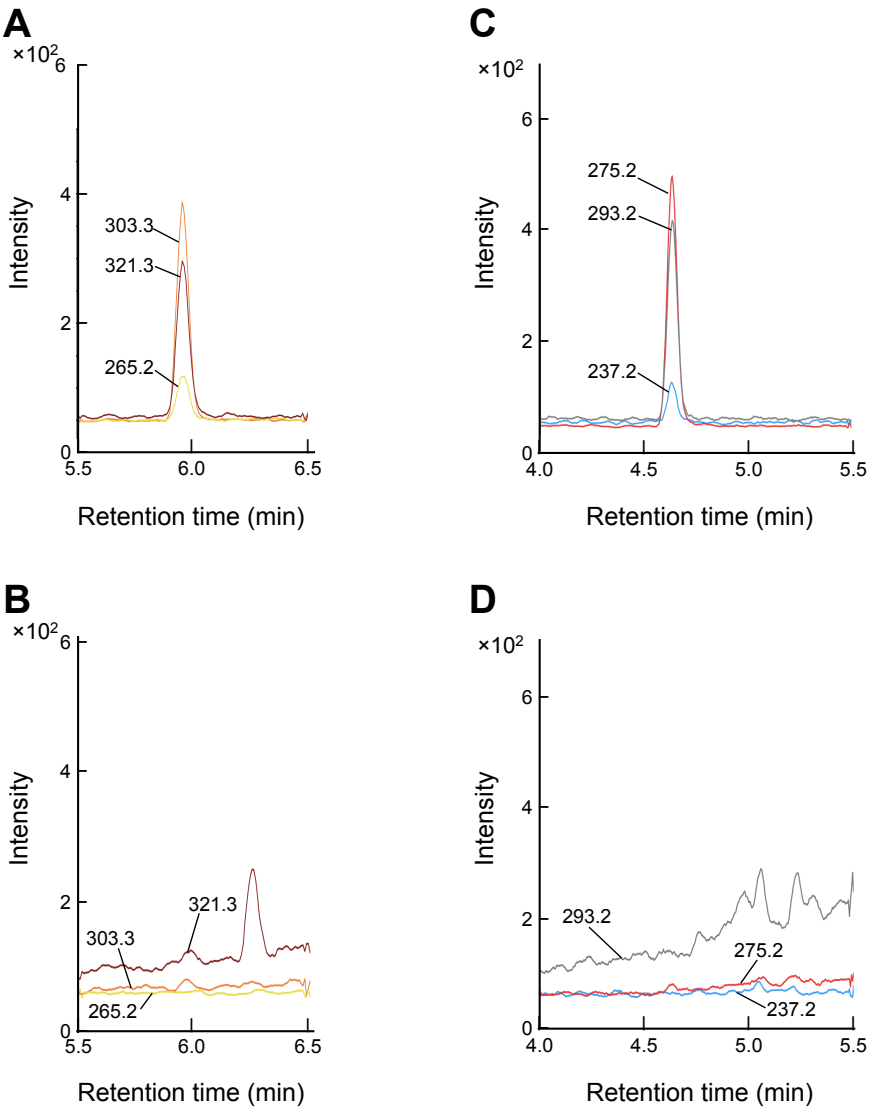

Figure S4. LC/ESI-MS/MS analysis of 7α-OH-Preg and 7α-OH-DHEA in the hippocampal extract of trained KO mice. Related to Figure 4.

MRM chromatograms of LC-ESI-MS/MS analysis of standard 7α-OH-Preg (**A**) and 7α-OH-DHEA (**C**) solutions (10 pg each) and the hippocampal extract in trained KO mice (**B**, **D**). (**A**, **B**) MRM transitions for detecting 7α-OH-Preg were 339.2->321.3 (claret), 339.2->303.3 (orange), and 339.2->265.2 (yellow). (**C**, **D**) MRM transitions for detecting 7α-OH-DHEA were 311.1->293.2 (gray), 311.1->275.2 (red), and 311.1->237.2 (blue).

Supplemental table

Table S1. Product ion species of 7α-OH-Preg and 7α-OH-DHEA employed in multiple reaction monitoring (MRM) analysis of UPLC/ESI-MS/MS. Related to Figure 4.

Compound Name	RT/ min	Precursor Ion	MS1 Res	Product Ion	MS2 Res	Dwell	Fragmentor	Collision Energy	Cell Accelerator Voltage
7α-OH-Preg	5.900	339.2	Widest	321.3	Unit	20	155	18	4
	5.901			303.3				20	
	5.900			265.2				24	
7α-OH-DHEA	4.579	311.1	Widest	293.2	Unit	20	155	19	4
	4.578			275.2				20	
	4.579			237.2				24	

Transparent Methods

Animals

Male mice (11-18 weeks) were housed under a 12 hr/12 hr light/dark cycle (LD) with the light provided by a white fluorescent lamp (300-400 lx at the level of the heads of mice) in a light-tight chamber at 23 ± 1 °C and constant humidity (55 ± 10 %) in cages with commercial chow (CLEA Japan, Inc.) and water available *ad libitum*. The time point when the light turned on was defined as zeitgeber time (ZT) 0 and the turned off as ZT12. In all the behavioral experiments, mice were housed individually in their home cages, and handled daily for at least one week before the tasks. All animal experiments described here adhered to local guidelines of the University of Tokyo, and all appropriate ethical approval and licenses were obtained.

Cyp7b1 knockout mice on the C57BL/6J genetic background were obtained from the Jackson Laboratory. In this mutant, deletion of exon 6 of the *Cyp7b1* gene eliminated CYP7B1 protein and its enzyme activity completely (Li-Hawkins *et al.*, 2000). Previously, it was reported that *Cyp7b1* KO mice have defects in prostate proliferation (Omoto *et al.*, 2005) or reproductive behaviors (Oyola *et al.*, 2015), but in our laboratory, *Cyp7b1* KO mice were fertile and apparently indistinguishable from WT mice in terms of survival and gross physical appearances as described by Li-Hawkins *et al*. (Li-Hawkins *et al.*, 2000).

To investigate locomotor activity, mice were housed individually in cages equipped with infrared area sensors (Elekit). Mice were entrained to a light/dark (LD) cycle for at least four weeks and released into constant darkness (DD). The total activities were recorded in five-min bins and analyzed with ClockLab analysis software (Actimetrics). The circadian period of the activity rhythms in DD was determined by using an extrapolation procedure with ClockLab. The time points of the onsets of mice activity were defined as circadian time (CT) 12.

RNA extraction and RT-qPCR

Total RNAs were extracted from the mouse brain regions by TRIzol reagent (Invitrogen). Total RNAs extracted from the pineal glands and the prefrontal cortexes were purified by using RNeasy MinElute Cleanup Kit (Qiagen), and those from the other brain tissues were purified by using RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. The RNAs were reverse-transcribed into cDNA using oligo $(dT)_{15}$ primer with GoScript Reverse Transcriptase (Promega). The cDNA was subjected to quantitative PCR using GoTaq qPCR Master Mix (Promega) with the StepOnePlus Real-time PCR system (Applied Biosystems) according to the manufacturer's protocol. The mRNA levels were quantified using a relative standard curve

method with hippocampal cDNAs as the standard. The gene-specific primers used for RT-qPCR were *StAR*-Fw (5'-GCTGG AAGCT CCTAT AGACA-3'), *StAR*-Rv (5'-AGCTC CGACG TCGAA CTTGA-3'), *Cyp11a1*-Fw (5'-ACATG GCCAA GATGG TACAG TTG-3'), *Cyp11a1*-Rv (5'-ACGAA GCACC AGGTC ATTCA C-3'), *Cyp7b1*-Fw (5'-CGGAA ATCTT CGATG CTCC-3'), *Cyp7b1*-Rv (5'-TAGCC CTATA GGCTT CCTGT CG-3'), *Cyp17a1*-Fw (5'-TGACC AGTAT GTAGG CTTCA GTCG-3'), *Cyp17a1*-Rv (5'-TCCTT CGGGA TGGCA AACTC TC-3'), *Rps29*-Fw (5'-TGAAG GCAAG ATGGG TCAC-3'), and *Rps29*-Rv (5'-GCACA TGTTC AGCCC GTATT-3').

Morris water maze test

The Morris water maze test was performed in a white Plexiglas circular pool (30 × ϕ100 cm; O'Hara & Co., Tokyo, Japan), which was set at a fixed position apart from the room walls and curtains and filled to a depth of 21 cm with 24 ± 1 °C water made opaque by adding TiO_2 (3 g). A circular escape platform (20 cm height, ϕ10 cm) was placed 1 cm below the water surface at a fixed position in a goal quadrant. All the experiments were performed under a flat white LED light at 4.0 ± 0.1 lx at the water surface. For the purpose of habituation to the platform before the water maze training, a mouse was placed on the platform surrounded by an equilateral triangle frame (40 cm on a side, 45 cm in height) without any visible cues and kept there for three min.

Male mice (11-18 weeks) of *Cyp7b1* KO and the littermate WT were composed of 12-15 individuals. The mice were trained for five consecutive days (Day 1 to Day 5) at ZT1 with four training trials per day at three to seven min inter-trial intervals. On each training trial, a start position was selected randomly from one of the three positions at the pool edge of the quadrants (right, left, and opposite to the goal quadrant). The mouse was gently placed in the water facing the wall. The trial was completed once the mouse found the hidden platform. If the mouse failed to reach the platform within 60 sec on a given trial, an experimenter gently guided the mouse onto the platform.

On the next day (Day 6) of the last training, spatial memory was assessed by a probe test (probe test I), in which the platform was removed from the pool. The mouse was allowed to search for the platform for 60 sec, and the time when the mouse stayed in each quadrant was measured. A similar probe test (probe test II) was performed at two weeks after the probe test I.

All the mouse behaviors were recorded by a video camera positioned right above the pool. The trace and the swimming speed were analyzed by a computer-operated system (TimeMWM;

O'Hara & Co., Tokyo, Japan), which also judged the goal time (escape latency) by detecting two-sec stay time on the platform. Rate of the recording was 25 frames/sec.

Golgi staining and dendritic spine analysis

Mice were trained in the Morris water maze task (trained) or maintained without training (untrained). Brains were isolated six hours after the last training and stained by using FD Rapid GolgiStain kit (FD NeuroTechnologies, Inc) according to the manufacturer's protocol. Briefly, the brains were rinsed with water and immersed in a mixture of FD Solution A/B (1/1, v/v, 2 mL) overnight at room temperature in the dark. On the next day, the mixed solution was renewed and brains were incubated at room temperature in the dark for two weeks. Then, brains were transferred to 2 mL FD Solution C. On the next day, the solution was renewed and brains were further incubated at room temperature in the dark for one week. The solution was removed and the brains were frozen at -80 °C. The cryoprotected brains were sliced into 100 μm-thick sections using a microtome-cryostat (Leica). Sections were put on 3% gelatin-coated slides and dried overnight before staining. The sections were rinsed twice with chilled water and immersed in a mixture of FD Solution D/E/water (1/1/2, v/v/v, 200 mL) for 10 min. After rinse twice with chilled water, sections were dehydrated in a series of ethanol solutions (50%, 75%, 95%, and 100% ethanols).

Dendrites spanning from hippocampal neurons of both hemispheres were drawn using a camera lucida attached to a Leica DMI6000B microscope (magnification 100×). The images were analyzed by using Neurolucida software (MBF Bioscience). In hippocampal CA1, regions between the first through second-order branches (12- to 100-μm length) of the dendrites located at 20- to 150-μm distances from the center of the cell body were traced and the dendritic spines were counted. Totally three basal dendrites per single neuron were selected from each mouse (n = 5; 12 neurons per each mouse). In the molecular layer of DG, regions between the first through second-order branches (17- to 300-μm length) of the dendrites located at 30- to 200-μm distances from the center of the cell body were traced and the spines were counted. Totally three dendrites per single neuron were selected from each mouse (n = 5; 12 neurons per each mouse). The spine densities (number per 1 μm) were averaged across animals in each group.

Extraction and purification of steroids

Mice were trained in the Morris water maze task (first day only, 4 trials), and sacrificed two hours after the last trial. Hippocampi isolated from two male mice were homogenized in ten

volumes (0.6 mL) of 0.25 M acetic acid solution with a glass-Teflon homogenizer on ice. Steroids were extracted by 0.6 mL ethyl acetate three times, and the extracts collected in glassware were evaporated under a gentle stream of nitrogen. The dried extract was dissolved in 0.75 mL acetonitrile/H_2O (50/50, v/v), collected in a 1.5 mL-tube and then centrifuged (3,000 × *g*, 5 min, 4 °C). The supernatant was diluted with 0.75 mL H_2O and subjected to purification with a solid-phase extraction column (HF Bond Elut, 50 mg, 1 mL; Agilent), which had been pre-washed two times with 0.5 mL acetonitrile/H_2O (80/20, v/v) and equilibrated with 1 mL acetonitrile/H_2O (5/95, v/v) successively. The sample was loaded on the column and the flow-through fraction was discarded. The column was then washed three times with 0.5 mL acetonitrile/H_2O (5/95, v/v), and the steroid fraction was eluted with 0.5 mL acetonitrile/H_2O (80/20, v/v) and evaporated under vacuum.

Liquid chromatography-mass spectrometry

7α-OH-Preg and 7α-OH-DHEA were separated by using an Agilent 1290 Infinity II LC system (Waldbronn, Germany) consisting of a binary pump, a micro vacuum degasser, a temperature controlled auto-sampler and a column oven. The sample storage temperature was 4 °C. The stationary phase was an Agilent Eclipse Plus C18 RRHD column (2.1×100 mm, particle size: 1.8 μm), which was maintained at 40 °C. The steroid sample was dissolved in methanol/H_2O (40/60, v/v; 12 μL), and 10 μL of the reconstituted sample was injected and eluted at a flow rate of 0.4 mL/min. The mobile phase consisted of 0.1% formic acid in H_2O (solvent A) and 0.1% formic acid in methanol (solvent B). The elution gradient was formed as follows: 40-80% B from 0 to 8 min, 80% B from 8 to 10 min, 80-40% from 10.0 to 10.1 min, and 40% B from 10.1 to 13.1 min. The post-column addition of 0.2 mM LiCl (final 0.1 mM) was done with Agilent 1100 binary pump as the auxiliary pump. Mass spectrometry experiments were performed with electrospray ionization (ESI) in positive ion mode. The capillary voltage was -3,500 V. The gas temperature was maintained at 210 °C. The sheath gas temperature was 275 °C and the flow rate was 12 L/min. The nebulizer gas flow was 13 L/min. Multiple reaction monitoring (MRM) was applied for highly selective and sensitive detection of 7α-OH-Preg and 7α-OH-DHEA. Three different MRM transitions were set and the measuring condition was optimized for each steroid as shown in Table S1. It was difficult to quantify small amounts of the steroids in the hippocampal extract due to noisy baseline (Figure 4C, F).

Intracerebroventricular infusion of steroids

Cyp7b1 KO male mice (11-15 weeks) were anesthetized by an intraperitoneal injection (20 μL/g body weight) of a mixture of ketamine (4.4 mg/mL; Daiichi Sankyo Propharma) and xylazine (0.44 mg/mL; Bayer Health Care) dissolved in bacteriostatic saline. The anaesthetized animals were placed in a stereotaxic frame (Narishige Inc.) keeping bregma and lambda at a horizontal level. A small hole was drilled in the skull, and a chronic indwelling stainless-steel cannula (ALZET Brain Infusion Kit 1; DURECT Co.) was placed into the right lateral ventricle using the following coordinates relative to bregma: 0.22 mm posterior; 1.0 mm right lateral; 2.3 mm below the horizontal plane of bregma. The cannula was fixed to the skull with adhesive and dental cement, and the external part of the intracerebroventricular cannula was connected with polyethylene tube to an ALZET osmotic minipump with an infusion rate of 0.11 μL/h for four weeks (model 1004; DURECT Co.) placed subcutaneously on the back. Steroids were dissolved in an artificial cerebrospinal fluid (aCSF) containing 1% DMSO. aCSF was prepared by mixing the same volume of solvent A containing 150 mM NaCl, 3 mM KCl, 1.4 mM $CaCl_2$, 0.8 mM $MgCl_2$ and solvent B containing 0.8 mM Na_2HPO_4, 0.2 mM NaH_2PO_4, and pH was adjusted at 7.4 with NaOH. The concentration of 7α-OH-Preg (Steraloids Inc.) or 7α-OH-DHEA (Steraloids Inc.) solution was 910 ng/μL, and that of the mixed steroid solution was 455 ng/μL each of 7α-OH-Preg and 7α-OH-DHEA. The dose of steroids was chosen based on the previous study (Yau *et al.*, 2006). All the mice were handled for at least one week before the surgery, housed individually after the surgery and allowed to recover at least three days before the Morris water maze test.

Elevated plus maze test

The elevated plus maze (EPM) was constructed of Plexiglas (O'Hara & Co., Tokyo, Japan) with two open arms (25×5 cm) and two enclosed arms with clear walls (15 cm height) at an elevation of 50 cm above the floor. The arms of the maze form a cross with the two open arms facing each other. The maze was washed and dried after each test.

Male mice of *Cyp7b1* KO and the littermate WT were composed of 10-16 individuals. The mice were placed on the center of the maze alone facing one of the closed arms under a flat white LED light at 4.0 lx ± 0.1 lx at the center of the apparatus. The behavior was recorded for 5 min by a video camera positioned above the maze. The rate of recording: 4 frame/s, moving criterion: 3 cm.

The numbers of entries into the open and closed arms and the time spent exploring the open and closed arms were analyzed using a computer-operated system (TimeEP1; O'Hara & Co., Tokyo, Japan).

Open field test

Male mice of *Cyp7b1* KO and the littermate WT were composed of 11-12 individuals. The mice were placed alone in the right front corner of a white Plexiglas open field arena (40×40 cm, 30 cm height) (O'Hara & Co., Tokyo, Japan) and allowed to explore for 5 min under a white LED light at 330 lx ± 10 lx at the center of the apparatus. The arena was cleaned up with disinfectant spray and water spray in this order after each test. The activity in the open field was recorded by a video camera positioned above the arena using a computer-operated system (OpenField plug-in; O'Hara & Co., Ltd.), a modified software based on the public domain Image J program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/ij). Total distance and the time spent in the center area of the arena were calculated. The rate of recording: 3 frames/s, center area: 30%.

Supplemental references

Omoto, Y., Lathe, R., Warner, M., and Gustafsson, J.-Å. (2005). Early onset of puberty and early ovarian failure in CYP7B1 knockout mice. Proc. Natl. Acad. Sci. U. S. A. *102*, 2814-2819.

Oyola, M.G., Zuloaga, D.G., Carbone, D., Malysz, A.M., Acevedo-Rodriguez, A., Handa, R.J., and Mani, S.K. (2015). CYP7B1 Enzyme Deletion Impairs Reproductive Behaviors in Male Mice. Endocrinology *156*, 2150-2161.