Supplemental Information

Supplemental Methods and Materials

Mice. AC3 KO mice and AC3 WT littermates were bred from heterozygotes and genotyped as previously reported[1, 2]. The mice used in behavioral analysis were age-matched 2.5 to 4 month-old males with comparable body weight, if not otherwise indicated. Mice were maintained on a 12-h light/dark cycle at 22°C and had access to food and water *ad libitum*. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Washington and performed in accordance with their guidelines. For behavioral tests, mice were handled by the investigator for 5-7 days to allow them to adjust to the investigator before starting the experiments.

Tail suspension test. The tail suspension test was used to assay depression behavior in mice. Mice were suspended upside-down from their tail for 6 min. The session was recorded by a video camera and the total time immobile was scored.

Forced swim test. Experiments were performed in a glass cylinder (14 cm inner diameter, with water level 13 cm deep) filled with room temperature water (22-23 °C). The legs of the mice were unable to touch the bottom of the cylinder. Mice were gently placed into the water and swimming behaviors were video-recorded for 6 min. The total duration of immobility was scored during the last 4 min. Immobility was defined as no movement of the front or back legs and no attempt to escape.

3-Chamber sociability test. The apparatus for sociability test was a rectangular, three-chamber box. Each chamber was 22 x 20 x 13 cm and the walls of chamber were made from Plexiglas. There were open holes between chambers that allowed subject mouse to freely explore each chamber. Two identical, wire mesh containers that were large enough to hold a single interacting target mouse were used. These two wire cups were placed inside the apparatus, one in left and one in right; none in the middle chamber. The mesh cup containers prevented direct physical interaction between the inside target mouse with the

outside subject mouse. We used a young (6-8 weeks) male C57BL/6 mouse that had no any prior contact with the subject mouse as the interacting target mouse. During the sociability test, we first placed a subject mouse in the apparatus for habituation for 5 min. Subsequently, one C57BL/6 target mouse was placed inside a wire cup in a chamber in one side. The other wire cup was left empty in the other side. The subject mouse then freely explored any of the three chambers. The exploration/interaction lasted 10 min and was video-recorded. The exploring time in each chamber was analyzed by Ethovision software.

Novelty-suppressed feeding. Exposure to a novel environment suppresses feeding behavior in rodents and this test has been widely used to assess depression and anti-depressants. Mice were food-deprived for 24 h before testing. A novel open container was used to present novel environments to mice. A piece of Whatman paper was placed in the center of the container with rodent chow placed on the paper. The trial duration was 15 min and video recorded. The latency to feeding (defined as nibbling the food pellet for 4 s) and the total feeding time during testing period were recorded.

Novelty-suppressed drinking. We examined novelty-suppressed drinking as a supplemental experiment to the novelty-suppressed feeding because drinking water doesn't need olfactory stimuli. Mice were deprived of water in their home cage overnight and then were placed into a novel open chamber with free water access. The trial duration was 15 min and it was video recorded. The latency to begin sipping water and the total time of sipping during the testing period were scored.

Nesting behavior test. Nesting behavior was tested on mice. Under regular housing condition, cages were changed around noon and a cotton nestlet was placed in each cage. Nest quality was evaluated at 4 time points (3, 6, 24 and 48 hours after cage change) using 5-points rating scale: score 1, the cotton square is intact; score 2, the cotton square is partially used; score 3, the cotton is scattered but no form of nest; score 4, the cotton is gathered but only with flat nest; score 5, the cotton is well nested into a ball with a small passage for entry (look like warm, cozy and safe). Nesting behavior of same groups of AC3

mice were also tested in novel environment, i,e. in transparent Plexiglas circular chambers (20 cm high and 25 cm diameter) with food and water freely accessible.

EEG/EMG headmount implantations, signal recordings and data analysis. Each mouse (3 to 4 months old, male) was implanted with an EEG/EMG headmount following the manufacturer's instructions (Pinnacle Technology, Lawrence, Kansas). Briefly, mice were anesthetized with a ketamine/xylazine cocktail and aligned in a stereotaxic apparatus. After exposing the skull surface, headmount was centered along the sagital suture with the front edge 3.5 mm anterior to bregma. Headmount was then secured with four stainless steel screws (also functioned as EEG electrodes) and coated in acrylic for insulation. When positioned properly, all four screws (two anterior: AP 3.0 mm, ML ± 1.75 mm; two posterior: AP - 4.0 mm, ML ± 1.75 mm, relative to Bregma) sit on the cerebral region of an adult mouse. Two EMG wires were inserted bilaterally into the trapezius muscles to monitor neck activity. After surgery, mice were allowed one week to recover in their home cages before tethering to the commutators in circular recording cages separately. Mice were adapted to the recording apparatus in free-moving condition for 2 days. After acclimation to the recording environment and tether wire, EEG/EMG signals were collected continuously for 24 hours using Sirenia software (Pinnacle Technology, Lawrence, Kansas). EEG/EMG signals were amplified 5,000 times and sampled with a rate of 400 Hz. EEG and EMG signals were low-pass filtered at 70 Hz and 100 Hz respectively. EEG/EMG data were scored off-line through manual visual inspection assisted with cluster scoring method of Sleep Pro Version 1.3 (Pinnacle Technology) to distinguish waking, REM sleep and NREM sleep based on standard criteria including the amplitude, frequency and regularity of EEG and EMG signal. EEG/EMG sleep/wake events in 10-s epoch were first scored as wake (low-amplitude, high frequency EEG and high-amplitude EMG), NREM sleep (high-voltage, low frequency EEG and low amplitude EMG) or REM sleep (low-voltage EEG mostly theta wave and EMG atonia). EEG/EMG scores were further

analyzed with MS Excel and Sirenia Sleep Pro V1.3 (Pinnacle Technology). After scoring, EEG power spectrum analysis of NREM sleep was performed with Sirenia Sleep Pro V1.3.

Volume measurement of hippocampal regions. Volume estimation of CA1 and DG subregion was conducted using the Cavalieri Estimator probe of Stereo Investigator software (MBF Bioscience) on a Zeiss Axio Imager with a ×5 objective (NA 0.16). Every fourth section in a series of 40-μm coronal section spanning between 1.34 and 3.58 mm posterior relative to bregma was stained with cresyl violet. A 150-μm^2 point-counting grid was randomly superimposed over each section by the stereology system. The volume of each subregion was determined by multiplying the total grid points for per subregion by the section thickness.

Sholl analyses of morphology of primarily cultured cortical neurons from AC3 WT or KO mice. Sholl analyses of neuronal morphology were performed as previously described with some modifications [3]. Briefly, cortical neurons were isolated and cultured from AC3 WT or AC3 KO newborn pups at postnatal day 1 [4]. To visualize cell morphology, cultured neurons were infected with low titer lentivirus expressing EGFP at DIV 7. At DIV 12, cultured neurons were fixed with 4% paraformaldehyde and incubated overnight at 4 °C with the following primary antibodies: mouse anti-GFP (A11120, 1:500; Invitrogen) and rabbit anti-type 3 adenylyl cyclase (SC-588, 1:500, Santa Cruz Biotechnology). Individual GFP positive neurons (from 5 AC3 WT or AC3 KO 4 newborn pups) selected from 3 cover slips (10-30 neurons per mouse) were photographed using an Zeiss 510 META confocal microscope fitted with a 40 x oil objective. All raw images of were re-sampled to obtain smaller, higher resolution grayscale binary images. The grayscale images were thresholded to create high-contrast tiff format image files. Images were then opened in Image J64 (http://rsbweb.nih.gov/ij) and analyzed using the Sholl Analysis plugin. The intersections of each cell's processes with concentric rings placed every 10 μm up to 200 μm from a point positioned in the center of each soma was counted,

and the means of the various treatment groups were compared using two-way ANOVA (Prism5 software, Graphpad).

Spatial learning and memory in the Morris water maze. The water maze was a circular metal pool 122 cm in diameter and 31 cm in height. The water temperature was maintained at 21°C–23°C. Nontoxic water-soluble paint was added to make the water opaque. A 10 x 5 cm rectangle escape platform was submerged 1 cm below the water surface (hidden platform) at a fixed location during the training. Mice were given three trials a day for 5 consecutive days with a 1.0 h inter-trial interval each day. During training, each mouse was allowed to swim freely for 120 s and allowed to climb onto the platform. During each trial, the mouse was placed facing along the edge of the wall and randomly released at different start points. Each trial ended when either the mouse had climbed onto the platform or 120 s had passed before the mouse located the platform and the mouse was placed on the platform. After locating the platform, each mouse was allowed to rest for 30 s on the platform. During each trial, the time elapsed for locating the platform was determined. A probe test was carried out on day 6. During the probe test, the escape platform was removed from the water maze. Mice were placed and released at the location opposite the site where the platform had been located and allowed to swim for 120 s. The probe test procedure was recorded by a video-recorder that was fixed on the ceiling above the pool. The time spent in each quadrant and the numbers of crossings of the platform were analyzed. After the probe test, mice were subjected to reversal training (3 trials per day). During reversal training, the escape platform was hidden in a different location, mice were allowed 120 s to swim and find the platform followed by 30 s of rest on the platform. The latency to reach new platform site of each trial was analyzed.

Local field potential recording in acute brain slices. Local field recording was performed as previously described [5]. Mice (2.5 to 3 months old) were decapitated and brain tissues were rapidly taken out and placed in oxygenated (95% O_2, 5% CO_2) ice-cold artificial cerebral spinal fluid (aCSF;

composition: 125 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 1.25 mM NaH_2PO_4, 25 mM $NaHCO_3$ and 10 mM D-glucose). Coronal hippocampal slices (400 μm) were cut with a Leica vibrotome (VT1200S) and were left at room temperature to recover for at least 1 h before being transferred to a submerged recording chamber. Population spikes were recorded from the CA1 pyramidal layer with stimulating electrode placed in the stratum radiatum region. Field excitatory post-synaptic potential (fEPSP) recordings were made from the stratum radiatum region of CA1 of the hippocampus using electrodes filled with aCSF (resistance 3–5MΩ). Baseline stimulation along the Schaffer-collateral pathway was accomplished with a bipolar tungsten electrode (Rhodes Medical Instruments) delivered via a Grass S11 Stimulator. Baseline stimulated pulses consisted of 0.1-ms square waves. The baseline period consisted of at least 15 min of stable recordings taken at ~ half-maximum response strength. After the baseline period, decremented LTP (D-LTP) was induced with a stimulation protocol consisting of 1 train of 100 stimuli delivered at 100 Hz in 1 s. Long-lasting LTP (L-LTP) was induced with a stimulation consisting of 4 trains of 1 second 100 stimuli delivered at 100 Hz every 5 min. After induction, fEPSP was recorded for more than 60 min (D-LTP) or more than 160 min (L-LTP). For analysis, potentiated responses were expressed as a percentage of the mean baseline fEPSP slope (normalized fEPSP) averaged in 1-min bins.

Whole cell blind-patch recording of pyramidal neurons in CA1 region. Hippocampal slices were cut from young AC3 WT and AC3 KO mice (4-6 weeks) under ice-cold high magnesium sucrose solution: 87 mM NaCl, 2.5 mM KCl, 7 mM MgCl2, 0.5 mM CaCl2, 1.25 mM NaH2PO4, 25 mM NaHCO3, 10 mM D-glucose and 75 mM sucrose. After cutting, slices were placed in aCSF to recover for 1-2 hours at room temperature. Recording pipettes were prepared from thin wall borosilicate glass capillaries (A-M system, Sequim WA).Patch clamp electrodes (pipette resistance 4-7 MΩ) were filled with intracellular solution containing the following: 135.5 mM potassium gluconate, 17.5 mM KCl, 0.2 mM ethyleneglycol-bis-(α-amino-ethyl ether) N, N′-tetra-acetic acid (EGTA), 10 mM HEPES and 2 mM

MgATP and 0.3 mM GTP, osmolarity 290-300, pH 7.2. Whole-cell mode was achieved using the ‘‘blind’’ patch method[6]. Pyramidal neurons were distinguished from interneurons based on membrane and electrical properties. The resting membrane potential of neurons was measured immediately after rupture of membrane in recording pipette via suction. Whole-cell voltage-clamp recordings from CA1 neurons within hippocampal slices were obtained at room temperature (22–24°C). Unless specified otherwise, the membrane potential was held at -70 mV throughout the recordings. Spontaneous EPSC signal was amplified with an Axopatch-200B amplifier (Molecular Devices, Sunnyvale, CA, USA). Electrophysiological signals were filtered at 2 kHz and digitized at 5–10 kHz by means of a Digidata 1440 processor and/or were simultaneously digitized through a MiniDigi 1A processor. The signals were acquired online with pClamp10 (Axon Instruments, Foster City, CA, USA) or/and with Axoscope 10 (Axon Instruments, Foster City, CA, USA).

Fiber fluorescence confocal endomicroscopy calcium imaging. We used FFE, an advanced *in vivo* calcium imaging system (Cellvizio Neuropak^{TM} deep brain imaging system, Mauna Kea Technologies) in combination with viral-mediated gene delivery to hippocampal CA1 region to monitor overall CA1 neuronal activation in response to stimulation[7-9]. During surgery, a small skull window (coordination, AP: -1.85 to -1.95mm; ML:-1.4mm) above dorsal CA1 region of AC3 mice was made to allow penetration of a special cannula (Mauna Kea Technologies) that accommodated an imaging probe. The cannula was then installed on mouse head-top using dental Jet acrylic. We then stereotaxically injected 0.5-1 μl GCaMP3 (a calcium-sensitive fluorescence indicator)-expressing AAV1 (Vector Core, University of Pennsylvania) through the cannula into CA1 region (coordination, AP: -1.85 mm to -1.95mm; ML: -1.4mm; DV -1.4 mm). Imaging was performed 3 weeks after surgery and GCaMP3 expression. Mice were first positioned on a stereotaxic apparatus under isoflurane anesthesia. A CerboflexJ^{tm} Neuropak endomicroscope fibre optic probe was hooked to a vertical micropipette guide of the stereotaxic apparatus that allows positioning of the imaging probe and moving through cannula until

image was seen. After imaging probe reached GCaMP3 expression site in CA1 region, it was tightly fixed to cannula by a screw. After wake up, mice could freely behave with the imaging probe (the endomicroscope) on its head-top. Mice were then put in a box with food and water *ad libitum* for recovery from anesthesia for more than 1 hour. Mice were then placed in a foot shock box and after 2 min exploration; 2-s 0.7 mA foot shock stimulation was delivered. Neuronal activity was monitored with or without foot shock by the imaging probe. Imaging data were acquired at 11.7 Hz by Cellvizio 488® (Mauna Kea Technologies) and analyzed with IC Viewer 3.8 (Mauna Kea Technologies) off-line in combined with Graphpad Prism 5. After imaging, Mice were sacrificed and brain tissues were fixed with 4% paraformaldehyde and then subjected to immunological histochemistry staining using primary antibodies against AC3 (1:500, Cat# SC-588, Santa Cruz) and against GFP (1:500, Cat# A-11120, Invitrogen).

Immunostaining of brain slice from conditioning knockout mice. Mice were perfused with a mixture consisting of 4% paraformaldehyde in PBS. The brains were then dissected and post fixed with the same fixing solution overnight at 4°C. Then the brains were cryoprotected with 30% sucrose for 48 h at 4°C. Brains were cut into 30 μm sections with a cryostat freezing microtome. Floating sections were blocked and then first incubated with AC3 antibody (1:500, SC-588, Santa Cruz Biotechnology, Inc.) overnight at 4°C followed by the secondary antibody, Alexa Fluor 488-conjugated donkey anti-rabbit IgG (Invitrogen, La Jolla, CA), for 2 h at room temperature. DNA was stained with Hoechst (5 μM). Stained sections were mounted onto microscopy slides and visualized by confocal microscopy (Zeiss 510 META).

Actogram monitoring in home cage. Mice are individually housed and a photobeam sensor was installed at the litter top of each cage. Mice were maintained on a 12-h light/dark cycle at 22°C and had access to food and water *ad libitum.* Activity counts were collected in every 5 min. Actogram (reflecting

activity) was acquainted in 14 days with Vitalview data acquisition system and analyzed with Actiview 1.2 (Mini Mitter Company, Bend, OR).

Open field test. The open-field test was conducted in an open plastic container (38 X 56 X 23 cm). At the start of each trial, the mouse was placed in a corner of the container and its movement was video recorded for 30 min. The Noldus Ethovision 3.0 tracking software was used to monitor the time spent in the center and peripheral zones.

Elevated plus maze test. The plus maze consisted of a plus-shaped apparatus with two open and two enclosed arms, each with an open roof, elevated 40 cm from the floor. Each mouse was put into center of the plus maze and its free movement was video recorded for 10 min. The Noldus Ethovision 3.0 tracking software was used to analyze the mouse's preference for the open arm or closed arms.

Novelty object recognition. The novel-object recognition test was used to test recognition memory. The mice were first habituated in a cage for 30 min before training, after which two similar plastic blocks (A1 and A2) were presented for 5 min of training for two times with a interval of 1 hour. Object recognition was scored by the time percentage spent in each object zone. When the mice were tested for memory retention 24 h later, one of the original blocks A2 was replaced by a new object (B), and the mouse was scored for recognition during 5 min of testing. Recognition index was present as ratio of time spent in A2 relative to time spent in A1 (A2/A1) during training and time spent in B relative to time spent in A1 (B/A1) for testing.

Electroolfactogram (EOG) Recording. EOG recording were performed as previously described [2]. Mice (2-4 months) were killed by decapitation. Each head was bisected through the septum with a razor blade and the septal cartilage was removed to expose the olfactory turbinates. Odorized air was produced by blowing nitrogen through a horizontal glass cylinder that was half-filled with an odorant mix. The odor mix was comprised of eugenol, octanal, r-(+)-limonene, 1-heptanol, s-(−)-limonene,

acetophenone, carvone, 3-heptanone, 2-heptanone, ethyl vanillin, butyric acid, and citralva, each at 50 μM in H_2O. The duration of air puff was 200 ms. The tip of the puff application tube had an inner diameter of 1.3 mm, which was directly pointed to the recording site on the main olfactory epithelia (MOE). Several sites were recorded in the turbinates the MOE to verify the responses. The EOG field potential was detected with an agar-filled and Ringer's solution-filled glass micro-electrode in contact with the apical surface of the olfactory epithelia in an open circuit configuration.

Olfactory habituation/dis-habituation test. All the tests were done in home cages as previously described [7]. The test mouse was singly housed. Odor stimulations were delivered with a cotton-tipped swab placed through the cage top ~ 8 cm above the bedding. After 10 min of habituation with a cotton-tipped swab without odor stimulant, the test mouse was stimulated by several application of odorants: water, citralva (10 μM), C57BL/6J male mouse urine (1:30 dilution), and eugenol (10 μM). Each stimulus was 2 min in duration with 1 min inter-trial interval. The sequence of the odor stimulation was as follows: water1, water2, water3, citralva1, citralva2, citralva3, urine1, urine2, urine3, eugenol1, eugenol2, eugenol3. Time spent sniffing the odorants was measured by manual observation with a stopwatch. Sniffing was only scored when the test mouse's nose was close from and pointing to swab. The biting of swab by mouse was excluded.

Data analysis. Data were analyzed with GraphPad Prism 5 or with Clampfit 10.0 software (Axon Instruments), using Student's t-test, one-way ANOVA with Tukey post hoc test and two-way ANOVA with Bonferroni's post hoc comparison or using Kolmogorov-Smirnov test to compare samples with probability distributions, If not specifically indicated in the Figure Legend; statistical analysis was unpaired student t-test with a two-tailed distribution. n.s. not significant, * $p < .05$, ** $p < .01$, *** $p < .001$. Data was considered as statistically significant if p value < .05 and values in the graph are expressed as mean ± standard error of the mean.

References

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Supplementary Figure Legends

Fig. S1 (related to Fig 4). Population-spikes recorded in CA1 layer was reduced in AC3 KO mice. Population-spikes (pop-spikes) recorded from CA1 pyramidal cell layer. Schaffer-collateral synapses were electrically stimulated. Pop-spike recordings were performed in acute hippocampal slices that were incubated in aCSF for 4-5 hours. Representative pop-spikes (a) and point-point voltage sum of pop-spikes of AC3 WT and KO mice (B) are shown. n = 5 for $AC3^{+/+}$; n =10 for $AC3^{-/-}$; $^{**}p < 0.01$, $^{***}p < 0.001$.

Fig. S2. Generation of AC3 floxed mouse. (A) Scheme of the targeting construct including the DNA inserts of AC3 gene. (B) The 5' arm has 4.6 kb and 3' arm 3.46 kb insert in the targeting construct that was confirmed by restriction enzymatic assay. (C) Targeting strategy of AC3 floxed mouse. Scheme of 5' arm, Exon 3 and 3' arm in genomic DNA, vector and targeted allele. 3' probe (D) and neo probe (E) were used in Southern blot to screen and verify ES clones with targeted allele. (F) PCR was used for genotyping to screen homologous AC3 floxed mice. 5' primer: ACCCTTTGAGGCCAGGGGCAA; 3' primer: CTGCGGTGAGAGCCTGGCACA.

Fig. S3. Sequence of the loxP and exon 3 of the *Adcy3* gene in the targeting vector. Top panel shows the scheme of targeting construct. Bottom presents the sequence in the vicinity of exon3 in the targeting construct. Blue lines underline two lox P sites and black line the exon 3 of the Adcy3 gene.

Fig. S4 (related to Fig. 6). AC3 was ablated specifically in the forebrain. AC3 was expressed in WT mice ($AC3^{+/+}$:Emx1-Cre), but not present in AC3 forebrain KO mice ($AC3^{fr/fr}$:Emx1-Cre). Immunostaining of AC3 in hippocampal CA1 region and cortex are shown.

Fig. S5 (related to Fig. 6). AC3 was retained in the hypothalamus in the AC3 Forebrain KO mice.

Fig. S6 (related to Fig. 6). Verification of Cre expression in the forebrain of Emx1-Cre mice. A TdTomato Cre-reporter strain was cross-bred with Emx1-Cre mice and that was used to monitor Cre expression in the Emx1-Cre mice. Emx1-Cre mouse strain has focal Cre-expression in the forebrain (note that the cortex was in red color), not outside of the forebrain, or in the main olfactory epithelia (no red color, green arrow). Td-, no TdTomato Cre-reporter allele; Td+, having TdTomato Cre-reporter allele.

Fig. S7 (related to Fig. 7). AC3 was largely removed in CA1 and cortex in AC3 inducible KO mice ($AC3^{fr/fr}$:Ubc-Cre) by administration of tamoxifen (Tamo), but not by vehicle (Veh).

Fig. S8 (related to Fig. 7). Activity of AC3 inducible KO mice is reduced in home cage. Representative actogram of 4 days are shown. Control mice ($AC3^{fr/fr}$:Ubc-Cre, vehicle-treated) have higher activity during night-time than AC3 inducible KO mice ($AC3^{fr/fr}$:Ubc-Cre, tamoxifen-treated).

Fig S1 (related to Fig 4)

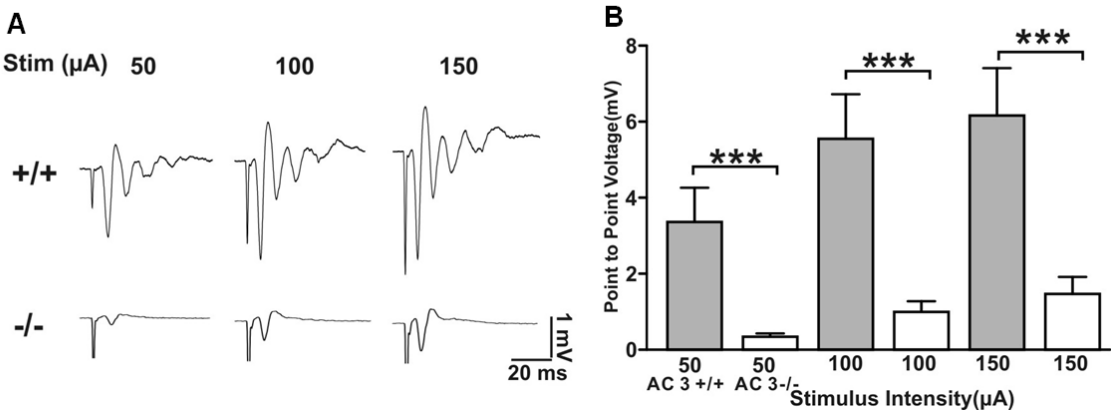

Fig. S2

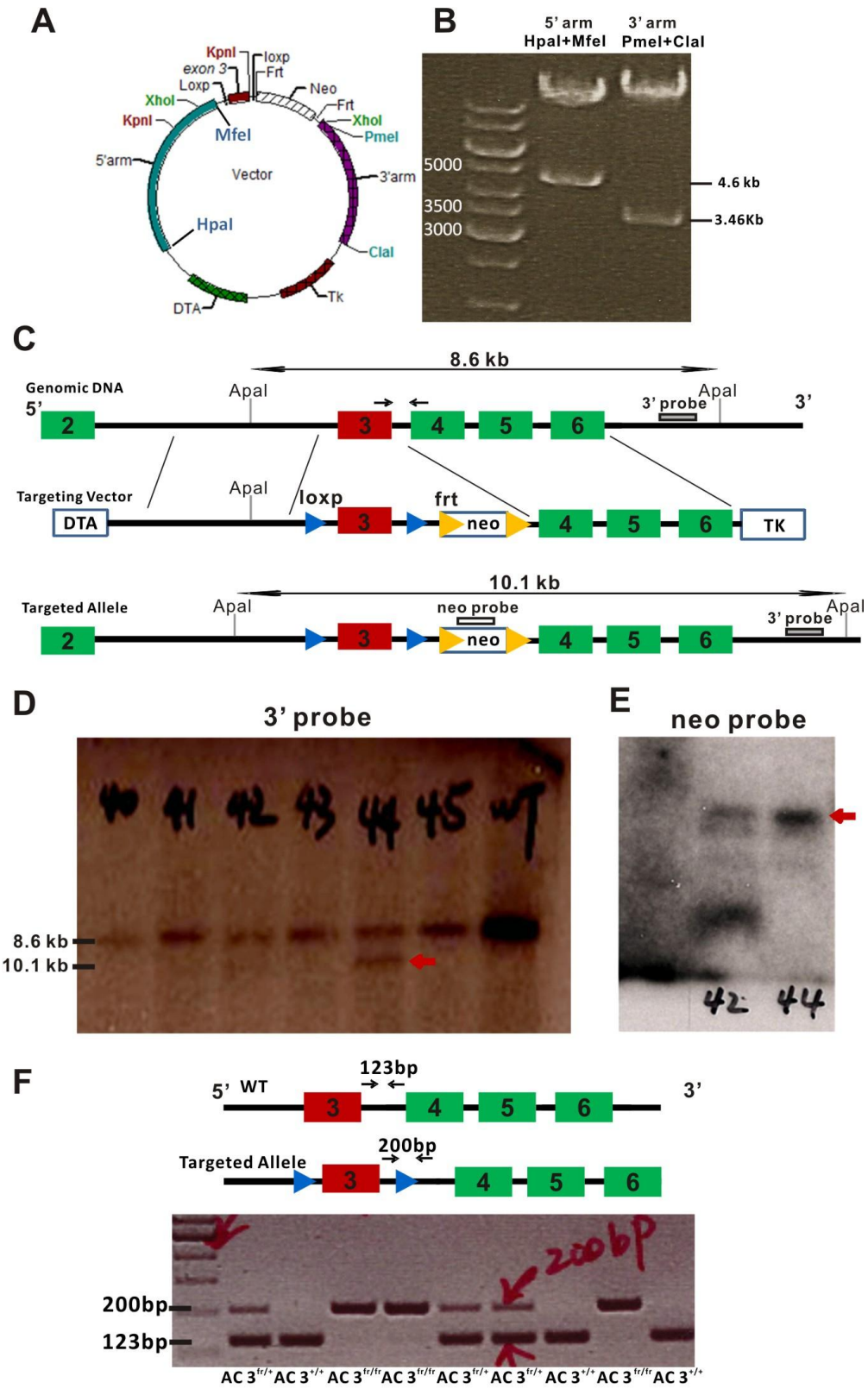
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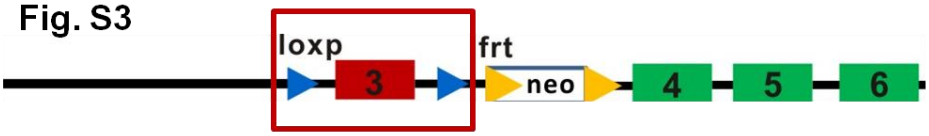
Fig. S3



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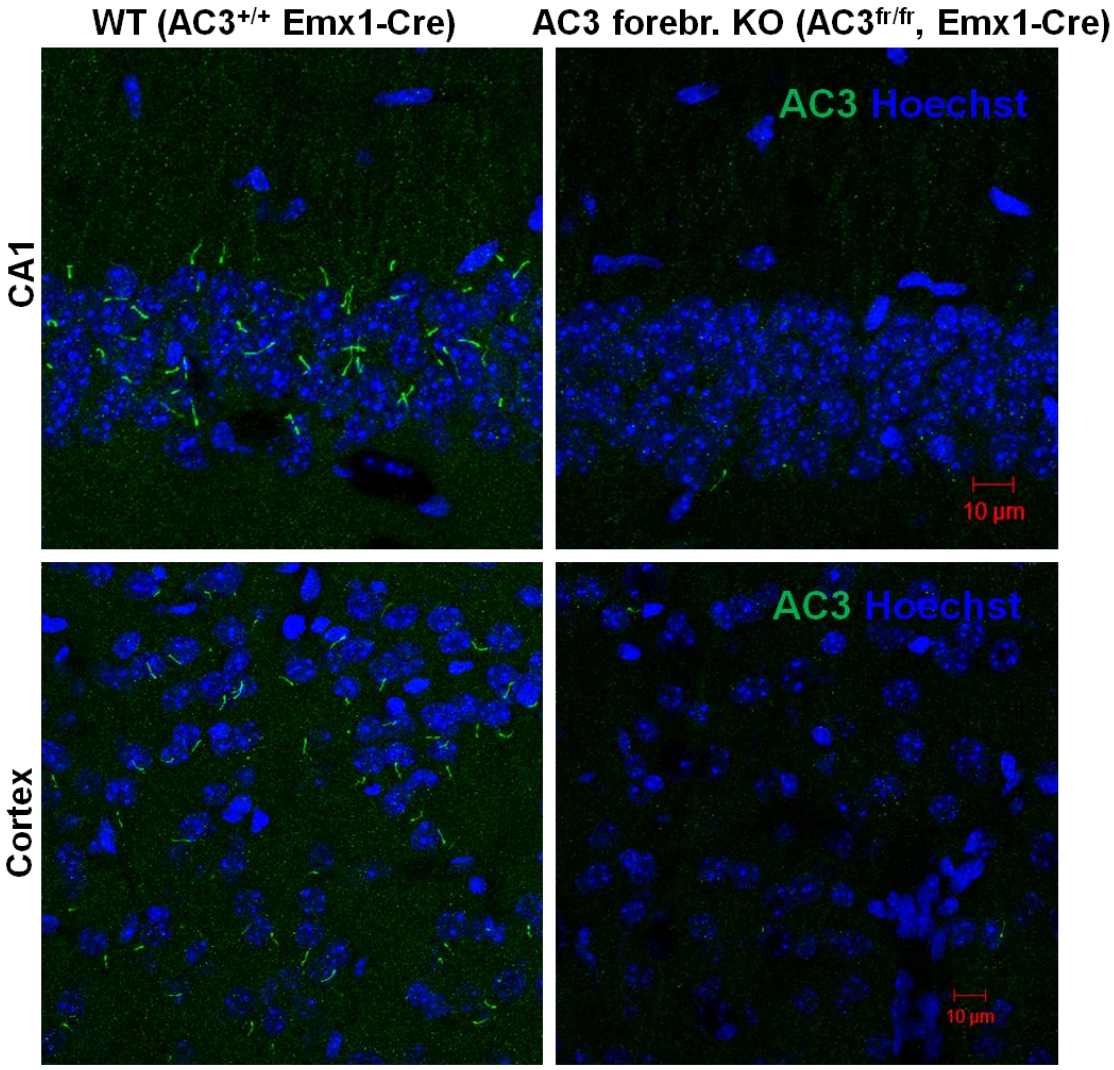

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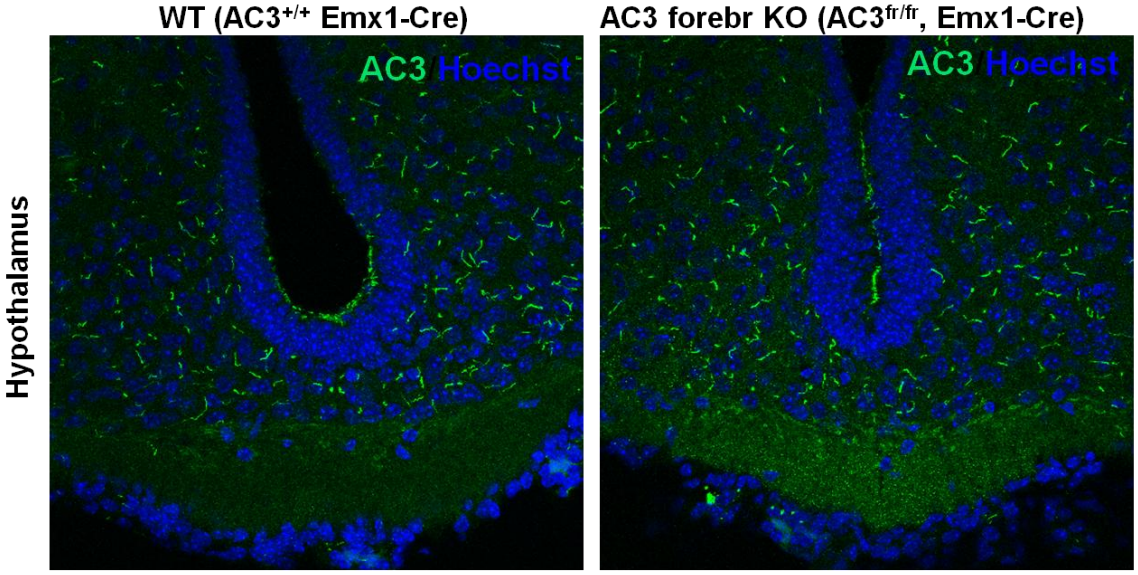
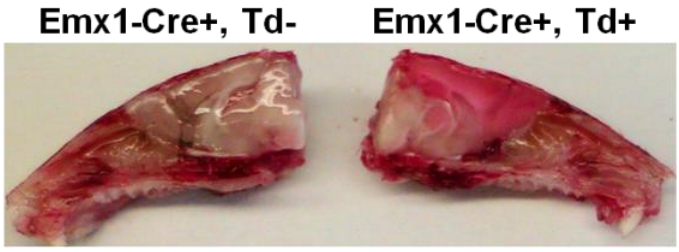


Fig. S6 (related to Fig. 6)



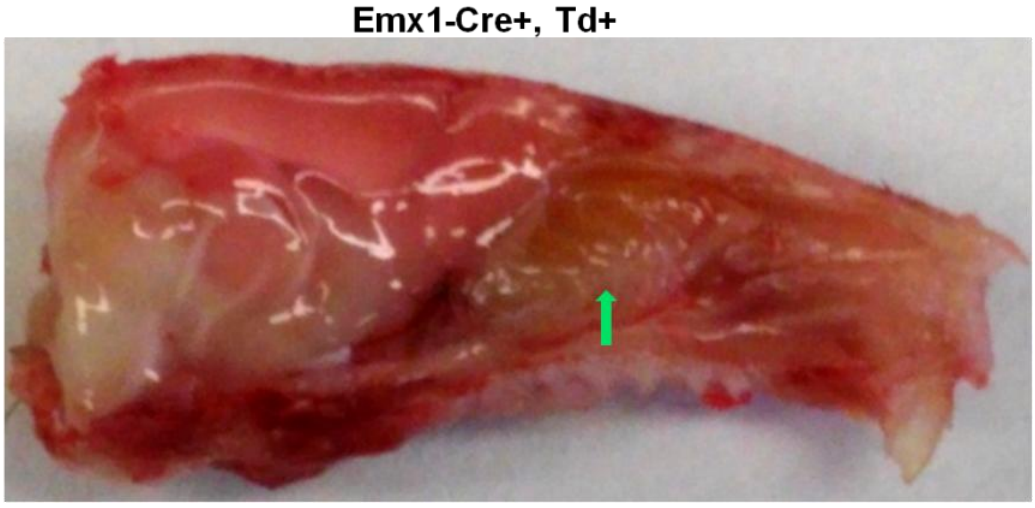


Fig. S7 (related to Fig. 7)

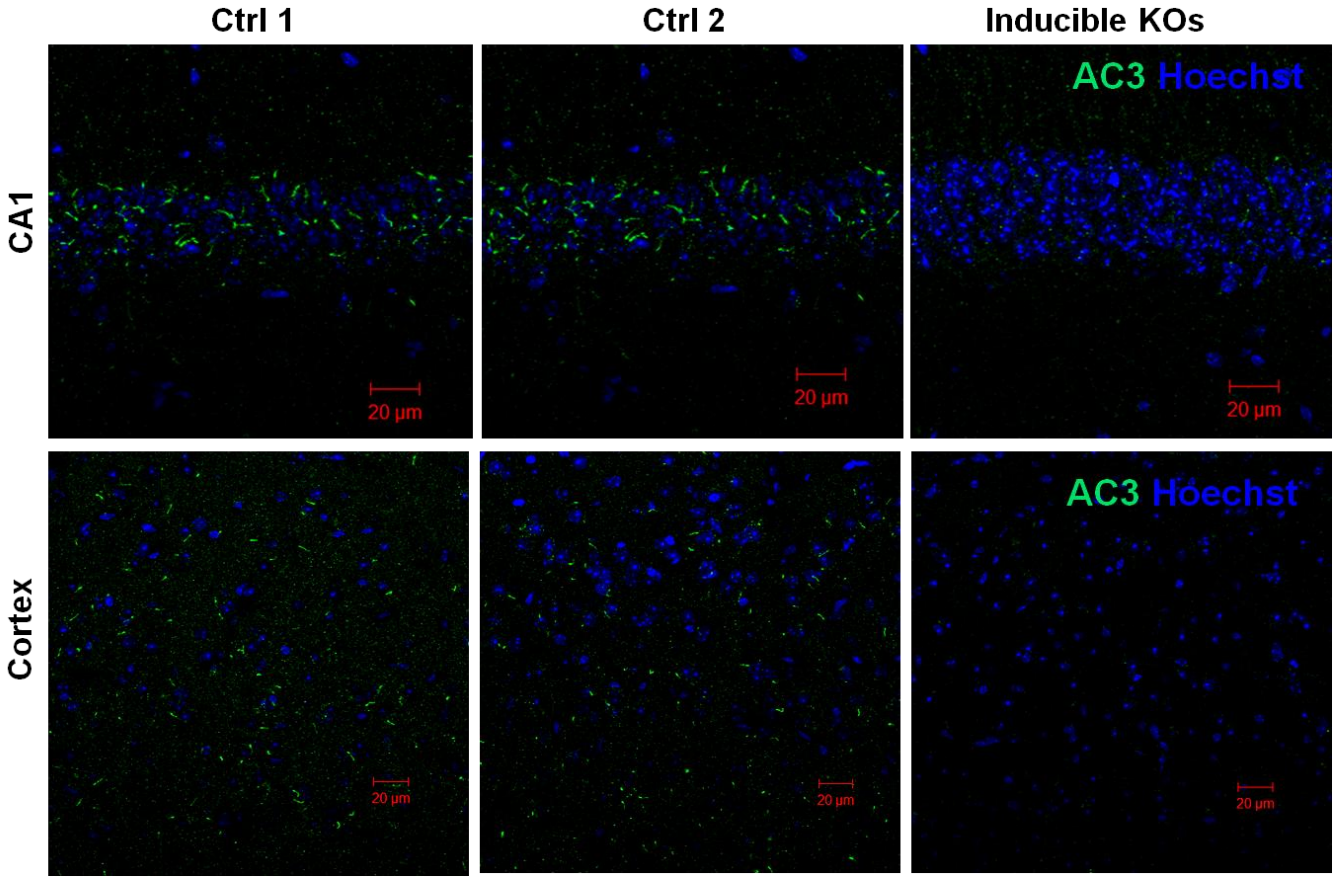


Fig. S8
(related to Fig. 7)