

Supporting Information

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SI Materials and Methods

Ethanol Induction/Group Assignment. Oral ethanol self-administration was induced using a procedure in which the concentration (%) or amount (g/kg) of ethanol in a palatable solution was gradually increased over a series of daily limited-access sessions that identify stable individual preferences for ethanol (1). All seven monkeys were initially given the opportunity to consume 1% (wt/vol) ethanol plus 6% (wt/vol) Tang (Kraft Foods) in tap water with an absolute ethanol limit of 0.5 g/kg per session via a drinking bottle placed on each animal's home cage. Ethanol concentrations were gradually increased to 6% (wt/vol) ethanol plus 6% (wt/vol) Tang with an absolute ethanol limit of 2.0 g/kg per session (Fig. 1B). Cage water was not available for the 1-h period preceding the alcohol session to reduce variability in initial fluid satiation; drinking water was restored after the session. Total intake in the final stage (2.0 g/kg of alcohol per session, 6% wt/vol solution in Tang) was used to balance the groups for individual ethanol preference. Alcohol sessions were thereafter discontinued in the control animals, and maintenance of drinking was continued in the alcohol group throughout the study.

Oral Ethanol Self-Administration Procedure. Solutions of 6% ethanol with 6% Tang (ethanol solution; 3.0 g/kg session limit) or 6% Tang in water (Tang solution) were made available in the home cage of the alcohol and control groups, respectively, during daily (Monday through Friday) 1-h sessions. The amount of the ethanol and Tang solution consumed during the sessions was recorded for the initial 5 min of the session, then 10, 15, 20, 30, and 60 min after ethanol availability. Consumption was measured at the specified time points during each session by examining the fluid level within the bottle, which had marked graduations on the side of it corresponding to volumes previously measured and validated. Drinking bottles were tested before each use to ensure that they did not leak before installation on the cage. Thereafter, the orange color of the solution made it easy for the investigators to detect any leaking or dripping during the sessions and to correct the lost volume. Similarly, if bottles were knocked off the cage by the subject, then the volume was restored to the previously recorded level and the drinking session was continued.

Blood Alcohol Levels. One and 10 months after the chronic maintenance phase was initiated for the alcohol group, a single 30-min alcohol session was scheduled with 3.0 g/kg of 6% ethanol available to all seven animals for the determination of BALs. Samples were drawn from the femoral vein under ketamine (10 mg/kg, intramuscular) anesthesia 30 min after the session. Serum was separated from blood cells by centrifugation and analyzed for ethanol content with an Analox AM1 (Analox Instruments).

Tissue Preparation. After completion of the study, monkeys were immobilized with 10 mg/kg ketamine/5 mg/kg xylazine and euthanized by 10 mg/kg pentobarbital followed by transcardial perfusion with ice-cold PBS. Brains were hemisectioned, transported on dry ice, and frozen at -80°C . Left or right hemispheres were used for histological analysis (control group, two right hemispheres and one left hemisphere; alcohol group, four left hemispheres). The frozen brain hemisphere was thawed on ice to -20°C , and blocks of brain tissue containing the hippocampal formation were dissected and processed for immunohistochemistry. The remaining brain tissue was refrozen for future use. Hippocampal-enriched blocks were immediately immersed in cold, freshly prepared paraformaldehyde solution (pH 7.4) and

left to postfix for 5–7 d at 4°C . Blocks of brain tissue were then cryoprotected in 30% sucrose, after which they were sectioned coronally on a freezing microtome into 40- μm sections. Hippocampal sections were serially collected in six wells and stored in PBS containing sodium azide (0.1%) for subsequent use. Every 30th section through the hippocampus was used for quantitative and qualitative analyses (2).

Antibodies and Immunohistochemistry. The following primary/secondary antibodies were used for immunohistochemistry (IHC): primary antibody (rabbit monoclonal anti- K_i -67; 1:1000; LabVision) and secondary antibody (biotinylated goat anti-rabbit; 1:200; Vector Laboratories) visualized using a DAB reaction for quantitative analysis and with tyramide signal amplification reaction for fluorescent qualitative analysis (TSA-fluorescein; PerkinElmer); primary antibody (chicken polyclonal anti-GFAP; 1:500; Abcam), secondary antibody (donkey anti-chicken cyanine 5; 1:200; Jackson ImmunoResearch), primary antibody (goat polyclonal anti-NeuroD1; 1:2,000; Santa Cruz Biotechnology), and secondary antibody (biotinylated horse anti-goat; 1:200; Vector Laboratories) visualized with DAB reaction for quantitative analysis and with cyanine 5 donkey anti-goat (1:200; Jackson ImmunoResearch) for fluorescent qualitative reaction; primary antibody (goat polyclonal anti-Sox2; 1:50; Santa Cruz Biotechnology), secondary antibody (cyanine 3 donkey anti-goat; 1:200; Jackson ImmunoResearch) for fluorescent qualitative reaction; primary antibody (mouse monoclonal anti-PSA-NCAM; 1:500; Millipore) and secondary antibody (biotinylated horse anti-mouse; 1:200; Vector Laboratories) visualized using a DAB reaction for quantitative analysis; and primary antibody (rabbit polyclonal anti-AC-3; 1:500; Cell Signaling Technology) and secondary antibody (biotinylated goat anti-rabbit; 1:200; Vector Laboratories) visualized using a DAB reaction for quantitative analysis. Every 30th section (approximately nine sections per monkey) through the left or right hippocampus were slide-mounted and dried overnight before IHC. Slides were coded before IHC, and the code was not broken until after analysis was complete. All incubations were performed at room temperature unless otherwise indicated. Slide-mounted sections were subjected to pretreatment steps as previously described (3). Slides were incubated with 0.3% H_2O_2 for 30 min to remove any endogenous peroxidase activity. Nonspecific binding was blocked with 5% serum and 0.5% Tween-20 in 0.1 M PBS for 60 min and incubated with the primary antibody (in 5% serum and 0.5% Tween-20) for 18–20 h. After washing with 0.1 M PBS, the sections were exposed to biotinylated secondary IgG for 1 h (1:200; Vector Laboratories). After secondary antibody incubation, slides were incubated in ABC for 1 h (Vector Laboratories), and staining was visualized with 3,3'-diaminobenzidine (DAB; Pierce Laboratories). Sections were counterstained with Fast Red (Vector Laboratories).

Microscopic Analysis and Quantification. Cells in the SGZ (K_i -67-IR, NeuroD1-IR, PSA-NCAM-IR, and AC-3-IR cells touching and within four cell widths inside the hippocampal granule cell-hilus border) were analyzed and quantified with a Zeiss Axiophot photomicroscope ($\times 400$) using the optical fractionator method. Cells from each bregma region were summed and multiplied by 30 to give the total number of cells. For type 1, type 2a, type 2b, and type 3 phenotype analysis, five sections through the hippocampus were triple labeled with one of the combinations GFAP/Sox2/ K_i -67 or Sox2/NeuroD1/ K_i -67. All K_i -67-IR cells in the

SGZ, ≈ 70 (control, 85 ± 11 ; alcohol, 62 ± 9) K_i -67-IR cells from each monkey, were scanned and analyzed for K_i -67⁺/Sox2⁺/GFAP⁺ (type 1), K_i -67⁺/Sox2⁺/NeuroD1⁻ (type 2a), K_i -67⁺/Sox2⁺/NeuroD1⁺ (type 2b), K_i -67⁺/Sox2⁻/NeuroD1⁺ (type 3), or K_i -67-alone labeling. All labeling was visualized and analyzed using a confocal microscope (LaserSharp 2000, version 5.2, emission wavelengths 488, 568, and 647 nm; Bio-Rad Laboratories).

For fluorescent labeling, antibody dilution was first titrated, and each marker (GFAP, NeuroD1, Sox2, and K_i -67) was tested in specific channels (GFAP-CY5, Sox2-CY3, NeuroD1-CY5, K_i -67-FITC for individual staining patterns). The fluorescent labeling pattern mimicked the DAB staining pattern for all antibodies tested. For triple-labeling fluorescent IHC (GFAP/Sox2/ K_i -67 or Sox2/ K_i -67/NeuroD1), five sections from each monkey were incubated with primary antibody followed by cyanine-conjugated (CY3 [Sox2], or CY5 [GFAP or NeuroD1]; Invitrogen) or tyramide signal amplification-conjugated (FITC [K_i -67]; PerkinElmer) secondary antibodies. For GFAP/Sox2/ K_i -67, staining was performed simultaneously (chicken anti-GFAP, goat anti-Sox2, and rabbit anti- K_i -67) in which all primary antibodies were incubated followed by sequential secondary antibody treatment. Sox2/ K_i -67/NeuroD1 staining was performed sequentially (goat anti-NeuroD1, rabbit anti- K_i -67, and goat anti-Sox2), in which each primary-secondary antibody-visualization step was separated by blocking treatment. Care was taken to avoid possible cross-reactivity between goat anti-Sox2 and goat anti-NeuroD1 staining. For example, staining for K_i -67 separated NeuroD1 and Sox2, such that goat serum was added during K_i -67 staining to prevent any cross-reactivity for the second anti-goat antibody. Omission or dilution of the primary antibodies resulted in a lack of specific staining, thus serving as a negative control for antibody experiments.

Confocal analysis was performed on individual K_i -67-IR cells at $\times 600$ magnification. Optical sectioning in the z-plane was performed using multitrack scanning with a section thickness of 0.45 μ m. Cells in the uppermost range of a section were omitted from analysis to avoid counting endcaps. Colocalization of antibodies was assessed with the confocal system by analysis of adjacent z-sections (gallery function). The percentage of K_i -67-IR cells that were GFAP/Sox2 positive, Sox2 positive, NeuroD1 positive, or Sox2/NeuroD1 positive in relation to the total number of K_i -67 cells was analyzed for each monkey. For calculated phenotype analyses, the ratio of K_i -67-IR cells that were type 1, type 2a, type 2b, and type 3 in relation to the total number of K_i -67 cells was analyzed from each monkey. The values for type 1, type 2a, type 2b, type 3, and none of these phenotypes were computed by multiplying the ratio of double- or triple-labeled K_i -67-IR cells and total number of K_i -67-IR cells from each monkey. All microscopic quantification and analyses were performed by an observer blind to the study.

Fluoro-Jade C Staining. Fluoro-Jade C staining was performed as previously described (4). Every 30th section through the hippocampus was slide mounted and air dried before Fluoro-Jade C treatment. First, slides were incubated with an alcohol-sodium hydroxide mixture for 5 min followed by 2-min washes with 70% alcohol and distilled water. Second, sections were incubated with potassium permanganate for 10 min and rinsed in distilled water for 2 min. Third, sections were incubated with FJC (0.0001% solution of FJC dye; Millipore) combined with 4',6-diamidino-2-phenylindole (DAPI; 0.0001% solution; Roche) dissolved in 0.1% acetic acid for 10 min followed by three distilled water washes. The slides were air dried and coverslipped. Fluoro-Jade C-IR cells in the granule cell layer of the hippocampus were visualized and quantified under a confocal microscope. The total number of cells from each monkey was multiplied by 30 and is reported as the total number of degenerating cells per animal.

Measurement of Hippocampal Granule Cell Number. Quantitative analysis to obtain unbiased estimates of the total number of hippocampal granule layer cell bodies (5) was performed on a Zeiss Axiophot microscope equipped with MicroBrightField Stereo Investigator software, a three-axis Mac 5000 motorized stage (Ludl Electronics), a digital CCD ZVS video camera (Zeiss), PCI color frame grabber, and personal computer workstation. All 40- μ m sections from the hippocampus were saved in strict anatomical order. Systematic random sampling of the hippocampus consisted of a 1-in-30 section analysis, and 9 sections were analyzed per monkey. All sections for quantitative analysis were counterstained with Nuclear Fast Red, and all portions of the granule cell layer were examined. Live video images were used to draw contours delineating the dentate gyrus granule cell layer. All contours were drawn at low magnification using a Zeiss Neofluar $\times 5$ objective, numerical aperture 0.15. After determination of mounted section thickness, z-plane values, and selection of contours, an optical fractionator analysis was used to determine hippocampal granule cell neuron number. A counting frame of appropriate dimensions denoting forbidden and nonforbidden zones ($10 \times 10 \times 2$ - μ m counting grid, and a 2- μ m top and bottom guard zone) was superimposed on the video monitor, and the optical fractionator analysis was performed using a Zeiss Plan Achromat $\times 63$ oil objective, numerical aperture 1.4 and a 1.4 auxiliary condenser lens. Cells were identified as neurons on the basis of standard morphology, and only neurons with a focused nucleus within the nonforbidden regions of the counting frame were counted. More than 700 cells per animal were counted. The total number of granule cells is presented as the average density of cells (cells/ μ m³) in Fig. 2L. Granule cell number estimates were made by an observer blind to the study.

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