Supporting Information

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SI Materials and Methods

Animals. Male Wistar rats (250–300 g) were used for all of the experiments. The rats were maintained on a 12 h/12 h light/dark cycle with ad libitum access to food and water. For the cognitive task, the rats were food-restricted and trained at 90% of their free-feeding body weight. All of the procedures were conducted in strict adherence to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by The Scripps Research Institute Institutional Animal Care and Use Committee.

Alcohol Self-Administration. This procedure was similar to the procedure in the work by Simms et al. (1). Briefly, the rats were given either continuous (24 h/d for 7 d/wk) or intermittent (24 h/d for 3 d/wk) access to alcohol (20% vol/vol) using a two-bottle choice procedure (ethanol vs. water) for 4 mo. During the intervening days, the ethanol bottle was replaced with a second bottle of water in the intermittent exposure group. Alcohol and water intake was monitored every day. Body weight was monitored two times per week.

Spontaneous Alternation and Anxiety-Like Behavior. After 5 mo of access to alcohol, 36 rats (12 continuous, 12 intermittent, and 12 naïve rats) were tested after 24 h of forced abstinence for spontaneous alternation in a Y-maze and 2 d later for anxiety-like behavior in an elevated plus maze. Working memory performance was evaluated with a Y-maze as described in the work by Gué et al. (2). The maze was constructed of black Plexiglas. Each arm was 40 cm long, 13 cm high, 3 cm wide at the bottom, and 10 cm wide at the top. The rats were tested for spontaneous alternation after 24 h of forced abstinence by being allowed to freely explore the maze for 10 min. Any consecutive exploration in which a rat explored all three arms was considered an alternation. The percentage of alternation was calculated as (actual alternations/maximum alternations) × 100, in which the number for maximum alternations was obtained by subtracting two from the total number of arm entries. Anxiety-like behavior was evaluated in an elevated plus maze made of black Plexiglas (100-cm elevation) that consisted of four arms (50 cm long × 10 cm wide), two arms with 40-cm-high dark walls (closed arms) and two arms with 0.5-cm-high ledges (open arms). The rats were positioned in the central area and allowed to explore the maze for 5 min under dim light conditions. The percentage of time spent in the open arms was calculated using a videotracking system (Clever Sys).

Delayed Nonmatching-to-Sample Task. An additional cohort of 24 rats was given intermittent or continuous access to alcohol as described above for 5 mo. The rats were then deprived of alcohol, and 4 d later, they were trained on the delayed nonmatching-to-sample task (DNMS) task using operant chambers (Med Associates). The rats were food-deprived (85–90% of body weight) and trained (30 min/d) to press a lever [fixed-ratio 1 (FR1)] to obtain a sucrose pellet (45 mg). After reaching the training criterion of 50 lever presses in 30 min, the rats were moved to the next phase (forced alternation). At the beginning of the session, the house light was turned on for 3 s, and the reward cue light above the food trough was turned on, indicating that the rat should nose-poke the trough. After the rat nose-poked, the cue light was turned off, and one lever was extended (right or left), indicating that the rat should press the lever. After the lever was pressed, one sucrose pellet was dropped into the food trough, and the reward cue light was immediately illuminated. After a second nose-poke to retrieve the sucrose pellet, the opposite lever was extended. This cycle continued for either 30 min or 50 lever presses, whichever occurred first. The criterion to move to the next phase (free alternation) was 50 lever presses. In the free alternation phase, the house light was turned on for 3 s. This light was followed by the reward cue light, eliciting a nose-poke response. The cue light was randomly assigned to remain on for a delay of at least 1–4 s. The rat had to continue nose-poking during this time. The light turned off when the rat nose-poked after the randomly assigned delay, at which point both levers were extended. Pressing either lever resulted in a reward. After the pellet dropped, there was a delay of 3 s before the cue light was turned back on, and another delay was initiated. This time, when the levers were extended, the rat had to choose the opposite lever to obtain the reward. If the rat chose the same lever, then a timeout period was initiated, with the house light turned on for 1 s, retraction of both levers, and no delivery of the pellet. If the rat continued to press the incorrect lever four consecutive times, then only one lever was extended (i.e., the correct lever), forcing the rat to make the correct decision, but the trial was not counted as correct. After this trial, the program returned to free alternation. The daily duration of this phase was 40 min or 50 trials, whichever occurred first. The rats were trained in this phase with progressively increasing delays of 1, 4, 8, and 12 s and then, 5, 10, 15, and 20 s; finally, they were tested at 0, 10, and 40 s and increasing timeout durations of 1, 2, and 3 s. The rats were tested for 5 wk, during which time they completed all phases of the experiment and obtained stable performance (>70% correct response at the 0-s delay). One rat was withdrawn from the continuous group ∼3 wk into the experiment because of extremely low performance. After the final phase of testing during protracted abstinence, the rats were reintroduced to alcohol (20% vol/vol) and their previous schedules of drinking. Both the intermittent and continuous groups were tested weekly using the DNMS task during acute withdrawal. Testing occurred two times per week after both groups were deprived of alcohol for 24 h or after 2 h of access to alcohol that followed 24 h of abstinence.

Immunohistochemistry. All of the animals were anesthetized with 3.5% chloral hydrate and perfused with saline followed by 4% paraformaldehyde/0.15 M phosphate buffer, pH 7.4. The brains were harvested and cryoprotected in 20% sucrose/PBS. Coronal sections (40 μm) cut on a cryostat were collected in strict anatomical order and stored in 0.1% NaN_3 in PBS at 4 °C. Every eighth section in each series was selected for free-floating immunohistochemistry. The sections were incubated with 0.3% H_20_2 for 20 min to remove any endogenous peroxidase activity and rinsed in PBS. To block nonspecific antigen binding, the sections were incubated for a minimum of 60 min in PBS, 0.3% TX100, 1 mg/mL BSA, and 5% normal donkey serum. The sections were then incubated overnight on a shaker plate at 4 °C with the following primary antibodies: FBJ murine osteosarcoma viral oncogene homolog (Fos) rabbit polyclonal (1:5,000; SCBT), GABAergic (GAD_{67}) mouse monoclonal (1:50,000; Millipore), and corticotropin-releasing factor (CRF; 1:500; SCBT). Primary antibodies were diluted in PBS, 5% serum, and 0.5% Tween. The sections were then rinsed several times in PBS. For Fos single labeling, the sections were incubated in Rabbit ImmPress Reagent (Vector Laboratories) for 60 min and rinsed several times in PBS. Fos immunoreactivity was visualized using 3,3-diaminobenzidine (DAB) enhanced with nickel (DAB Substrate Kit; Vector Laboratories).

Double-labeling experiments were performed as above with the following modifications. The sections were simultaneously incubated with Fos and CRF or GAD_{67} antibodies. Fos immunoreactivity was visualized as described above. For the development of CRF/GAD_{67} immunoreactivity, the sections were rinsed in PBS, blocked for 60 min in PBS/5% normal donkey serum, and incubated for 60 min in Mouse ImmPress Reagent (Vector Laboratories). After several rinses in PBS, CRF/GAD_{67} immunoreactivity was visualized using DAB (DAB Substrate Kit; Vector Laboratories). The sections were then slide-mounted, air-dried, dehydrated in ethanol, and coverslipped with Permount.

Microscopy. Quantitative analysis to obtain unbiased estimates of the total number of Fos+, CRF+, and GAD_{67}+ cell bodies was performed on a Zeiss Axiophot Microscope equipped with MicroBrightField Stereo Investigator software and a Q Imaging Retiga 2000R color digital camera. Systematic random sampling of medial prefrontal cortex (mPFC), central nucleus of the amygdala (CeA), and hippocampus consisted of a one-in-eight section analysis, and three sections per region were analyzed bilaterally per animal by an investigator blind to the different groups. Live video images were used to draw contours that delineated each structure. All contours were drawn at low magnification using a Zeiss Neoflaur 2.5× objective, N.A. 0.15. After the determination of mounted section thickness, *Z*-plane values, and the selection of contours, optical fractionator analysis was used to determine Fos+, CRF+, and GAD_{67}+ neuron number. To ensure that all Fos+, CRF+, and GAD_{67}+ cells were counted, a frame of appropriate dimensions that denoted forbidden and nonforbidden planes was superimposed on the video monitor, and optical fractionator analysis was performed using a Zeiss Plan Apochromat 20× objective, N.A. 0.60, and a 1.4 auxiliary condenser lens. Cells were identified as neurons based on standard morphology, and only neurons with a focused nucleus within the nonforbidden regions of the counting frame were counted.

Blood Alcohol Determinations. The rats were gently restrained under the technician's arm while the tip of the tail (2 mm) was removed with a clean razor blade. Tail blood (0.5 mL) was collected into Eppendorf tubes that contained evaporated heparin and kept on ice. The samples were centrifuged, and the serum was decanted into new Eppendorf tubes. The serum was then injected into an oxygen-rate alcohol analyzer (Analox Instruments) for blood alcohol level determination.

SI Results

Effect of Alcohol Drinking on Fos Activation. The analysis of Fos activation in the PFC revealed significant effects of abstinence ($F_{1,20} = 43.4$, $P = 0.0001$) and group ($F_{1,20} = 4.6$, $P = 0.04$) and a significant abstinence × group interaction ($F_{1,20} = 5.0$, $P = 0.036$). The analysis of Fos activation in the CeA revealed significant effects of abstinence ($F_{1,20} = 36.47$, $P = 0.0001$) and group ($F_{1,20} = 44.9$, $P = 0.0001$) and a significant abstinence × group interaction ($F_{1,20} = 17.2$, $P = 0.0001$). The number of Fos+ neurons in the dentate gyrus (Fig. 2*M*) was similar across all conditions ($F_{1,19} = 0.01$, $P = 0.94$). No difference was observed between the intermittent and continuous groups in the CA1 (intermittent abstinence = 18 ± 9, continuous abstinence = 16 ± 4, intermittent alcohol = 7 ± 2, continuous alcohol = 6 ± 2; abstinence effect: $F_{1,19} = 4.05$, $P = 0.06$; group effect: $F_{1,19} = 0.12$, $P = 0.73$; abstinence × group interaction: $F_{1,19} = 0.009$, $P = 0.93$) or CA3 (intermittent abstinence = 16 ± 5, continuous abstinence = 10 ± 5, intermittent alcohol = 9 ± 6, continuous alcohol = 5 ± 1; abstinence effect: $F_{1,19} = 1.93$, $P = 0.18$; group effect: $F_{1,19} = 1.53$, $P = 0.23$; abstinence × group interaction: $F_{1,19} = 0.05$, $P = 0.82$) subregion of the hippocampus. Moreover, the number of Fos+ neurons in the dentate gyrus (Fig. 2*N*) and nucleus accumbens (NAc) was not correlated with alcohol drinking. In contrast, the number of Fos+ neurons in the NAc core and shell increased during abstinence, but no difference was observed between continuous and intermittent rats (NAc core: intermittent abstinence = 46 ± 14, continuous abstinence = 39 ± 11, intermittent alcohol = 10 ± 3, continuous alcohol = 11 ± 3; abstinence effect: $F_{1,20} = 12.24$, $P = 0.002$; group effect: $F_{1,20} = 0.15$, $P = 0.70$; abstinence × group interaction: $F_{1,20} = 0.20$, $P = 0.66$; NAc shell: intermittent abstinence = 51 ± 15, continuous abstinence = 32 ± 9, intermittent alcohol = 15 ± 6, continuous alcohol = 12 ± 3; abstinence effect: $F_{1,20} = 8.29$, $P = 0.009$; group effect: $F_{1,20} = 1.27$, $P = 0.27$; abstinence × group interaction: $F_{1,20} = 0.66$, $P = 0.42$).

Nonparametric Statistical Analysis.

Analysis of Fig. 1*A*: Intermittent access was significantly higher than continuous access on days 33–40. Kruskal–Wallis: all $H_{1,24} > 4.09$, all $P < 0.05$.

Analysis of Fig. 1*B*: Intermittent access was significantly higher than continuous access at 2 and 24 h. Kruskal–Wallis: 2 h, $H_{1,24} = 3.9$, $P < 0.05$; 24 h, $H_{1,24} = 7.5$, $P < 0.01$.

Analysis of Fig. 1*C*: Intermittent access was significantly higher than continuous access at 2 h but not 24 h. Kruskal–Wallis: 2 h, $H_{1,24} = 4.6$, $P < 0.05$; 24 h, $H_{1,24} = 0.12$, $P > 0.05$.

Analysis of Fig. 2*C*: During abstinence, intermittent access was significantly higher than continuous access: $H_{1,12} = 4.3$, $P < 0.05$.

Analysis of Fig. 2*D*: $H_{2,38} = 7.7$, $P < 0.05$.

Analysis of Fig. 2*H*: During abstinence, intermittent access was significantly higher than continuous access: $H_{1,12} = 8.3$, $P < 0.01$.

Analysis of Fig. 2*I*: $H_{2,37} = 1.5$, $P > 0.05$.

Analysis of Fig. 2*M*: During abstinence, intermittent access was the same as continuous access: $H_{1,12} = 0.0$, $P = 1.0$.

Analysis of Fig. 4*A*: 24 h, $H_{1,24} = 3.9$, $P < 0.05$; 72 h, $H_{1,24} = 7.1$, $P < 0.01$; 16 d, $H_{1,24} = 0.4$, $P > 0.05$; 68 d, $H_{1,24} = 0.5$, $P > 0.05$.

Analysis of Fig. 4*B*: 0 s, Wilcoxon $Z = 2.9$, $P < 0.01$; 10 s Wilcoxon $Z = 1.7$, $P > 0.05$; 40 s, Wilcoxon $Z = 0.4$, $P > 0.05$.

Analysis of Fig. 4*C*: 0 s, Wilcoxon $Z = 1.8$, $P > 0.05$; 10 s, Wilcoxon $Z = 1.96$, $P < 0.05$; 40 s, Wilcoxon $Z = 0.78$, $P > 0.05$.

Analysis of Fig. 4*D*: Kruskal–Wallis for 0, 10, and 40 s: all $H_{1,22} < 0.48$, all $P > 0.05$.

Analysis of Fig. 5*A*: $H_{3,23} = 2.02$, $P > 0.05$.

Analysis of Fig. 5*B*: $H_{3,23} = 13.02$, $P < 0.01$.

Analysis of Fig. 5*D*: $H_{3,23} = 11.3$, $P < 0.01$.

Analysis of Fig. 5*E*: $H_{3,23} = 5.6$, $P > 0.05$.

1. Simms JA, et al. (2008) Intermittent access to 20% ethanol induces high ethanol consumption in Long-Evans and Wistar rats. *Alcohol Clin Exp Res* 32(10):1816–1823.

2. Gué M, et al. (2004) Sex differences in learning deficits induced by prenatal stress in juvenile rats. *Behav Brain Res* 150(1-2):149–157.

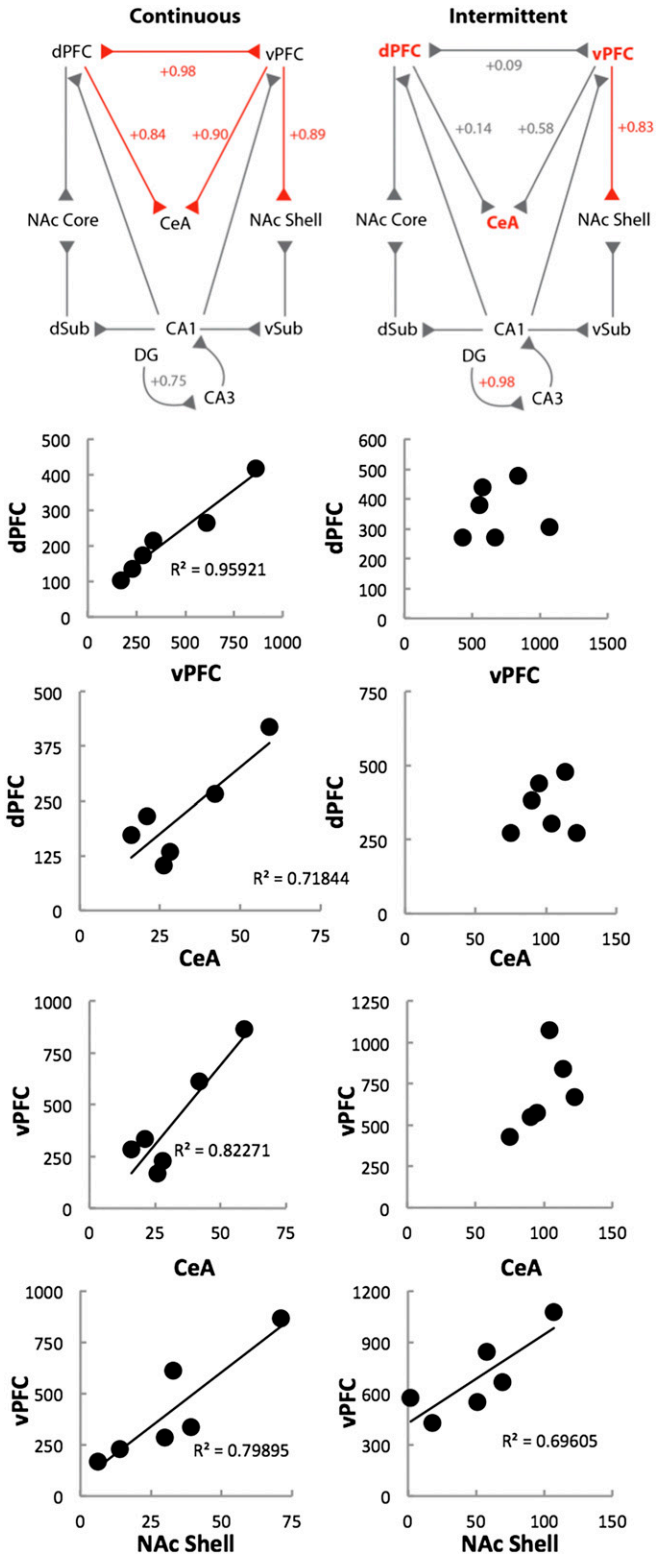

Fig. S1. Abstinence from alcohol in rats with intermittent access is associated with a functional disconnection of the PFC and CeA. Correlational analysis of Fos+ cell number between anatomically connected structures. The connections with a significant correlation ($P < 0.05$) are represented in red with the respective R value of the Pearson correlation coefficient. Nonsignificant correlations are in gray. Note that, for comparison purposes, nonsignificant R values of the connections that are significant in the other condition are also represented in gray, despite being nonsignificant.