# Supplemental Information

## Methods and Materials

### Subjects

Male Wistar rats (Charles River, Raleigh, NC; 225-250 g on arrival) were housed 2-3/cage on a reversed 12 h light/dark cycle (lights off at 0800) in a temperature- and humidity-controlled vivarium with *ad libitum* access to food and water. All procedures were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute.

### Ethanol Self-administration Training

Rats were trained to orally self-administer ethanol in 30 min daily sessions inside sound-attenuated operant conditioning chambers (Med Associates, St. Albans, VT) using a sweet solution-fading procedure, as previously described (1). Briefly, responses at the active lever were initially reinforced by 0.1 ml of saccharin (0.2% w/v) on a fixed-ratio 1 schedule of reinforcement. After acquisition, ethanol (5% w/v) was added to the saccharin solution. During subsequent training, ethanol concentrations were gradually raised to 10% (w/v), while saccharin was slowly eliminated. A second but inactive lever was then introduced at which responses were recorded but had no programmed consequences.

**Ethanol Dependence Induction**

Once stable ethanol self-administration was obtained at 10% (w/v) ethanol, rats were made ethanol dependent (*n*=102) via repeated intragastric ethanol intubations as previously described (2). Briefly, ethanol solution (final concentration 20% w/v) was made up in Sustacal® (Mead Johnson, Glenview, IL) and intubated four times per day for 5 consecutive days in a total volume of 22 ml/kg/day, with each daily dose administered at 4 h intervals (see Figure S1 for details). Non-dependent controls (*n*=97) received intragastric administration of Sustacal® vehicle at volumes and time intervals identical to those in ethanol-treated rats. Blood alcohol levels (BALs) were determined from tail blood samples (200 µl) taken 1 h after the first (Bleed 1) and second (Bleed 2) intragastric administrations on day 5. Behavioral signs of withdrawal were measured 12 h after the final ethanol administration (see Figure S1 for details).

One week after the completion of ethanol dependence induction, daily ethanol self-administration sessions resumed for non-dependent and post-dependent animals until stable levels of intake were re-established (±10% over three consecutive sessions). At this time, behavioral testing began in rats designated for tests of ethanol reinforcement, whereas rats assigned to testing for stress-induced reinstatement entered an extinction phase.

**Extinction Training**

After re-establishing stable ethanol self-administration, animals designated for tests of stress-induced reinstatement were subjected to extinction conditions in daily 30 min sessions for a total of 3 weeks during which time ethanol was withheld.

## Results

### Extinction

During the first extinction session, rats emitted 35.3 ± 2.2 (non-dependent) and 37.84 ± 2.4 (post-dependent) responses (mean ± SEM). After 20 extinction sessions, responding decreased to 11.0 ± 0.9 (non-dependent) and 10.3 ± 0.8 (post-dependent) responses (Figure S2), with no differences between groups ($F_{1,157}$ = 0.2, NS). Additionally, there were no differences among subgroups of non-dependent and post-dependent rats designated for testing with different LY379268 or MTEP doses (*ethanol dependence history* -- LY379268: $F_{1,75}$ = 0.6, NS, MTEP: $F_{1,83}$ = 0.1, NS; *dose* -- LY379268: $F_{3,75}$ = 0.5, NS, MTEP: $F_{3,83}$ = 2.7, NS; *ethanol dependence history x dose interaction* -- LY379268: $F_{3,75}$ = 0.4, NS, MTEP: $F_{3,83}$ = 0.0, NS).

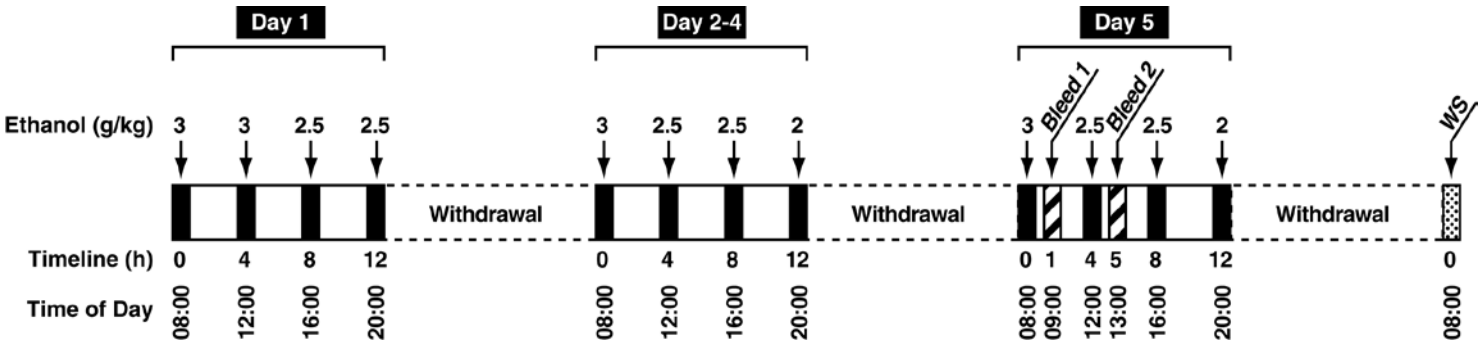


**Figure S1.** Diagram illustrating the procedure for inducing ethanol dependence. On day 1, rats were administered a total of 11.0 g/kg ethanol in four fractional doses of 3.0, 3.0, 2.5, and 2.5 g/kg ethanol, with doses administered at 4 h intervals. On days 2-5, 12 h after the last intubation on the preceding day, rats received a total of 10.0 g/kg ethanol in four fractional doses of 3.0, 2.5, 2.5, and 2.0 g/kg ethanol, again separated by 4 h intervals. Non-dependent controls received intragastric administration of vehicle at volumes and time intervals identical to those in ethanol-treated rats. On day 5, tail blood (approximately 200 µl) was collected 1 h after the first (Bleed 1) and second (Bleed 2) intragastric administrations. Samples were immediately centrifuged (10 min, 5000 rpm, 4ºC), and a 5 µl plasma sample was assayed for alcohol content by injection into an oxygen-rate alcohol analyzer (Analox Instruments, Lunenburg, MA). Twelve hours after the final ethanol administration, behavioral signs of withdrawal (withdrawal scoring, WS) were rated by an experimenter blind to treatment conditions. Using a withdrawal scale adapted from Macey and colleagues (1996), ethanol withdrawal signs, including ventromedial limb retraction, irritability upon touch (vocalization), tail rigidity, abnormal gait, and body tremors, were scored (3). Each sign was assigned a score of 0-2, based on the following severity scale: 0 = no sign, 1 = moderate, 2 = severe. The sum of the five observations scores (0-10) was used as a quantitative measure of withdrawal severity.

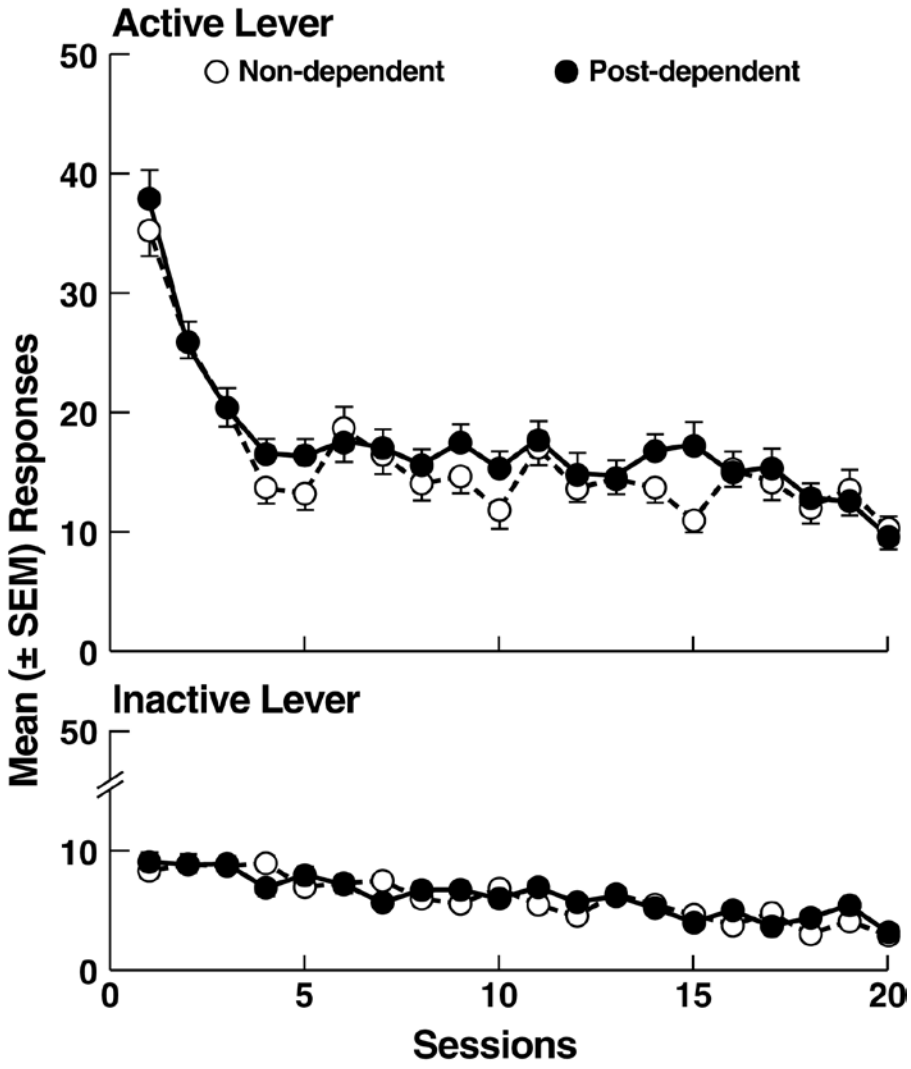


**Figure S2.** Active and inactive lever responses during extinction sessions.

1. Ciccocioppo R, Angeletti S, Weiss F (2001): Long-lasting resistance to extinction of response reinstatement induced by ethanol-related stimuli: role of genetic ethanol preference. *Alcohol Clin Exp Res* 25:1414-1419.

2. Braconi S, Sidhpura N, Aujla H, Martin-Fardon R, Weiss F, Ciccocioppo R (2010): Revisiting Intragastric Ethanol Intubation as a Dependence Induction Method for Studies of Ethanol Reward and Motivation in Rats. *Alcohol Clin Exp Res* 34.

3. Macey DJ, Schulteis G, Heinrichs SC, Koob GF (1996): Time-dependent quantifiable withdrawal from ethanol in the rat: Effect of method of dependence induction. *Alcohol* 13:163-170.