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Chronic Ethanol Intake Alters Circadian Phase Shifting and Free-Running Period in Mice

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Abstract

Chronic alcohol intake is associated with widespread disruptions in sleep and circadian rhythms in both human alcoholics and in experimental animals. Recent studies have demonstrated that chronic and acute ethanol treatments alter fundamental properties of the circadian pacemaker—including free-running period and responsiveness to photic and nonphotic phase-shifting stimuli—in rats and hamsters. In the present work, the authors extend these observations to the C57BL/6J mouse, an inbred strain characterized by very high levels of voluntary ethanol intake and by reliable and stable free-running circadian activity rhythms. Mice were housed individually in running-wheel cages under conditions of either voluntary or forced ethanol intake, whereas controls were maintained on plain water. Forced ethanol intake significantly attenuated photic phase delays (but not phase advances) and shortened free-running period in constant darkness, but voluntary ethanol intake failed to affect either of these parameters. Thus, high levels of chronic ethanol intake, beyond those normally achieved under voluntary drinking conditions, are required to alter fundamental circadian pacemaker properties in C57BL/6J mice. These observations may be related to the relative ethanol insensitivity displayed by this strain in several other phenotypic domains, including ethanol-induced sedation, ataxia, and withdrawal. Additional experiments will investigate chronobiological sensitivity to ethanol in a range of inbred strains showing diverse ethanol-related phenotypes.

Keywords

circadian; wheel running; ethanol; alcohol; inbred mice; C57BL/6J

Chronic alcohol intake is associated with dramatic and widespread disruptions of sleep-wake cycles and other daily biological rhythms in both human alcoholics (Brower, 2003; Kuhlwein et al., 2003; Sano et al., 1993) and experimental animals (Ehlers and Slawecki, 2000; Mukherjee and Simasko, 2009; Wasielewski and Holloway, 2001). In turn, chronobiological dysregulation may promote or sustain excessive alcohol intake and contribute to the negative health consequences associated with alcohol abuse disorders (Danel and Touitou, 2004; Rosenwasser, 2001; Spanagel et al., 2005b).

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Although most studies on the chronobiological effects of alcohol have been conducted under entrained conditions, recent animal experiments have begun to explore the effects of ethanol on the phase and period of free-running circadian rhythms, parameters that directly reflect the phase and period of the underlying circadian pacemaker (Rosenwasser, 2001; Turek, 1987). Thus, chronic ethanol intake alters free-running period (Mistlberger and Nadeau, 1992; Dwyer and Rosenwasser, 1998; Rosenwasser et al., 2005a) and attenuates the phase-shifting and/or period-altering effects of brief light pulses presented during late (but not early) subjective night (Rosenwasser et al., 2005c; Seggio et al., 2007) in both rats and hamsters. Similarly, acute ethanol pretreatment also selectively attenuates the phase-shifting effects of late-night light pulses in hamsters (Ruby et al., 2009). Taken together, these findings indicate that ethanol alters the period and photic responsiveness of the circadian pacemaker.

Although the neurobiological mechanisms underlying these effects have not been fully elucidated, chronic ethanol treatment alters gene expression and neuropeptide levels within the SCN, the site of the central circadian pacemaker (Chen et al., 2004; Madeira et al., 1997). Further, GABA-A and *N*-methyl-D-aspartate (NMDA) receptors are well-known molecular targets for ethanol action in the central nervous system (Davis and Wu, 2001; Faingold et al., 1998) and play critical roles in regulation of the SCN pacemaker (Rosenwasser, 2003). Like ethanol, GABAergic benzodiazepines alter free-running circadian period and selectively attenuate the phase-shifting effects of late-night but not early-night light pulses (Ralph and Menaker, 1986, 1989; Subramanian and Subbaraj, 1996). Further, recent experiments have shown that direct ethanol application to the SCN can attenuate the phase-shifting effects of light and glutamate, in vivo (Ruby et al., 2009) and in vitro (Prosser et al., 2008).

The primary aim of the present experiments was to extend these observations to inbred C57BL/6J mice. This aim was motivated by a desire to establish a mouse model that could be used to investigate neurogenetic linkages between ethanol preference and circadian pacemaker phenotype. Such relationships are indicated by data showing that selective breeding for ethanol preference alters circadian phenotype in both rats (Rosenwasser et al., 2005b) and mice (Hofstetter et al., 2003), whereas mutation of the critical circadian clock gene *per2* modifies ethanol preference in mice (Spanagel et al., 2005a). We chose to focus initially on the C57BL/6J strain due to its very high levels of innate ethanol preference (Belknap et al., 1993; Yoneyama et al., 2008) and its robust and highly stable circadian activity rhythms (Daan and Pittendrigh, 1976; Schwartz and Zimmerman, 1990).

MATERIALS AND METHODS

Experiment 1: Effects of Voluntary and Forced Ethanol Intake on Light-Induced Circadian Phase Shifting

Eight-week-old male C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and housed individually in running-wheel cages (Coulbourn Instruments, Whitehall, PA; wheel diameter 11.5 cm) with food and fluid (either plain water or plain water and 10% [vol/vol] ethanol solution; see below) provided ad libitum. Cages were placed 2 per shelf within light- and sound-shielded enclosures equipped with exhaust fans and programmable lighting provided by incandescent lamps. Running-wheel activity was recorded and analyzed using the ClockLab interface system (Actimetrics Co., Wilmette, IL), and fluid intakes were determined at weekly intervals.

The mice were initially maintained in a light-dark (LD) 12:12 cycle, and following the establishment of stable, light-entrained rhythms, divided randomly into groups. Experiment 1 utilized 2 separate ethanol-treatment groups: a free-choice ethanol group ($n = 9$), which

received both plain water and 10% ethanol (vol/vol) solution in 2 separate drinking bottles, and a forced ethanol group (n = 10), maintained on 10% ethanol solution as the only drinking fluid. Each of these ethanol-treated experimental groups was compared with its own water-only control group (n = 10 per group). After a 21-day baseline period to allow ethanol intakes to stabilize in the experimental groups, all animals were tested sequentially (4 tests per animal; see below) for responses to both phase-advancing and phase-delaying light pulses (15 min, 30–50 lux) using the Aschoff type II protocol (Mistlberger, 1996; Mrosovsky, 1996). In this protocol, animals are kept under an entraining LD cycle until the day of light pulse presentation, and then maintained subsequently for several days in constant darkness (DD) for assessment of the phase of the free-running rhythm. Light pulses were administered at phases expected to yield maximal phase advances (i.e., ZT 21; 9 h following the last light-to-dark transition, designated by tradition as ZT 12) and phase delays (ZT 15; 3 h after the last light-to-dark transition) in this mouse strain (Daan and Pittendrigh, 1976; Schwartz and Zimmerman, 1990). Successive phase-response tests were separated by at least 3 weeks of re-exposure to the LD cycle, ensuring stable entrainment prior to the delivery of all light pulses.

Two tests were conducted at each ZT, 1 during ongoing ethanol access and a 2nd following 24 h of ethanol deprivation. Following the 1st test conducted under ethanol deprivation, ethanol access was restored for at least 4 weeks prior to the second deprivation test. Thus, each animal was tested a total of 4 times in the same sequence: 1) ZT 15 light pulse, 2) ZT 21 light pulse, 3) ZT 15 light pulse during acute withdrawal; and 4) ZT 21 light pulse during acute withdrawal. Two potential limitations of this design should be mentioned here: First, the different test conditions followed different numbers of days of ethanol drinking experience (ranging from 30 to more than 150), and second, animals experienced a 1-week ethanol deprivation 2 weeks prior to the final phase-shift test that could have altered subsequent alcohol intake (i.e., the “alcohol deprivation effect”; Melendez et al., 2006). It is unlikely that either of these factors influenced the results presented here, however, inasmuch as the alcohol deprivation effect is generally not robust following a single 1-week deprivation episode (Melendez et al., 2006) and inasmuch as weekly ethanol intakes showed no systematic change with time after the initial 2 weeks of drinking experience (data not shown).

The magnitude and direction of circadian phase responses were determined using ClockLab's automated activity-onset detection algorithm. Prestimulus phase was estimated as the mean time of activity onset over the last 5 days of LD entrainment, and poststimulus phase was estimated by a regression line fit to activity onsets over 6 to 7 free-running circadian cycles following the test stimulus, excluding the 1st 2 activity onsets due to the possible occurrence of “transients” prior to the establishment of a steady-state free-running phase (Daan and Pittendrigh, 1976). Phase responses were then determined as the difference between these 2 phase estimates extrapolated to the 1st poststimulus activity onset.

Experiment 2: Effects of Voluntary and Forced Ethanol Intake on Free-Running Circadian Rhythms

C57BL/6J male mice were obtained from the Jackson Laboratory and individually housed in running wheel cages under prolonged constant darkness. Following a water-only baseline period of 21 days, the animals were divided randomly into 3 groups and exposed to 1 of the following drinking conditions for an additional 148 days: 1) A free-choice ethanol group was concurrently offered 10% v/v ethanol and water via separate drinking bottles (n = 12); 2) a forced ethanol group was presented 10% v/v ethanol solution as the only drinking fluid (n = 12); and 3) a control group continued to be maintained on plain water throughout the experiment (n = 11). Wheel-running activity was monitored using the ClockLab interface system, and fluid intakes were determined weekly.

Circadian rhythm parameters were determined for each of eight 3-week experimental epochs (one 3-week baseline epoch followed by seven 3-week epochs in which animals were maintained under the different drinking conditions). Free-running circadian period was determined using well-established methods implemented in the ClockLab analysis routines, including both the χ^2 (nonparametric) and the Lomb-Scargle (parametric) periodogram analyses, which were averaged to yield the period estimates reported here. In addition, the peak magnitude of the Lomb-Scargle periodogram was used to estimate the robustness of free-running rhythmicity (Ruf, 1999). Finally, the total number of daily wheel turns was also determined for each animal and for each epoch of the experiment.

RESULTS

Photic Phase Shifting

As expected from previous studies, control mice showed reliable phase delays to light pulses presented at ZT 15 and phase advances to light pulses presented at ZT 21, whereas the overall magnitude of phase delays was greater than phase advances (Figs. 1, 2). Voluntary ethanol intake had no effect on circadian phase shifting at either test phase, whether tested during maintained drinking or under acute withdrawal (Fig. 2, top). In contrast, forced ethanol intake resulted in a significant attenuation of photic phase shifting at ZT 15, but not at ZT 21, both under continued drinking ($t_{18} = 2.33$, p = 0.032) and during acute withdrawal ($t_{18} = 2.53$, $p = 0.021$) conditions (Fig. 1; Fig. 2, bottom). There were no significant differences between tests conducted during continued drinking and tests conducted under withdrawal at either ZT or for either group.

Free-Running Period

Repeated-measures analysis of variance (ANOVA) including all 8 experimental epochs detected a significant main effect of time ($F_{7,224} = 14.38$, $p < 0.001$), but no effect of group nor any group-by-epoch interaction, indicating that free-running periods generally lengthened over the course of long-term exposure to constant darkness in all groups (Figs. 3, 4). To examine the immediate response to the introduction of ethanol treatment, a similar analysis was conducted using only the baseline and the first 3-week ethanol treatment epoch; this analysis revealed a significant group-by-epoch interaction ($F_{2,32} = 3.46$, $p = 0.044$), indicating that the introduction of forced ethanol intake shortened the free-running period. Follow-up pairwise comparisons using least significant difference (LSD) tests showed no differences among groups during baseline conditions, but free-running periods in the forced ethanol group were significantly shorter than in the free-choice ethanol group or the water-only control group during the 1st, 2nd, and 4th ethanol treatment epochs (Fig. 4). In contrast, there were no significant differences between the free-choice group and the control group during any epoch. Thus, forced ethanol intake resulted in a shortening of free-running period that persisted for about 3 months of continued treatment.

Periodogram Amplitude

ANOVA revealed significant main effects of experimental epoch ($F_{7,224} = 34.39$, $p < 0.001$) and treatment group ($F_{2,32} = 4.08$, $p = 0.026$) on the robustness of free-running rhythmicity, assessed by periodogram peak amplitude. The significant main effect of treatment group reflected the fact that both ethanol-treated groups showed generally lower periodogram amplitude during ethanol treatment (Fig. 4, middle). Thus, follow-up LSD tests detected significant differences between the forced ethanol group and the control group during treatment epochs 5 and 6 and between the free-choice ethanol group and the control group during epochs 3, 6 and 7, but no differences between the 2 ethanol treatment groups in any epoch.

Activity Level

ANOVA revealed a significant main effect of experimental epoch ($F_{7,224} = 38.77$, $p < 0.001$), reflecting the gradually decreasing activity levels displayed by both ethanol-exposed and control groups over the course of the experiment (Fig. 4, bottom). However, there was no main effect of ethanol treatment, nor any treatment-by-epoch interaction.

Ethanol Intake

Figure 5 shows mean daily water and/or 10% ethanol intakes by volume for all groups in both experiments (for these analyses, the separate water-only control groups from the forced ethanol and free-choice ethanol comparisons in experiment 1 have been combined). Although adequate fluid intake was maintained in all groups, pairwise *t* tests showed that forced intake of 10% ethanol resulted in significant reductions in daily fluid intake relative to both free-choice ethanol and water-only control groups, in both experiments (Fig. 5, top). In addition, daily fluid intake was significantly higher in the free-choice ethanol group than in the water-only controls in experiment 2, but not in experiment 1 (all $p < 0.05$; Fig. 5, top). Comparison of water and ethanol intakes in the free-choice groups revealed that voluntary ethanol intake was higher and water intake was lower in experiment 2 than in experiment 1 (Fig. 5, middle); ethanol preference ratios (i.e., 10% ethanol intake divided by total fluid intake) averaged about 35% in experiment 1 and about 57% in experiment 2. Comparison of daily ethanol in free-choice and forced ethanol groups showed that forced ethanol intake was significantly higher than voluntary intake in both experiments (Fig. 5, bottom). Indeed, animals under forced ethanol intake conditions consumed about twice as much ethanol as did those under free-choice conditions.

DISCUSSION

The present study demonstrated that chronic forced (but not free-choice) ethanol intake alters photic phase shifting and free-running circadian period in C57BL/6J mice. Such effects could reflect direct pharmacological targeting of circadian clock cells in the SCN, and indeed, recent studies have shown that ethanol application to the SCN attenuates the phase-shifting effects of light pulses in vivo (Ruby et al., 2009) and of glutamate in vitro (Prosser et al., 2008).

C57BL/6J mice were used in these studies partly because they display the highest levels of voluntary ethanol intake among all inbred strains tested to date (Belknap et al., 1993; Yoneyama et al., 2008). Nevertheless, we observed alterations in circadian pacemaker function only under conditions of forced ethanol intake, which yielded ethanol intakes about twice those seen under free-choice conditions. Inasmuch as blood ethanol concentrations were not obtained in this study, we cannot speculate regarding the blood levels necessary to produce such effects. These observations may be related to the relative ethanol insensitivity of C57BL/6J mice in other domains, including ethanol-induced sedation, ataxia, and withdrawal (Crabbe et al., 2006; Metten and Crabbe, 2005). Thus, future studies will examine the chronobiological effects of ethanol in inbred mouse strains characterized by lower preference for and greater physiological sensitivity to ethanol. Such studies will clarify the relationship between strain differences in chronobiological sensitivity to ethanol and other, better-studied ethanol-response phenotypes.

The present results are consistent with previous studies showing that both chronic (Seggio et al., 2007) and acute (Ruby et al., 2009) ethanol exposure attenuates photic phase shifting in Syrian hamsters. Similar attenuation of photic phase shifting has also been reported for other sedative-anxiolytic drugs (Duncan et al., 1998; Dwyer and Rosenwasser, 1998; Subramanian and Subbaraj, 1996). Nevertheless, mice and hamsters apparently differ in the circadian

phase dependence of such effects. Thus, ethanol selectively attenuates phase advances to late-night light pulses in hamsters (Seggio et al., 2007; Ruby et al., 2009) and selectively attenuates phase delays to early-night light pulses in mice (present study). Although the mechanism underlying phase-dependent sensitivity to ethanol is unknown, it should be noted that hamsters generally show more robust phase advances than delays, whereas the opposite is true in mice. Thus, ethanol selectively inhibits photic phase shifting during the temporal window of maximal responsiveness in both species. In addition, direct in vitro ethanol application to the SCN inhibits both the phase-advancing and phase-delaying effects of glutamate in brain slices prepared from C57BL/6J mice (Prosser et al., 2008). This result suggests that modulatory signals originating outside the SCN are responsible for conferring phase specificity to the in vivo effects of ethanol on the photic entrainment pathway.

Despite the significant attenuation of photic phase shifting under forced ethanol conditions, we failed to observe any effect of acute ethanol withdrawal relative to maintained drinking. This test was conducted in anticipation of a possible “rebound” potentiation of photic phase shifting during acute withdrawal, a hypothesis based on the known ability of chronic ethanol treatment to up-regulate excitatory NMDA-glutamate receptors and down-regulate inhibitory GABA-A receptors, leading to central nervous system hyperexcitability that is unmasked only during ethanol withdrawal (Davis and Wu, 2001; Faingold et al., 1998). Although glutamatergic and GABAergic mechanisms are known to reciprocally modulate the circadian pacemaker's response to photic stimuli (Rosenwasser, 2003), it is not known whether adaptations to chronic ethanol occur specifically within the SCN and/or other components of the circadian timing system, or what the time course of such adaptations might be. Future experiments will examine whether other, withdrawal-sensitive inbred mouse strains exhibit ethanol withdrawal-related potentiation of photic phase shifting.

The present results are also consistent with previous studies showing that chronic ethanol intake modulates the free-running circadian period in constant darkness in hamsters (Mistlberger and Nadeau, 1992) and rats (Dwyer and Rosenwasser, 1998; Rosenwasser et al., 2005a). Importantly, effects on the free-running period occurred despite the fact that ethanol had only minor effects on the overall robustness of free-running rhythmicity, and was without significant effect on total daily activity levels, similar to our previous studies with rats (Rosenwasser et al., 2005a). Nevertheless, effects on the free-running period have been somewhat variable across studies, possibly due to species or strain differences in ethanol responsiveness. Thus, whereas Mistlberger and Nadeau (1992) originally reported period lengthening during voluntary ethanol intake in hamsters, our laboratory observed period shortening during voluntary ethanol intake in Wistar rats (Dwyer and Rosenwasser, 1998) and both lengthening and shortening of the free-running period in Long-Evans rats during forced ethanol intake (Rosenwasser et al., 2005a). Taken together, the results of these studies resemble the inconsistent effects on the free-running period seen during treatment with other anxiolytic and antidepressant drugs (Duncan et al., 1998; Rosenwasser, 1996; Subramanian and Subbaraj, 1996; Wollnik, 1992).

Animals in the free-choice ethanol groups displayed ethanol preference ratios of about 0.35 in experiment 1 and about 0.57 in experiment 2, differing both from each other and from published reports of preference ratios of 0.60 to 0.80 for this strain (Belknap et al., 1993; Yoneyama et al., 2008). These differences could be due, in part, to differences in housing conditions among the various experiments. As is common in behavioral chronobiology but uncommon in studies of ethanol preference, animals in the present study were housed individually and had continuous access to running wheels. Both running-wheel access (McMillan et al., 1995; Ozburn et al., 2008; Werme et al., 2002) and social housing (Araujo et al., 2005; Reed et al., 2001; Wolffgramm, 1990) have been shown to affect voluntary ethanol intake. Further, the relatively higher ethanol preference observed in experiment 2

may have been due, in part, to the use of prolonged exposure to constant darkness for assessment of free-running activity rhythms, inasmuch as previous research has shown that maintenance in constant darkness or exposure to short photoperiods increases ethanol preference in several rodent species (Burke and Kramer, 1974; Geller, 1971; Millard and Dole, 1983; Reiter et al., 1974; Smith et al., 1980).

In summary, these results confirm and extend previous work on the chronobiological effects of chronic ethanol intake in rats and hamsters to include the C57BL/6J inbred mouse, and provide additional evidence that ethanol alters fundamental properties of the underlying circadian pacemaker. Further studies will be required to identify possible strain differences in the chronobiological effects of ethanol and ethanol withdrawal, and to determine how these effects are related genetically or physiologically to other behavioral and neurobiological effects of ethanol.

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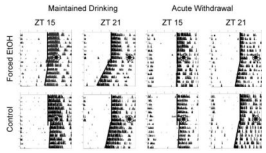

Figure 1.

Representative actogram segments showing light-induced phase shifts for forced ethanol and control animals, at ZT 15 and ZT 21, during either maintained drinking or at 24 h after ethanol replacement by plain water (acute withdrawal). Bold lines superimposed on each chart connect successive activity onsets prior to and following each light pulse; stars indicate the approximate times of light pulse delivery.

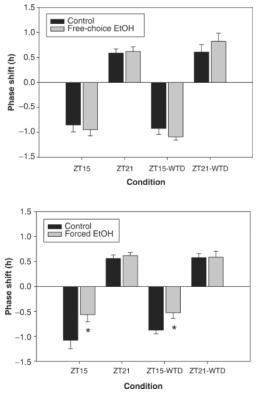

Figure 2.

Mean (± SEM) light-induced phase shifts (free-choice ethanol vs. controls, top; forced ethanol vs. controls, bottom). Each animal was tested a total of 4 times: at ZT 15 and ZT 21, during maintained drinking and at 24 h following ethanol withdrawal (WTD). Asterisks indicate significant attenuation of phase shifting.

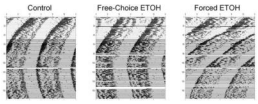

Figure 3.
Representative actograms showing free-running activity rhythms under long-term DD from 1 animal in each of the 3 groups (forced ethanol, free-choice ethanol, and water-only controls). All animals were maintained on plain water for the 1st 3 weeks of the experiment, after which ethanol was continuously available in the forced and free-choice ethanol groups (horizontal line indicates beginning of ethanol treatment on day 22).

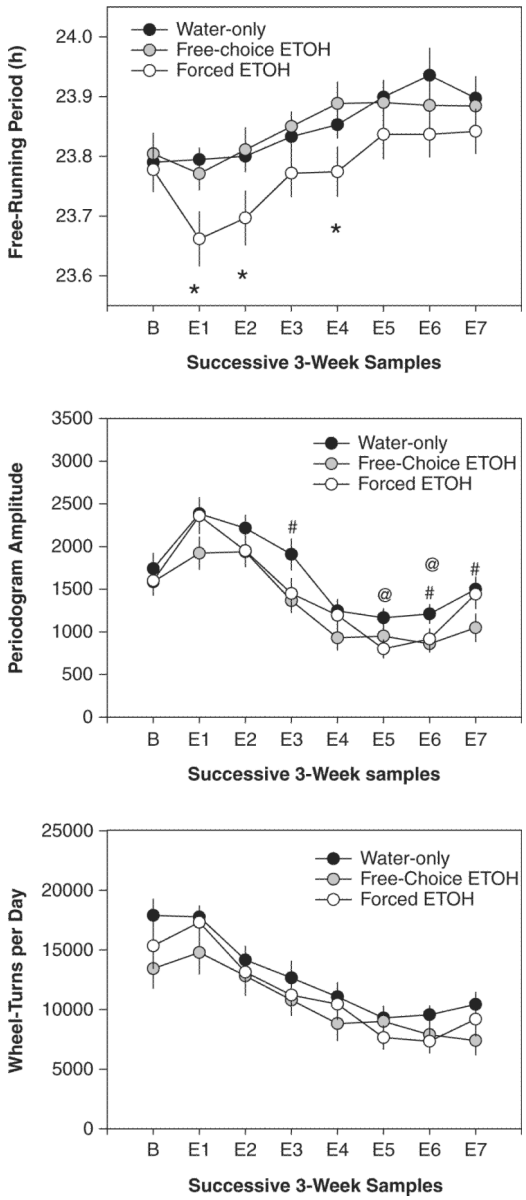

Figure 4.

Mean (± SEM) free-running period (top), periodogram amplitude (middle), and daily activity (bottom) for all 3 groups in successive 3-week data samples. “B” indicates the initial water-only baseline, and “E1” through “E7” indicate successive 3-week samples in which ethanol was continuously available in the forced and free-choice ethanol groups. * = controls significantly different from both ethanol-treated groups; @ = controls significantly different from forced ethanol group; # = controls significantly different from free-choice ethanol group.

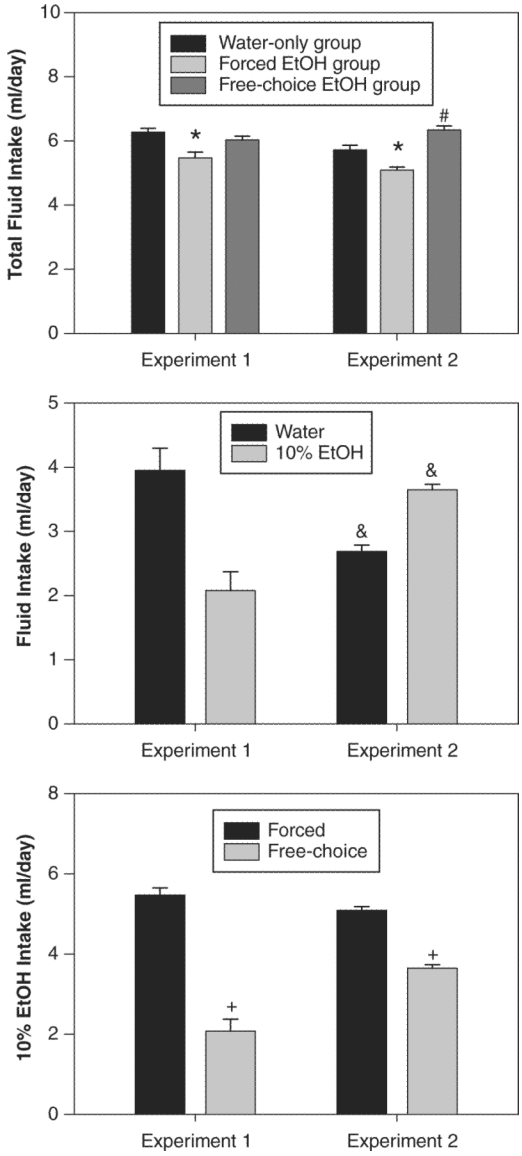

Figure 5.

Mean (± SEM) fluid intakes in both experiments. (Top) Total fluid intake for forced and free-choice ethanol groups and combined water-only controls. (Middle) Water and 10% ethanol intake in free-choice ethanol groups. (Bottom) Ten percent ethanol intake in forced and free-choice ethanol groups. * = forced ethanol group significantly different from both controls and free-choice ethanol groups; # = free-choice ethanol group significantly different from controls; & = experiment 2 significantly different than experiment 1; + = free-choice ethanol group significantly different from forced ethanol group.