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## Chronic daily ethanol and withdrawal: 6. Effects on rat sympathoadrenal activity during “abstinence”

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### Abstract

We have reported that repetitive daily ethanol consumption increased anxiety-like behavior in rats 4 weeks after ethanol consumption had ceased, consistent with the persistently increased anxiety exhibited by abstinent alcoholics. Increased anxiety is associated with sym-pathoadrenal activation, so we have now also investigated ethanol-induced persistent changes in basal and stress-induced plasma epinephrine (E) and norepinephrine (NE) levels. Male Sprague-Dawley rats received liquid diet containing ethanol versus pair-fed isocaloric control liquid diet for 9 weeks. After 5 weeks’ subsequent “abstinence” (i.e., no ethanol in the diet), the control rats exhibited low basal plasma E and NE, which were both increased by 150–300% within 5 min after transfer to a novel cage in a novel room, returning toward basal levels within 15 min. “Abstinent” ethanol-treated rats exhibited elevated basal E levels (195% of controls,  $P < .05$ ), which were not significantly altered by transfer to novel environment; basal NE levels tended ( $P < .07$ ) to be elevated and likewise were not altered by novel environment. These results suggest that daily ethanol consumption can induce persistent increases in sympathoadrenal activation during subsequent “abstinence,” which are relatively refractory to further stimulation.

### Keywords

Alcohol; Ethanol; Stress; Epinephrine; Norepinephrine; Abstinence

### 1. Introduction

We have previously developed a well-characterized liquid diet model in which (1) rats drink sufficient ethanol to achieve daily nocturnal plasma ethanol levels of 125–150 mg/dl, alternating with daily withdrawal to undetectable diurnal ethanol levels; (2) physical dependence on ethanol is established, with daily diurnal withdrawal episodes; and (3) hypothalamo-pituitary-adrenal, hypothalamo-pituitary-gonadal, hypothalamo-pituitary-thyroid, and behavioral (response to novelty, sucrose preference) changes are all consistent with those of actively drinking and subsequently abstinent alcoholics (Rasmussen, 2003a; Rasmussen et al., 2000, 2001, 2003). This model also increased water consumption and anxiety-like behavior during imposed “abstinence” long (>4 weeks) after removal of ethanol from the diet, consistent with the persistently increased fluid consumption exhibited by abstinent alcoholics (Doring et al., 2003) and with evidence that increased anxiety has a key

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role in negative reinforcement contributing to relapse by abstinent alcoholics (Koob & LeMoal, 1997). Since diuretic actions of ethanol have been reported to involve activation of sympathetic autonomic mechanisms (Pohorecky & Packard, 1986), and sympathetic activity has likewise been reported to be increased in anxiety (Sullivan et al., 2000), we hypothesized that chronic ethanol abuse may induce changes in central and peripheral sympathetic regulation which persist long into abstinence and which may contribute to relapse. Consequently, we have now used the same liquid diet model to investigate “abstinence”-induced changes in plasma norepinephrine (NE) concentrations as an indirect index of overall sympathoneuronal activity, and changes in plasma epinephrine (E) concentrations as an index of primarily sympathoadrenal activity. We reasoned that this model, which produces not only daily oral ethanol consumption but also neuroendocrinal and behavioral changes characteristic of actively drinking and subsequently abstinent alcoholics, would allow evaluation of abstinence-induced changes in sympathetic regulation especially relevant to alcoholics and alcohol abusers.

## 2. Methods

### 2.1. Animals

Adult male Sprague-Dawley rats obtained from Simonsen Laboratories (Gilroy, CA, USA) were individually housed in 12 h light/12 h dark (lights off at 1800 h) for 2 weeks before and then throughout the study. The rats weighed 260–280 g at the start of the study. All procedures were performed under a University of Washington IACUC-approved protocol in accord with the NIH Guide for Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996), with appropriate measures to minimize pain or discomfort.

### 2.2. Liquid diet

Bio-Serv Liquid Rat Diet L/D’82 (Bio-Serv, Frenchtown, NJ, USA) was provided daily during the final 15 min of the light period in graduated Liquidiet Feeding Tubes (Bio-Serv, Frenchtown, NJ, USA) and removed the next day at approximately 3 h after lights on. On weekends, the liquid diet was likewise provided at the end of the light period but not removed until fresh diet was provided at the same time on the next day. Consequently, on 5 days/week the liquid diet  $\pm$  ethanol available during the dark period was removed early in the subsequent light period, whereas on the weekends it was available continuously. Supplemental water was available at all times for all rats. Each rat was also provided with a nonnutritive nylon Nylabone (Bio-Serv, Frenchtown, NJ, USA) to maintain gnawing and chewing behaviors.

### 2.3. Daily ethanol consumption/daily withdrawal

The paradigm used in these studies was essentially the same as we have previously characterized (Rasmussen et al., 2002), and is illustrated in Fig. 1. There were two treatment groups ( $n = 10$ /group): ethanol versus pair-fed control. For the ethanol rats, ethanol was slowly introduced into liquid diet over a 4-week period (1 day 0% ethanol, 1 day 0.8%, 1 day 1.7%, and then 4 days each at 2.5, 2.9, 3.3, 3.8, 4.2, and 4.6% before finally achieving 5% ethanol; w/v, prepared with 95% ethanol). This 5% ethanol in liquid diet was then provided for an additional 4 weeks, followed by a 1-week withdrawal period in which ethanol was gradually removed from the diet (to avoid withdrawal-associated seizure activity), daily decreasing ethanol concentrations in reversed order from the introduction (i.e., one day each at 4.6, 4.2, 3.8, 3.3, 2.9, 2.5, 1.7, and 0% ethanol). Each pair-fed control rat received the same amount of isocaloric control liquid diet (in which the calories from ethanol were replaced by maltose dextrin) as consumed by a matched ethanol rat on the previous day. After complete removal of ethanol from the diet, all rats received ad libitum rodent chow for the remaining 5 weeks of the study.

## 2.4. Catheterization

At 4 weeks after removal of ethanol from the liquid diet, each rat received a surgically implanted jugular vein catheter (Harms & Ojeda, 1974). On each of 3–4 days during the following week, the implanted catheter was connected for at least 90 min to extension tubing extending outside the home cage, commencing at 0800 h, and several 0.35 ml blood samples were withdrawn and then flushed back in, to familiarize the rats with the blood-sampling procedure. The catheter was flushed with 0.25 ml of gentamicin sulfate (GentaMax 100; Phoenix Pharmaceutical, Inc.; St. Joseph, MO, USA) in sterile saline (80 µg/ml) at the end of each of these familiarization trials.

## 2.5. Novel environment test

One week after catheterization (i.e., 5 weeks after cessation of ethanol consumption), the implanted catheter of each rat was again connected to extension tubing at 0800 h. After 90 min, a blood sample was collected from each undisturbed rat in its home cage before transfer to a novel environment (i.e., a clean cage with wire mesh floor instead of the bedding present in the home cage, visually isolated from other rats, in an unfamiliar room, with unfamiliar “white” 60 dB background noise), and additional blood samples were collected after 5 and 15 min.

## 2.6. Blood samples

Each 0.35 ml blood sample was added to 10 µl of heparin sodium solution (1,000 u/ml in saline; Elkins-Sinn, Inc.; Cherry Hill, NJ, USA) and 14 µl of dithiothreitol solution (0.25 M in water; Sigma-Aldrich, Co.; St. Louis, MO, USA) in a polypropylene microfuge tube on ice. The samples were immediately mixed and kept on ice until completion of sampling. Plasma was then collected and stored at –80°C until assay.

## 2.7. Assays

Plasma E and NE levels were determined by a sensitive single-isotope radioenzymatic method (Evans et al., 1978). The minimum detectable levels of both E and NE were approximately 20 pg/ml, and the intra-assay coefficients of variation were approximately 11%.

## 2.8. Data analysis

The data are presented as mean ± S.E. E and NE data were analyzed by two-way repeated measures analysis of variance (treatment × time) with subsequent specific comparisons by Student’s *t*-test (control vs. ethanol at individual times) or one-way analysis of variance for repeated measures, with Newman–Keuls or Mann–Whitney Rank Sum test post-hoc comparisons (between times within a treatment).  $P < .05$  was considered statistically significant.

## 3. Results

Five weeks after termination of daily ethanol versus pair-fed control treatment, “abstinent” ethanol rats exhibited basal plasma NE levels which tended to be elevated ( $P = .07$ ) compared to control rats which had never consumed ethanol (Fig. 2). Plasma NE concentrations in the control rats increased by  $296 \pm 88\%$  ( $P < .01$ ) 5 min after transfer to a novel cage in a novel room, returning toward basal levels within 15 min (Fig. 2). In contrast, NE levels in the rats which had previously consumed ethanol were not significantly altered either 5 or 15 min after transfer to the novel environment (Fig. 2). Plasma NE levels 5 min after transfer to the novel environment were significantly higher in the control rats than in the “abstinent” ethanol rats ( $P < .05$ ).

The rats that had previously consumed ethanol likewise exhibited basal plasma E levels which were elevated by 95% ( $P < .05$ ) compared to pair-fed control rats, which had never consumed ethanol (Fig. 2). Plasma E concentrations in the control rats increased by  $174 \pm 39\%$  ( $P < .001$ ) 5 min after transfer to a novel cage in a novel room, returning toward basal levels within 15 min. In contrast, the elevated basal levels in “abstinent” rats, which had previously consumed ethanol were not further increased ( $P = .18$ ) following transfer to the novel environment (Fig. 2). Plasma E levels were not significantly different between the “abstinent” ethanol versus control rats after transfer to the novel environment.

Body weights of ethanol versus control rats were not significantly different at the end of ethanol treatment (ethanol:  $388 \pm 13$  g; control:  $398 \pm 10$  g) or at the time of the novel environment test (ethanol:  $441 \pm 12$  g; control:  $438 \pm 12$  g).

#### 4. Discussion

These results suggest that “abstinent” rats tested 5 weeks after cessation of daily consumption/withdrawal of ethanol in liquid diet exhibited increased daytime plasma E, and probably ( $P < .07$ ) also NE, levels. Extensive efforts were made to assure that the baseline plasma E and NE concentrations represented unstressed levels (e.g., venous catheterization a week prior to the experimental trial; remote blood sampling via catheter extension tubing extending outside of cage; familiarization with the blood-sampling procedure on each of 3–4 days prior to the experimental trial; connection of the catheter extension tubing 90 min before withdrawing blood in both the familiarization and experimental trials). However, it is not possible to completely eliminate the possibility that the increased baseline E levels in “abstinent” ethanol rats may have reflected increased sensitivity and/or decreased habituation to acute stresses associated with the experimental procedures, rather than chronically sustained increases in activity. It is also possible that the increased plasma E concentrations may have been due to decreased clearance rather than increased adrenal secretion. In any case, the increased baseline plasma E concentrations in this rat model of abstinence are consistent with increased basal plasma E levels likewise demonstrated in nondepressed abstinent (3 weeks) alcoholic patients (Ehrenreich et al., 1997).

E and NE plasma levels in these “abstinent” ethanol rats were not further increased by novel environment stress, in contrast to the novelty-induced acute increases of both E and NE levels in control rats. The “abstinent” ethanol rats appeared to be clearly agitated after transfer to the novel environment, consistent with our previous report that “abstinent” rats in this same daily ethanol liquid diet consumption/withdrawal model exhibited increased (relative to pair-fed controls) locomotor activity in a similar novel environment (Rasmussen et al., 2001). Furthermore, we have determined in another study, again using this same liquid diet model, that air-puff startle elicited significantly greater heart rate increases (monitored by radiotelemetry) in “abstinent” ethanol rats compared to pair-fed controls (Rasmussen, 2003b). Even though significant changes in the plasma E and NE indices were not observed in the “abstinent” ethanol rats in this study, the results from these complementary studies suggest that novel environment stress-induced sympathetic activation probably did occur. Considered in the context of the elevated basal plasma E concentrations, and the trend for elevated basal NE, the lack of further significant plasma E and NE responses to acute novel environment stress in the “abstinent” ethanol rats suggests that increases in basal sympathetic activity may have left these plasma indices refractory to further significant change. We speculate that this refractoriness may be due to (1) relative depletion of readily releasable reserves of E and NE; (2) already increased circulating pools of E and NE (diluting the impact of further acute increases in E and NE release); (3) increased rapid metabolic clearance; or (4) some combination of these potential mechanisms. Since the novel environment stress-induced peak E levels in the “abstinent” ethanol rats were not significantly different from those of the

control rats, resolution of acute sympathoadrenal responses in the “abstinent” rats may also have been compromised by a “ceiling” effect.

This study did not include rats consuming ad libitum standard chow diet throughout the experiment. Consequently, it cannot be determined if nutritional or other factors associated with the liquid diet may have altered blood E or NE levels. Nonetheless, the results do demonstrate that under consistent and closely controlled isocaloric conditions, chronic ethanol consumption did alter E and NE plasma concentrations during prolonged imposed abstinence, under basal conditions and in response to transfer to a novel environment.

The brain noradrenergic system and the peripheral sympathetic system are functionally integrated, plasma NE concentrations generally correlate well with cerebrospinal fluid (CSF) NE, and this correlation is not due to peripheral catecholamines gaining access to CSF (Peskind et al., 1986; Roy et al., 1988; Ziegler et al., 1977). Plasma NE and E responses to stress are mediated at least in part by noradrenergic mechanisms in the brain, and stress-induced increases in plasma NE and E concentrations have been demonstrated to be consistent with corresponding changes in extracellular concentrations of NE in the hypothalamic paraventricular nucleus which integrates many stress responses (Tjurmina et al., 1999). Furthermore, evidence for a close temporal relationship between activation of locus coeruleus neurons (which provide much of the noradrenergic innervation of the central nervous system) and activation of peripheral sympathetic neurons likewise suggest that under at least some circumstances peripheral sympathetic activity may re-reflect central noradrenergic activation (Elam et al., 1981; Reis et al., 1988). Since increased brain noradrenergic and peripheral sympathetic activation are associated with increased anxiety (Jensen et al., 1997; Sullivan et al., 1999), the apparent tonic sympathetic activation in the “abstinent” ethanol rats in this study is consistent with increased anxiety-like behavior which we have previously demonstrated in identically-treated “abstinent” rats (Rasmussen et al., 2001), as well as with the increased anxiety which is a major risk factor for relapse by abstinent alcoholics and is characteristic of type I alcoholism (Cloninger, 1987; Kushner et al., 2000). The evidence of shared vulnerability between alcoholism and anxiety disorders has suggested that the comorbidity between alcoholism and anxiety may be in part attributable to common or shared etiologic factors, and it has been suggested that the two disorders may both represent manifestations of similar underlying factors (Merikangas et al., 1994, 1998; Sinha et al., 1998). Increased sympathetic activation during abstinence may be one of these underlying factors.

Since alcohol can be sympatho-suppressive, anxiolytic, and sedating, it has been suggested that sympathetic activation and anxiety can lead to alcohol abuse as a form of self-medication (Edwards et al., 1972; Kushner et al., 1990, 1999; Shirao et al., 1988). However, the current results, together with our previous work using this same experimental model, clearly suggest that chronic alcohol abuse can also conversely *induce* neuroadaptive changes responsible for subsequent long-term increased basal sympathoadrenal activity and anxiety during abstinence, as exhibited by abstinent alcoholics, thereby creating conditions favoring renewed drinking, i.e., relapse. Consequently, sympathetic activation and anxiety may not only facilitate initiation of alcohol abuse but also contribute to the transition to dependence.

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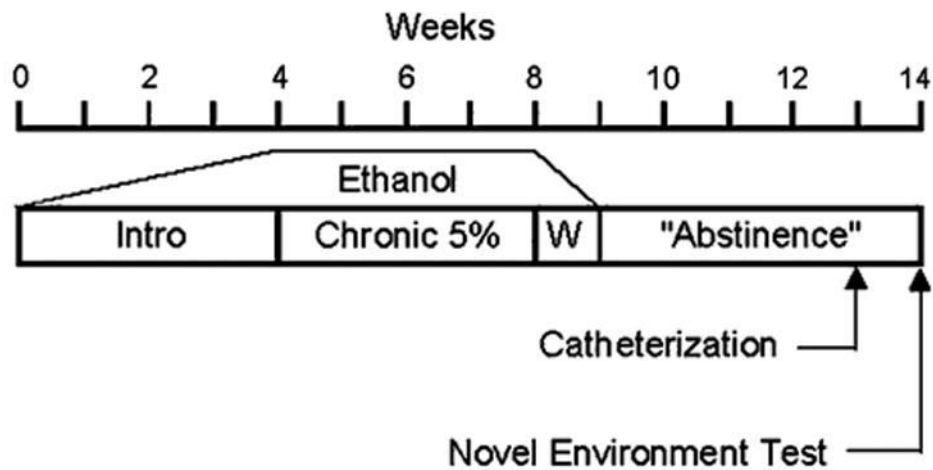
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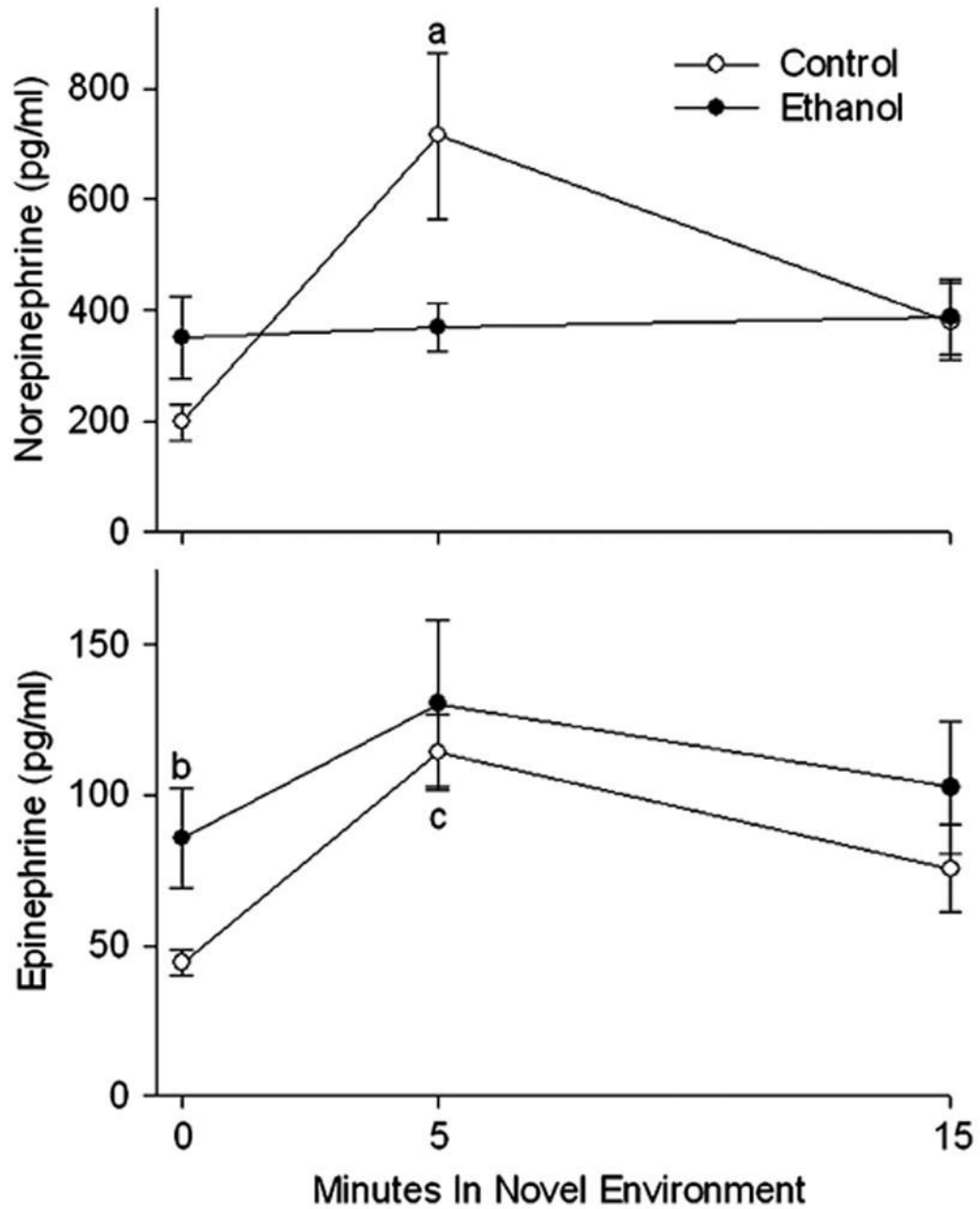
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**Fig. 1.**

Experimental design. Ethanol was slowly introduced into liquid diet over a 4-week period (Intro), achieving a 5% (w/v) maximum concentration. This 5% ethanol-containing diet was then provided for an additional 4 weeks (Chronic 5%), followed by a 1-week withdrawal period (W) in which ethanol was gradually removed from the diet, and then switched to standard rodent chow for 5 additional weeks ("Abstinence"). After 4 weeks of this forced "abstinence," each rat received a jugular catheter and was acclimated to home-cage remote blood sampling. One week after catheterization, a blood sample was collected from each rat while undisturbed in its home cage, and subsequent blood samples were collected 5 and 15 min after transfer to a novel environment.



**Fig. 2.** Plasma norepinephrine (upper panel) and epinephrine (lower panel) concentrations in “abstinent” ethanol rats and pair-fed control rats under basal conditions (time 0; samples collected from undisturbed rats in their home cages) and 5 and 15 min after transfer to a novel environment. <sup>a</sup> $P < .05$  versus corresponding pair-fed control, and  $P < .01$  versus time 0. <sup>b</sup> $P < .05$  versus corresponding pair-fed control. <sup>c</sup> $P < .001$  versus time 0. Each point represents the mean  $\pm$ S.E. of 10 rats.