Endocrine, *vol. 22, no. 2, 143–150, November 2003* 0969–711X/03/22:143–156/$25.00

Chronic Daily Ethanol and Withdrawal

4. Long-Term Changes in Plasma Testosterone Regulation, But No Effect on GnRH Gene Expression or Plasma LH Concentrations

Dennis D. Rasmussen,[1] Dipak K. Sarkar,[2] James L. Roberts,[3] and Andrea C. Gore[4]

[1]VA Puget Sound Health Care System Mental Illness Research, Education and Clinical Center and the Department of Psychiatry and Behavioral Sciences, University of WA, Seattle, WA; [2]Department of Animal Sciences, Rutgers University, New Brunswick, NJ; [3]University of Texas Health Science Center, San Antonio, TX; and [4]University of Texas at Austin, TX

Although ethanol has been repeatedly demonstrated to inhibit the hypothalamo–pituitary–testes axis by multiple mechanisms, plasma testosterone levels can be normal in alcoholics who do not exhibit severely compromised liver function and even increased in some abstinent alcoholics, suggesting that adaptive changes to chronic alcohol abuse may alter these regulatory mechanisms. To address this variability, we have investigated the effects of chronic ethanol and withdrawal on rat testosterone regulation using a well-characterized liquid diet model that we have previously demonstrated to (1) provide daily oral ethanol consumption that produces behaviorally relevant plasma ethanol levels during the active (awake) stage of the photoperiod; (2) establish physical dependence on ethanol; and (3) produce not only hypothalamo–pituitary–adrenal axis, but also behavioral (anxiety-like behavior, response to novelty, sucrose preference) changes consistent with those of actively drinking and subsequently abstinent alcoholics. The results demonstrate that chronic daily episodes of ethanol consumption and withdrawal by male Sprague-Dawley rats decreased ($p < 0.01$) plasma testosterone levels late in the afternoon (by 70% relative to *ad libitum*–fed controls and 63% relative to pair-fed controls), but not in the morning. During gradual cessation of daily ethanol consumption, morning plasma testosterone levels increased, and this 90–115% ($p < 0.05$) increase was maintained for 3 d after complete cessation of ethanol consumption. Three weeks after cessation of ethanol consumption, plasma testosterone levels were again increased by approx 100% ($p < 0.01$). Plasma luteinizing hormone (LH) concentrations and anterior hypothalamus/preoptic area gonadotropin-releasing hormone (GnRH) mRNA levels were not altered at any of these time points. Thus, chronic daily ethanol consumption and daily withdrawal induced changes in circulating testosterone regulation that (a) were time of day dependent and (b) included adaptive changes persisting long after consumption of ethanol ceased. Accordingly, resolution of changes in testosterone regulation and their potential roles in alcohol abuse and relapse will require evaluating changes throughout the circadian cycle during, shortly after, and long after active alcohol abuse.

Key Words: Alcohol; ethanol; testosterone; withdrawal; abstinence; circadian.

Introduction

Ethanol can suppress activity of the hypothalamo–pituitary–gonadal (HPG) axis through inhibition of hypothalamic gonadotropin-releasing hormone (GnRH) and/or pituitary luteinizing hormone (LH) secretion *(1–9)* as well as directly at the testes, through inhibition of testosterone biosynthesis *(10–14)*. In addition, there is evidence that ethanol-induced acute inhibition of testosterone secretion can also be mediated by a direct brain–gonadal neural pathway *(15)*. Nonetheless, normal serum levels of testosterone have been reported in noncirrhotic alcoholics and in cirrhotic alcoholics without hepatic decompensation (i.e., individuals with long-term intensive ethanol use but without the confounding influence of severely compromised liver function), as reviewed by Heinz et al. *(16)*. Furthermore, testosterone levels have been reported to be elevated during abstinence by subjects exhibiting type II alcoholism *(17)*. This variability suggests that long-term ethanol use may induce adaptive changes in testosterone regulation.

To address the variability of ethanol-induced changes in testosterone levels, we have investigated the effects of chronic ethanol and withdrawal on rat testosterone regulation using a well-characterized liquid diet model that we have previously demonstrated *(18,19)* to (1) provide reproducible daily oral ethanol consumption that produces behaviorally relevant plasma ethanol levels during the active (awake)

Received May 22, 2003; Revised July 18, 2003; Accepted August 7, 2003.
Author to whom all correspondence and reprint requests should be addressed: Dennis D. Rasmussen, PhD, VA Puget Sound Health Care System Medical Center, S-116-MIRECC, 1660 S. Columbian Way, Seattle, WA. E-mail: drasmuss@u.washington.edu

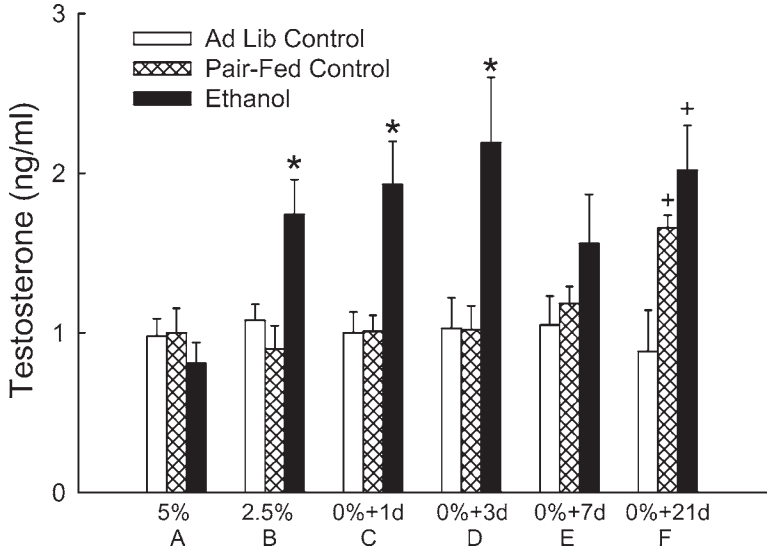

Fig. 1. Effect of chronic ethanol consumption and withdrawal on morning plasma testosterone levels after rapid (1 wk) introduction of ethanol to the liquid diet (Experiment 1). The rats were killed 3 h after lights-on at intervals during the experimental trial: (A: 5%) after 1 wk gradual introduction and 4 wk of 5% (w/v) ethanol consumption; (B: 2.5%) midway through subsequent withdrawal of ethanol from the diet (i.e., after 1 d of 2.5% ethanol), and at 1, 3, 7, or 21 d after complete withdrawal of ethanol from the diet (C: 0% + 1 d, D: 0% + 3 d, E: 0% + 7 d, and F: 0% + 21 d, respectively). Each bar represents the mean ± SE of 9–10 rats. *$p < 0.05$ vs both *ad libitum*–fed controls and pair-fed controls. +$p < 0.05$ vs *ad libitum*–fed controls.

stage of the photoperiod; (2) establish physical dependence on ethanol; and (3) produce not only hypothalamo–pituitary–adrenal axis, but also behavioral (anxiety-like behavior, response to novelty, sucrose preference) changes consistent with those of actively drinking and subsequently abstinent alcoholics. We reasoned that using this model, which has been confirmed to produce not only daily oral ethanol consumption but also both neuroendocrine and behavioral changes characteristic of actively drinking and subsequently abstinent alcoholics, would allow determination of chronic ethanol- and withdrawal-induced changes in testosterone regulation especially relevant to alcoholics and alcohol abusers.

Results

Experiment 1:
Rapid (1 Wk) Introduction of Ethanol, Liquid Diet Continuously Available

We reported in a previous article in this series that the addition of 5% (w/v) ethanol to liquid diet suppressed weight gain for approx 3 wk in the rats evaluated in this study (data previously presented in ref. *18*). This suppression of weight gain was associated with an initial 50% decrease in liquid diet consumption, which gradually stabilized over 3 wk to a level approx 90% that of the *ad libitum*–fed control rats; after stabilization of liquid diet consumption, the ethanol-fed rats and their pair-fed counterparts again gained weight at a rate similar to that of the *ad libitum*–fed control rats *(18)*. There were no significant differences in body weights of pair-fed control rats versus ethanol-treated rats at any time point in the study *(18)*. Daily provision of 5% ethanol in the liquid diet produced nocturnal plasma ethanol levels of 141 ± 24 mg/dL at the time of decapitation (i.e., 3 h after lights-on) on the last day of chronic ethanol consumption (i.e., at point A in Fig. 1); corresponding plasma ethanol levels in the *ad libitum*–fed and pair-fed rats were less than the 10 mg/dL detection threshold *(18)*. During this final week of ethanol treatment, rats receiving ethanol drank significantly more supplemental water than did *ad libitum*–fed and pair-fed controls *(18)*.

After 4 wk of daily 5% ethanol consumption, plasma testosterone levels at 3 h after lights-on were not significantly altered (Fig. 1). However, plasma testosterone levels began to increase during gradual removal of ethanol from the diet (i.e., greater than 60% increase relative to both *ad libitum*–fed controls and pair-fed controls, $p < 0.05$, when the liquid diet ethanol concentration was 2.5%) and remained elevated by 90–115% relative to both *ad libitum*–fed controls and pair-fed controls ($p < 0.05$) for 3 d after complete removal of ethanol (Fig. 1). At 21 d after removal of ethanol from the liquid diet, plasma testosterone levels were again increased ($p < 0.05$) in the rats that had been treated with ethanol (130% relative to *ad libitum*–fed controls) as well as in the pair-fed control rats (88% relative to *ad libitum*–fed controls) (Fig. 1).

Morning plasma LH levels and anterior hypothalamus/preoptic area GnRH mRNA concentrations were not significantly altered by long-term daily ethanol consumption,

Table 1
Chronic Daily Ethanol, Withdrawal, and Forced "Abstinence" Effects on Morning Plasma LH and Hypothalamic GnRH mRNA Levels After Rapid (1 wk) Introduction of Ethanol to the Liquid Diet (Experiment 1)[a]

		LH (ng/mL)	GnRH mRNA (fg/pg 1B15 mRNA)
A: 5%	Ad Lib Control	3.0 ± 0.4	0.88 ± 0.05
	Pair-Fed Control	4.1 ± 0.5	0.90 ± 0.04
	Ethanol	3.4 ± 0.5	0.84 ± 0.04
B: 2.5%	Ad Lib Control	3.4 ± 0.4	0.94 ± 0.05
	Pair-Fed Control	3.5 ± 0.8	0.92 ± 0.07
	Ethanol	3.2 ± 0.6	0.83 ± 0.03
C: 0% + 1 d	Ad Lib Control	3.2 ± 0.4	0.85 ± 0.06
	Pair-Fed Control	3.6 ± 0.2	0.98 ± 0.05
	Ethanol	4.6 ± 0.5	0.79 ± 0.05
D: 0% + 3 d	Ad Lib Control	4.2 ± 0.5	0.80 ± 0.04
	Pair-Fed Control	3.7 ± 0.5	0.86 ± 0.08
	Ethanol	3.3 ± 0.4	0.83 ± 0.04
E: 0% + 7 d	Ad Lib Control	3.6 ± 0.7	0.91 ± 0.07
	Pair-Fed Control	3.0 ± 0.3	0.94 ± 0.06
	Ethanol	3.6 ± 0.3	0.85 ± 0.03
F: 0% + 21 d	Ad Lib Control	4.4 ± 0.7	0.73 ± 0.08
	Pair-Fed Control	4.1 ± 0.6	0.81 ± 0.03
	Ethanol	3.0 ± 0.5	0.84 ± 0.04

[a]The rats were killed 3 h after lights-on at intervals during the experimental trial: (A: 5%) after 1 wk gradual introduction and 4 wk of 5% (w/v) ethanol consumption; (B: 2.5%) midway through subsequent gradual withdrawal of ethanol from the diet (i.e., after 1 d of 2.5% ethanol), and at 1, 3, 7 or 21 d after complete withdrawal of ethanol from the diet (C: 0% + 1 d, D: 0% + 3 d, E: 0% + 7 d, and F: 0% + 21 d, respectively). Each value represents the mean ± SEM of 9–10 rats.

gradual withdrawal of ethanol, or subsequent complete removal of ethanol from the liquid diet for 21 d (Table 1).

Experiment 2:
More Gradual (3 Wk) Introduction of Ethanol, With Liquid Diet Available Only During the Dark Period

We reported in a previous article in this series that the gradual addition of 5% (w/v) ethanol to the liquid diet over a 3 wk period maintained continuous weight gain by the rats evaluated in this study, although at a moderately decreased rate compared with *ad libitum*–fed controls (data previously presented in ref. *18*). This decrease in rate of weight gain was associated with an initial 15–25% decrease in liquid diet consumption, which stabilized at 10–15% decrease after approx 2 wk consumption of 5% ethanol *(18)*. There were no significant differences in body weights of pair-fed control rats versus ethanol-treated rats at any time point in the study *(18)*. Daily provision of 5% ethanol in the liquid diet produced nocturnal plasma ethanol levels of 135 ± 10 mg/dL, and daily removal of the liquid diet early in the light period produced complete plasma ethanol withdrawal by the end of the light period (i.e., plasma ethanol concentrations were undetectable) *(18)*. As in Experiment 1, daily water consumption was significantlyincreased in the ethanol-treated rats *(18)*. The model used in this experiment is illustratedin Fig. 2.

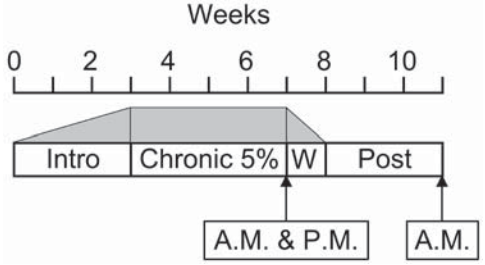

Fig. 2. Experimental model used in Experiment 2. The rats were killed after 3 wk of gradual introduction of ethanol (represented by the shaded area) to the diet (Intro) and four subsequent weeks of 5% w/v ethanol (Chronic 5%), with the liquid diet available from the start of the dark period until 3 h after lights-on. The ethanol was then gradually withdrawn from the liquid diet over a 1 wk period (W), followed by maintaining all rats on ad libitum chow with no ethanol for a 3 wk post-treatment period (Post). A.M. rats were killed at 3 h after lights-on; P.M. rats were killed at lights-off.

After gradual addition of 5% ethanol to the liquid diet over a 3 wk period followed by 4 subsequent weeks of chronic daily ethanol and withdrawal, morning plasma testosterone levels were not significantly altered ($p = 0.24$) (Fig. 3). However, afternoon plasma testosterone levels were decreased ($p < 0.01$) by 70% relative to *ad libitum*–fed controls and 63% relative to pair-fed controls (Fig. 3). A diurnal change in plasma testosterone levels, with afternoon lower than morning levels, was present in the *ad libitum*–fed control rats (27% change, $p < 0.05$) and accentuated in the ethanol-treated rats (68% change, $p < 0.01$), but not in the pair-fed rats (Fig. 3).

Three weeks after complete cessation of ethanol consumption, morning plasma testosterone levels were increased ($p < 0.01$) by approx 100% relative to both *ad libitum*– and pair-fed controls (Fig. 4).

Discussion

These results demonstrate that chronic daily episodes of ethanol consumption and withdrawal by male Sprague-Dawley rats decreased plasma testosterone levels late in the afternoon, but not in the morning. During gradual cessation of daily ethanol consumption, morning plasma testosterone levels increased, and this increase was maintained for 3 d after complete cessation of ethanol consumption. Three weeks after cessation of ethanol consumption, plasma testosterone levels were again increased. Thus, chronic daily ethanol consumption and daily withdrawal can induce changes in circulating testosterone regulation that (a) are time of

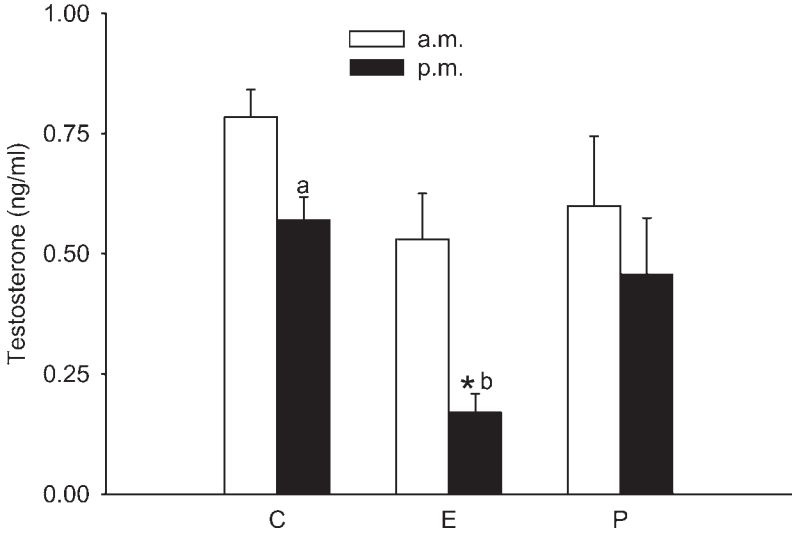

Fig. 3. Effect of chronic daily ethanol consumption and withdrawal on morning and afternoon plasma testosterone levels after prolonged (3 wk) gradual introduction of ethanol to liquid diet available during the dark period (Experiment 2). C, *ad libitum*–fed controls; E, ethanol-treated; P, pair-fed controls. The rats were killed after 3 wk of gradual introduction of ethanol to the diet and four subsequent weeks of 5% ethanol (w/v), with the liquid diet available from the start of the dark period until 3 h after lights-on. The a.m. rats were killed at 3 h after lights-on; p.m. rats were killed at lights-off. Each bar represents the mean ± SE of nine rats. *$p < 0.01$ vs *ad libitum*–fed controls (C) and pair-fed controls (E). [a]$p < 0.05$ vs a.m. [b]$p < 0.01$ vs a.m.

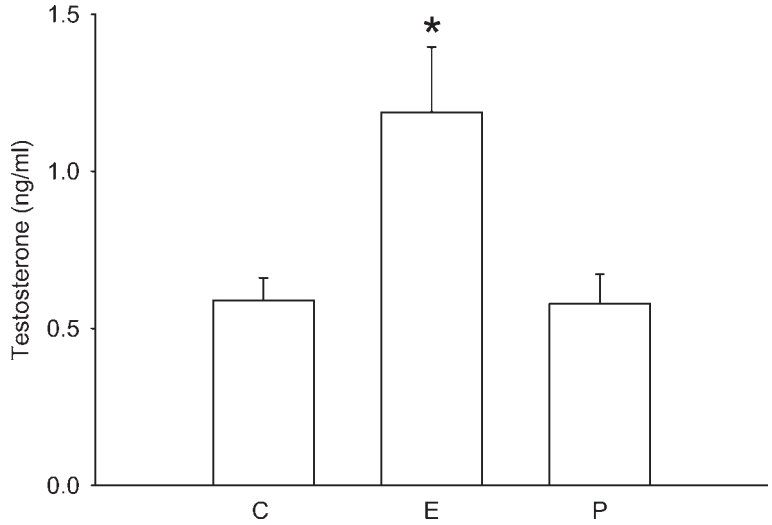

Fig. 4. Effect of prolonged (3 wk) gradual introduction of ethanol to liquid diet and subsequent chronic daily ethanol consumption and withdrawal on morning plasma testosterone levels after 21 d forced "abstinence" (Experiment 2). The rats were killed 3 h after lights-on at 21 d after complete withdrawal of ethanol from the diet following 3 wk of gradual introduction of ethanol to the diet, four subsequent weeks of 5% ethanol (w/v), and 1 wk of gradual withdrawal of ethanol from the diet. C, *ad libitum*–fed controls; E, ethanol-treated; P, pair-fed controls. Each bar represents the mean ± SE of nine rats. *$p < 0.01$ vs *ad libitum*–fed controls (C) and pair-fed controls (E).

day dependent and (b) include adaptive changes persisting long after consumption of ethanol ceases.

The two experimental models used in these studies produced similar changes in plasma testosterone levels 3 wk after withdrawal from chronic ethanol treatment, but different testosterone changes in response to pair-feeding. When ethanol was introduced to the diet in increments over 1 wk (i.e., Experiment 1), consumption of the ethanol-containing liquid diet (and thus consumption of control liquid diet by pair-fed rats) was markedly decreased and body weight gain was suppressed for 2–3 wk (data previously reported in ref. *18*). This reduction in eating and associated suppression of weight gain likely induced chronic hypothalamo–pituitary–adrenal (HPA) axis activation in the pair-fed rats, as revealed by increased adrenal and decreased thymus weights in a follow-up experiment using the same proto-

col, also reported in ref. *18*. In contrast, more gradual introduction of ethanol over 3 wk (Experiment 2) produced less suppression of eating, maintained continuous weight gain, and did not induce HPA activation in the pair-fed rats *(18)*. This suggests that the differences in testosterone regulation in pair-fed control rats 3 wk after cessation of the chronic ethanol treatment may have been due to the prior stresses associated with hunger, malnutrition, or both in the rapid ethanol introduction model (Experiment 1). In our previous analysis of HPA regulation in these same rats *(18)*, we determined that anterior pituitary proopiomelanocortin (biosynthetic precursor of adrenocorticotropin and related peptides) mRNA content was markedly decreased at 3 wk after withdrawal in both ethanol-treated and pair-fed control rats in Experiment 1, but only in ethanol-treated rats in Experiment 2, i.e., consistent with but opposite in direction to the changes in plasma testosterone levels reported here. This suggests that the decreases in HPA activity may have had a role in allowing the increased testosterone levels, consistent with evidence that HPA activity can have an inhibitory role in regulation of the HPG axis *(20–22)*. Conversely, because testosterone has been reported to inhibit the HPA axis in rats *(23)*, it is also possible that the increased testosterone levels 3 wk after withdrawal may have been responsible for the decreased HPA activity. In any case, the consistency of HPA and testosterone changes in response to the different conditions of Experiments 1 and 2 suggest potential commonality or interaction of mechanisms between these endocrine functions.

Because the testosterone changes after 3 wk of forced "abstinence" which were specifically attributable to chronic daily ethanol consumption (i.e., the ethanol-induced changes in Experiment 2) resembled the changes in testosterone levels that were presumably due to previous stresses (i.e., the changes in pair-fed rats in Experiment 1), even the long-term changes in testosterone most specific to ethanol consumption may have represented, entirely or in part, persistent responses to previous chronic stress associated with daily ethanol and withdrawal. We recently used the same model as used in Experiment 2 to demonstrate that rats receiving this chronic daily ethanol/withdrawal treatment experienced marked physical withdrawal each day in the afternoon, i.e., before they were again provided with ethanol diet at the start of the next dark period *(19)*. Consequently, either the daily increases in plasma ethanol or the daily withdrawals, or both, may have been stressful. Repetitive ethanol intoxication, dysphoria associated with repetitive withdrawal, commonly associated nutritional deficits, and the psychosocial consequences of ethanol abuse are likewise stressful for chronic alcohol abusers. Consequently, if stress did contribute to the subsequent changes in testosterone regulation during long-term forced "abstinence" as demonstrated here, these changes are likely to be clinically relevant.

Many studies have demonstrated suppression of the HPG axis in response to acute administration of moderate or high dosages of ethanol (e.g., refs. *6,15,24–27*), whereas some have conversely demonstrated increased plasma testosterone levels by moderate to low dosage ethanol *(24,28,29)*. In the current experiments there were no chronic ethanol-induced changes in plasma testosterone levels in the morning, at the end of nocturnal ethanol consumption. These results suggest a chronic adaptive response to the testosterone-altering effects of ethanol, although transient changes may have occurred during the night, such as when plasma ethanol levels were rising. The lack of detected response is compatible with reports of normal serum levels of testosterone in noncirrhotic alcoholics and in cirrhotic alcoholics without hepatic decompensation (reviewed in ref. *16*). This putative adaptive response to long-term ethanol consumption would also be consistent with the subsequent increased plasma testosterone levels for several days following withdrawal of ethanol in Experiment 1.

Although there were no changes in morning plasma testosterone levels in response to long-term daily ethanol and daily withdrawal, the levels in the afternoon were decreased. Because this is a time at which we have previously demonstrated plasma ethanol to be undetectable and physical withdrawal to be expressed *(19)*, it is reasonable to suggest that the afternoon decrease in plasma testosterone reflected, at least in part, a response to stress associated with the daily withdrawal from ethanol, consistent with low plasma testosterone levels in men during hangover *(30)*. We have previously reported that although afternoon plasma total or free corticosterone levels were not altered in these same ethanol-consuming rats, morning plasma free corticosterone levels were slightly increased, thymus size was decreased, and adrenal size was increased, suggesting chronic hypercorticosteronemia *(18)*. The afternoon decrease in plasma testosterone levels could thus reflect a time-of-day-dependent inhibition by the chronically increased HPA activity. It has also been reported that chronic ethanol consumption and withdrawal disrupts the rhythms of various brain functions and behaviors and produces irreversible changes in the functioning of the suprachiasmatic nucleus, which is the dominant pacemaker of the circadian system *(31)*. Consequently, the diurnal differences in testosterone response to chronic daily ethanol/withdrawal in the current study could also be due to changes in circadian regulation. These results clearly illustrate that determination of plasma testosterone responses at one time of day does not adequately reveal changes in function of the HPG axis, which may be experienced repeatedly by chronic alcohol abusers.

The changes in plasma testosterone levels in Experiment 1 were not accompanied by significant changes in plasma LH, which regulates gonadal testosterone secretion, or in anterior hypothalamus/preoptic area concentrations of mRNA for GnRH, the brain peptide that regulates LH secretion. LH is secreted in a distinctly pulsatile manner, so determinations at a single time point in the relatively small number of animals may have been insufficient to resolve responses

to the ethanol treatment, if they occurred. Although the lack of changes in GnRH gene expression in association with the sustained and relatively large amplitude changes in testosterone levels suggest that the chronic ethanol and withdrawal effects were not mediated at the level of GnRH secretion, it is also feasible that determination of GnRH mRNA at the selected single circadian timepoint may have been inadequate. Resolution of neuroendocrine, direct neuronal, metabolic, and/or intratesticular mechanisms mediating the changes in plasma testosterone will require additional studies.

Testosterone has important and varied roles in modulating behavior *(32)*, and has also been reported to decrease liver alcohol dehydrogenase activity and metabolism of ethanol *(9,33–35)*, but it remains to be determined whether adaptive changes in testosterone regulation are implicated in clinical or psychological symptoms during chronic alcohol use or subsequent abstinence. Since high concentrations of total testosterone have been reported *(17)* to be related to the type II form of alcoholism [distinguished by Cloninger et al. *(36)* to be characterized by family history of alcoholism, early onset, and criminality], our results suggest that chronic alcohol/withdrawal induced persistently increased testosterone levels, or mechanisms mediating this increase, could potentially contribute to increased risk of relapse during abstinence. However, our results also reveal that resolution of changes in testosterone regulation and their potential roles in alcohol abuse and relapse will require evaluating changes throughout the circadian cycle during, shortly after, and long after active alcohol abuse.

Materials and Methods

Relationship to Previous Study

The animals and their corresponding plasma samples and brains investigated in this study are the same as used in our previous analysis of chronic daily ethanol and withdrawal effects on regulation of the HPA axis *(18)*.

Animals

Adult male Sprague-Dawley rats obtained from Simonsen Laboratories (Gilroy, CA) were individually housed in 12 h light/12 h dark (lights off at 1700 h) for 2 wk before and throughout the study. A total of 255 rats weighing 260–280 g at the start of ethanol treatment were used in these studies (174 in Experiment 1 and 81 in Experiment 2). 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 (1985).

Liquid Diet

At the start of the experiment, all rats were switched from rat chow to Bio-Serv Liquid Rat Diet L/D'82 (Bio-Serv, Frenchtown, NJ) provided in graduated Liquidiet Feeding Tubes (Bio-Serv, Frenchtown, NJ), with supplemental water available at all times. Each rat was also provided with a non-nutritive nylon Nylabone (Bio-Serv, Frenchtown, NJ) to maintain gnawing and chewing behaviors. Fresh liquid diet was provided daily during the final 15 min of the light period.

Treatments, Experiment 1

Ethanol-treated rats were gradually introduced to 5% ethanol (w/v, prepared with 95% ethanol) in the liquid diet over a 1 wk period (i.e., 1 d 0% ethanol, 2 d 1.25%, 2 d 2.5%, 2 d 3.75%, then 5%) and then maintained on this ethanol diet for 4 wk. One group of control rats were individually pair-fed with isocaloric control liquid diet in which the calories from ethanol were replaced by maltose-dextrin; another group was fed *ad libitum* with the same control diet. Pair feeding was accomplished by daily measurement of the amount of diet consumed by each ethanol-treated rat and provision of that amount of control diet to the individually matched pair-fed control animal for the subsequent day. After 4 wk at 5% ethanol the ethanol-treated rats were gradually withdrawn from ethanol over 1 wk (i.e., decreasing ethanol concentrations in reversed order from the gradual introduction) and then maintained on control liquid diet for an additional week before switching to chow. There were 57–59 rats in each of the three treatment groups (i.e., ethanol, pair-fed control, *ad libitum*–fed control) at the start of the study. Rats from each treatment group were decapitated at 3 h after lights-on at intervals during the experimental trial (i.e., A: after 4 weeks of 5% ethanol treatment; B: midway through gradual withdrawal of ethanol from the diet, and C–F: 1, 3, 7, and 21 d after complete removal of ethanol from the diet, respectively).

Treatments, Experiment 2

At completion of Experiment 1 it was noted that some responses (e.g., decreases in thymus and spleen weight) of pair-fed control rats were similar to those of the ethanol-treated rats and different from the *ad libitum*–fed controls, suggesting potential confounding by malnutrition or other stresses associated with this model. Further investigation demonstrated that although the relatively rapid (i.e., 1 wk) ethanol introduction protocol used in Experiment 1 induced increased adrenal and decreased thymus weight in pair-fed rats, more gradual introduction of ethanol did not, and it allowed consistent weight gain in both ethanol-treated and pair-fed rats. [These results have been published elsewhere *(18)* and are addressed here in the Discussion section.] Consequently, we reinvestigated the responses to chronic ethanol treatment in a model modified to minimize stress (e.g., hunger) for the pair-fed controls by extending the period during which ethanol was gradually added to the liquid diet to 3 wk (4 d 0% ethanol, 1 d 0.8%, 1 d 1.7%, and then 3 d each at 2.5, 2.9, 3.3, 3.8, 4.2, and 4.6% before finally achieving 5%). After an additional 4 wk at 5% ethanol the ethanol-treated rats were gradually withdrawn from ethanol

over a 1 wk period (1 d each at 4.3, 3.3, 2.5, 1.7, 0.8, and 0% ethanol) and then switched to *ad libitum* chow. The model used in this study is illustrated in Fig. 2.

To ensure that the ethanol-treated rats were experiencing both daily ethanol exposure and daily withdrawal in this experiment, consistent with the repeated withdrawal episodes commonly experienced by alcohol abusers and alcoholics *(37)*, liquid diet which was provided immediately before the start of each 12 h dark period was subsequently removed the next day at 3 h after lights on (in contrast to Experiment 1, in which the liquid diet was always available). On the weekends, the liquid diet was provided at the start of the dark period but not removed until fresh diet was provided at the same time on the next day. Consequently, on 5 d/wk the liquid diet ± ethanol available during the dark period was removed early in the subsequent light period, whereas on the weekends it was available continuously.

There were 27 rats in each of the three treatment groups (i.e., ethanol, pair-fed control, *ad libitum*–fed control). As in Experiment 1, nine rats from each treatment group were decapitated at 3 h after lights-on at the end of the 4 wk chronic 5% ethanol period and at 3 wk after complete withdrawal of ethanol from the diet; nine additional rats from each treatment group were also sacrificed at lights-off (i.e., at 1700 h) at the end of the 4 wk chronic daily ethanol treatment.

Sample Collection at the Time of Sacrifice

At decapitation, trunk blood was collected into chilled polypropylene tubes containing 100 µL of 100 mg ethylenediaminetetraacetic acid per mL of water; plasma aliquots were stored at –70°C in polypropylene tubes. In Experiment 1, the brain was immediately removed and immersed in ice-cold saline. A Rodent Brain Matrix (Activational Systems, Warren, MI) was then used to remove a coronal section extending 3 mm rostral from the caudal edge of the optic chiasm. This coronal section was placed on a stainless steel plate on ice and a triangular piece of tissue (base bounded by the lateral olfactory tracts, apex at the midline of the corpus callosum) including the anterior hypothalamus, preoptic area, and part of the diagonal band was dissected, snap frozen in isopentane on dry ice, and stored at –70°C until solution hybridization/RNase protection assay for GnRH mRNA concentrations.

Radioimmunoassays (RIA)

Plasma testosterone concentrations in Experiments 1 and 2 were assayed with Active™ Testosterone RIA Assay kits from Diagnostic Systems Laboratories, Inc. (Webster, TX). Plasma LH concentrations in Experiment 1 were determined by radioimmunoassay using the rat double-antibody kit provided by the National Hormone and Pituitary Program, with values expressed in terms of NIDDK rLH-RP-3 equivalents. All samples from individual experiments were assayed in a single assay. The detection limits for the testosterone and LH assays were 0.08 ng/mL and 0.39 ng/mL, respectively. The intraassay coefficients of variation for all assays were <8%.

Quantitation of GnRH and Cyclophilin (1B15) mRNAs

Total cytoplasmic RNA was extracted from the anterior hypothalamus/preoptic area and then assayed for proGnRH and 1B15 mRNA contents by solution hybridization/RNase protection assay as we have previously described in detail *(38)*. The 1B15 mRNA contents were not significantly altered at any of the treatment × time combinations and served as internal references for expressing changes in proGnRH mRNA concentrations, controlling for gel-loading variability.

Statistical Analyses

Data are presented as mean ± SEM. Unless otherwise noted, comparisons were performed by two-way analysis of variance followed, when appropriate, by one way ANOVA and Newman–Keuls multiple comparisons, with $p < 0.05$ considered significant.

Acknowledgments

This research was supported by the Department of Veterans Affairs and by National Institute on Alcohol Abuse and Alcoholism/National Institutes of Health grant AA 10567 (DDR).

References

1. Dees, W. L., McArthur, N. H., Farr, K. L., Culler, M. D., and Harms, P. G. (1983). *Biol. Reprod.* **28,** 1066–1070.
2. Hiney, J. K. and Dees, W. L. (1991). *Endocrinology* **128,** 1404–1408.
3. Adams, M. L. and Cicero, T. J. (1991). *Alcoholism* **15,** 685–692.
4. Canteros, G., Rettori, V., Franchi, A., et al. (1995). *Proc. Natl. Acad. Sci. USA* **92,** 3416–3420.
5. Chapin, R. E., Breese, G. R., and Mueller, R. A. (1980). *J. Pharmacol. Exp. Ther.* **212,** 6–10.
6. Emanuele, M. A., Tentler, J., Reda, D., Kirsteins, L., Emanuele, N. V., and Lawrence, A. M. (1989). *J. Endocrinol.* **121,** 37–41.
7. Uddin, S., Emanuele, M. A., Emanuele, N. V., Reda, D., and Kelley, M. R. (1994). *Endocr. Res.* **20,** 201–217.
8. Rivier, C., Rivest, S., and Vale, W. (1992). *Alcoholism* **16,** 935–941.
9. Cicero, T. J. (1982). *Alcohol. Clin. Exp. Res.* **6,** 207–215.
10. Johnston, D. E., Chiao, Y. B., Gavaler, J. S., and Van Thiel, D. H. (1981). *Biochem. Pharmacol.* **30,** 1827–1831.
11. Cicero, T. J., Bell, R. D., Meyer, E. R., and Badger, T. M. (1980). *J. Pharmacol. Exp. Ther.* **213,** 228–233.
12. Adams, M. L., Meyer, E. R., and Cicero, T. J. (1997). *Alcohol Clin. Exp. Res.* **21,** 684–690.
13. Akane, A., Fukushima, S., Shiono, H., and Fukui, Y. (1988). *Alcohol Alcohol.* **23,** 203–209.
14. Orpana, A. K., Harkonen, M., and Eriksson, C. J. P. (1990). *Alcohol Alcohol.* **25,** 499–507.
15. Rivier, C. (1999). *Alcohol. Clin. Exp. Res.* **23,** 38–45.
16. Heinz, A., Rommelspacher, H., Gräf, K.-J., Kürten, I., Otto, M., and Baumgartner, A. (1995). *Psychiatry. Res.* **56,** 81–95.
17. Stålenheim, E. G., Eriksson, E., von Knorring, L., and Wide, L. (1998). *Psychiatry Res.* **77,** 79–88.

18. Rasmussen, D. D., Boldt, B. M., Bryant, C. A., Mitton, D. R., Larsen, S. A., and Wilkinson, C. W. (2000). *Alcohol. Clin. Exp. Res.* **24,** 1836–1849.
19. Rasmussen, D. D., Mitton, D. R., Green, J., and Puchalski, S. (2001). *Alcohol. Clin. Exp. Res.* **25,** 999–1005.
20. Dubey, A. K. and Plant, T. M. (1985). *Biol. Reprod.* **33,** 423–431.
21. Briski, K. P. and Sylvester, P. W. (1994). *Neuroendocrinology* **60,** 601–608.
22. Gambacciani, M., Yen, S. S. C., and Rasmussen, D. D. (1986). *Neuroendocrinology* **43,** 533–536.
23. Viau, V. and Meaney, M. J. (1996). *J. Neurosci.* **16,** 1866–1876.
24. Cicero, T. J. and Badger, T. M. (1977). *J. Pharmacol. Exp. Ther.* **201,** 427–433.
25. Mendelson, J. H., Mello, N. K., and Ellingboe, J. (1977). *J. Pharmacol. Exp. Ther.* **202,** 676–682.
26. Eriksson, C. J. P., Fukunaga, T., and Lindman, R. (1994). *Nature* **369,** 711.
27. Steiner, J. C., Holloran, M. M., Jabamoni, K., Emanuele, N. V., and Emanuele, M. A. (1996). *Alcohol. Clin. Exp. Res.* **20,** 1368–1374.
28. Sarkola, T. and Eriksson, C. J. (2003). *Alcohol. Clin. Exp. Res.* **27,** 682–685.
29. Alomary, A. A., Vallée, M., O'Dell, L. E., Koob, G. F., Purdy, R. H., and Fitzgerald, R. L. (2003). *Alcohol. Clin. Exp. Res.* **27,** 38–43.
30. Ylikahri, R., Huttunen, M., Härkönen, M., et al. (1974). *J. Steroid Biochem.* **5,** 655–658.
31. Madeira, M. D., Andrade, J. P., Lieberman, A. R., Sousa, N., Almeida, O. F. X., and Paula-Barbosa, M. M. (1997). *J. Neurosci.* **17,** 1302–1319.
32. Rubinow, D. R. and Schmidt, P. J. (1996). *Am. J. Psychiatry* **153,** 974–984.
33. Cicero, T. J., Bernard, J. D., and Newman, K. (1980). *J. Pharmacol. Exp. Ther.* **215,** 317–324.
34. Mezey, E. and Potter, J. J. (1985). *Biochem. Pharmacol.* **34,** 369–372.
35. Rachamin, G., MacDonald, J. A., Wahid, S., Clapp, J. J., Khanna, J. M., and Israel, Y. (1980). *Biochem. J.* **186,** 483–490.
36. Cloninger, C. R., Bohman, M., and Sigvardsson, S. (1981). *Arch. Gen. Psychiatry* **38,** 861–868.
37. Hilbom, M. E. (1990). In: *Alcohol and seizures: basic mechanisms and clinical concepts*. Porter, R. J., Mattson, R. H., Cremer, J. A., and Diamond, I. F. (eds.). A. Davies: Philadelphia, PA.
38. Gore, A. C. and Roberts, J. L. (1995). *Endocrinology* **136,** 889–896.