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Chronic Daily Ethanol and Withdrawal

5. Diurnal Effects on Plasma Thyroid Hormone Levels

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We previously demonstrated that chronic daily ethanol consumption and daily withdrawal by male rats in a modified ethanol liquid diet paradigm produced (a) chronically increased adrenal glucocorticoid activity; (b) decreased plasma testosterone; (c) decreased forebrain proopiomelanocortin gene expression; and (d) corresponding alterations in plasma leptin levels—all of which are consistent with reported changes during alcohol abuse and alcoholism. Each of these systems interact with hypothalamo–pituitary–thyroid (HPT) regulation, and links between chronic alcohol abuse and thyroid dysfunction have been suggested by both human and rat studies. Accordingly, we have begun to investigate potential HPT mediation of, or response to, alterations in these systems by investigating plasma thyroid hormone levels in the same chronic daily ethanol/withdrawal paradigm. Chronic daily episodes of ethanol consumption and withdrawal by male Sprague–Dawley rats decreased plasma levels of free (non-protein-bound) triiodothyronine (T3) ($p < 0.01$) and free thyroxine (T4) ($p < 0.05$) in the morning but not in the afternoon, relative to both *ad libitum*-fed and pair-fed controls ($n = 9$/treatment). Plasma total T4 levels were likewise suppressed ($p < 0.01$) in the morning, whereas total T3 levels were increased ($p < 0.05$) in the afternoon. These changes eliminated normal diurnal patterns (higher in the morning) of plasma free T3, free T4, and total T3 concentrations. Three weeks after cessation of ethanol consumption, morning plasma levels of free and total T3 and T4, as well as plasma thyroid-stimulating hormone (TSH), were all not significantly changed by the prior ethanol consumption or pair-feeding. These results reveal that plasma thyroid hormone concentrations are suppressed in a time of day dependent manner by chronic daily ethanol consumption and daily withdrawal in this model of chronic ethanol abuse. During subsequent long-term "abstinence," these thyroid hormones returned to control levels. These results are consistent with evidence that thyroid function is commonly diminished in alcoholism, with variable reports of recovery during abstinence. Further investigations with this rat model of daily ethanol consumption and daily withdrawal will help resolve interactions and roles of the HPT axis in alcohol abuse.

Key Words: Alcohol; ethanol; thyroid; T3; T4; TSH; diurnal.

Introduction

We have previously demonstrated that chronic daily ethanol consumption and daily withdrawal by male rats in a modified ethanol liquid diet paradigm produced (a) chronically increased adrenal glucocorticoid activity (small increases in morning plasma corticosterone levels coupled with increased adrenal weight and decreased thymus weight); (b) decreased plasma testosterone; (c) decreased forebrain proopiomelanocortin (POMC) gene expression; and (d) corresponding alterations in plasma leptin levels *(1–3)*. During subsequent long-term (3-4 wk) "abstinence" (i.e., removal of ethanol from the diet), all of these changes were reversed; adrenal glucocorticoid activity was decreased relative to controls, forebrain POMC gene expression was increased, plasma testosterone levels were increased, and plasma leptin levels tended to be increased, all consistent with reported changes in abstinent alcoholics *(1–3)*. There is evidence that each of these systems interact with hypothalamo–pituitary–thyroid (HPT) regulation *(4–7)*, and links between chronic alcohol abuse and thyroid dysfunction have been suggested by both human and rat studies *(8,9)*. Accordingly, we have begun to investigate potential thyroid axis mediation of, or response to, alterations in these systems by investigating plasma thyroid hormone levels in the same chronic daily ethanol/withdrawal paradigm.

Results

We reported in a previous article in this series that the gradual addition of 5% (w/v) ethanol to liquid diet in increments over a 3 wk period maintained continuous weight gain by the rats evaluated in this current study, although at

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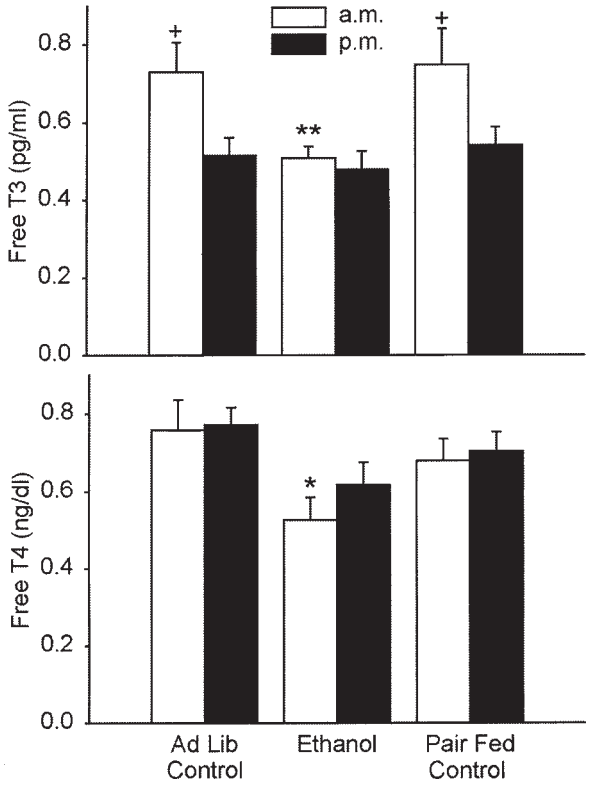

Fig. 1. Effects of chronic daily ethanol consumption and daily withdrawal on morning and afternoon plasma free T3 (upper panel) and free T4 (lower panel) levels. The rats were killed after 3 wk of gradual introduction of ethanol to the diet and four subsequent weeks of 5% ethanol (w/v), with the liquid 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 immediately before lights-off. Each bar represents the mean ± SE of nine rats. The data were analyzed by two-way analysis of variance (time of day × treatment) with subsequent specific comparisons with Student's *t*-test (a.m. versus p.m.) or one-way analysis of variance (between treatments). $^{+}p < 0.05$ versus a.m.; $^{**}p < 0.01$ versus both ad lib control and pair-fed control.

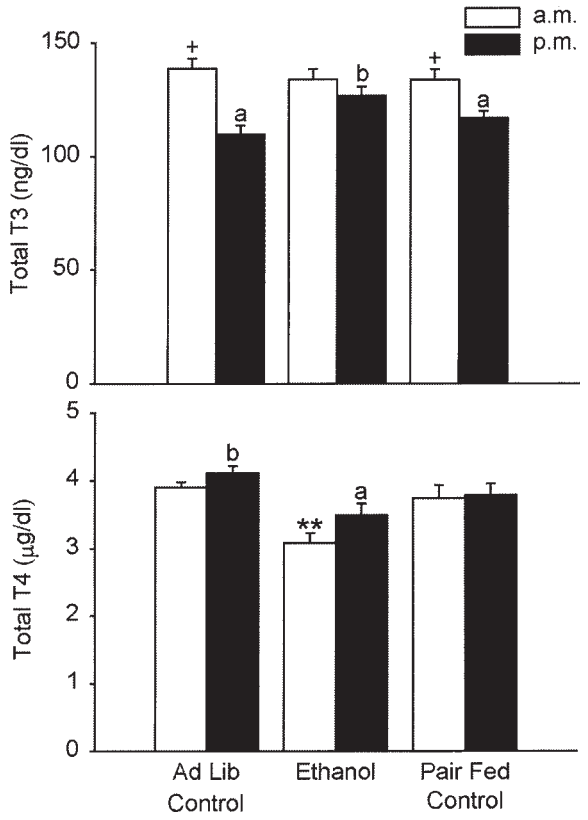

Fig. 2. Effects of chronic daily ethanol consumption and daily withdrawal on morning and afternoon plasma total T3 (upper panel) and total T4 (lower panel) levels. The rats were killed after 3 wk of gradual introduction of ethanol to the diet and four subsequent weeks of 5% ethanol (w/v), with the liquid 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 immediately before lights-off. Each bar represents the mean ± SE of nine rats. The data were analyzed by two-way analysis of variance (time of day× treatment) with subsequent specific comparisons with Student's *t*-test (a.m. versus p.m.) or one-way analysis of variance (between treatments). $^{+}p < 0.05$ versus corresponding p.m.; a<b, $p < 0.05$; $^{**}p < 0.01$ vs both ad lib control and pair-fed control.

a moderately decreased rate compared with *ad libitum*-fed controls (data previously presented in ref. *1*). 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 *(1)*. There were no significant differences in body weights of pair-fed control rats versus ethanol-treated rats at any time point in the study *(1)*. 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) *(1)*.

Both *ad libitum*-fed controls and pair-fed controls exhibited increased plasma free (i.e., nonprotein-bound) triiodothyronine (T3) levels in the morning relative to the afternoon ($p < 0.05$) (Fig. 1, upper panel). The gradual addition of 5% ethanol to the liquid diet over a 3 wk period followed by four subsequent weeks of chronic daily ethanol and daily withdrawal eliminated this diurnal change by decreasing morning free T3 levels ($p < 0.01$) (Fig. 1, upper panel); afternoon free T3 levels were not significantly different between the treatments (Fig. 1, upper panel). Morning plasma concentrations of free thyroxine (T4) were likewise decreased ($p < 0.05$) by the chronic daily ethanol and daily withdrawal treatment, with no significant afternoon changes (Fig. 1, lower panel).

A diurnal pattern was also evident in total (free + protein bound) T3 in both the *ad libitum*-fed and pair-fed controls, with higher ($p < 0.05$) levels in the morning relative to the afternoon (Fig. 2, upper panel). There was no significant effect of the 7 wk of daily ethanol consumption on morning total T3 concentrations, but afternoon total T3 levels were increased ($p < 0.05$), eliminating the diurnal pattern (Fig. 2,

Table 1
Effects of Forced "Abstinence" on Plasma Thyroid Hormone Levels 3 Wk after Cessation of Daily Ethanol Consumption and Withdrawal[a]

	Free T3 (pg/mL)	Free T4 (ng/dL)	Total T3 (μg/dL)	Total T4 (μg/dL)	TSH (ng/mL)
Ad libitum control	0.46 ± 0.04	0.71 ± 0.06	101.4 ± 4.0	3.62 ± 0.22	4.17 ± 0.53
Ethanol	0.59 ± 0.08	0.63 ± 0.05	119.6 ± 9.9	3.86 ± 0.48	5.64 ± 0.63
Pair-fed control	0.46 ± 0.09	0.87 ± 0.08	110.9 ± 5.7	3.94 ± 0.15	4.69 ± 0.62

[a]The rats were killed 3 h after lights-on, 3 wk after complete cessation of ethanol consumption. Each value represents the mean ± SEM of nine rats. There were no significant changes ($p > 0.05$) in any of the hormone concentrations when independently evaluated by one way analyses of variance.

upper panel). Total T4 levels were decreased ($p < 0.01$) in the morning relative to both *ad libitum*-fed and pair-fed controls (Fig. 2, lower panel). Afternoon total T4 was decreased ($p < 0.05$) relative to *ad libitum*-fed controls, but was not significantly different from pair-fed controls (Fig. 2, lower panel).

Three weeks after complete cessation of ethanol consumption, morning plasma free T3, free T4, total T3, total T4, and thyroid-stimulating hormone (TSH) levels were all not significantly changed by the prior ethanol consumption or pair-feeding (Table 1).

Discussion

These results reveal that plasma thyroid hormone levels are suppressed in a time of day dependent manner by chronic daily ethanol consumption and daily withdrawal in this model of chronic ethanol abuse. After subsequent long-term (3 wk) "abstinence," these thyroid hormones were restored to control levels. These results are consistent with evidence that thyroid function is commonly diminished in alcoholics, with variable reports of recovery during abstinence, as recently reviewed by Hermann et al. *(8)*.

In a previous study by Mason et al. *(9)* which likewise used a liquid diet model with rats, small differences in plasma thyroid hormone levels were noted in rats on ethanol diet compared to pair-fed controls. However, these results may have been confounded by the fact that, when compared to *ad libitum*-fed chow controls, not only rats receiving the ethanol diet but also pair-fed control rats exhibited much larger changes in levels of these hormones. In a previous study *(1)*, we determined that relatively rapid introduction of ethanol into liquid diet (i.e., over a 1 wk period) disrupted eating and blocked weight gain for nearly 3 wk (similar to the initial 3 wk suppression of body weight in the Mason et al. study, in which ethanol was introduced all at once), producing hypothalamo–pituitary–adrenal (HPA) activation in not only ethanol-treated rats but also in pair-fed controls *(1)*. Furthermore, effects on HPA activity in both the ethanol-consuming and pair-fed control rats persisted throughout four subsequent weeks of treatment *(1)*. We further determined that if the ethanol was more gradually introduced in increments spread over 3 wk instead of 1 wk, continuous weight gain was maintained and HPA indices of the pair-fed control rats were indistinguishable from those of *ad libitum*-fed control rats *(1)*. The plasma for the current analysis was obtained from rats in this apparently less stressful 3 wk gradual introduction study *(1)*. Our results reveal that thyroid hormone concentrations were altered only in the ethanol-consuming rats, whereas levels in pair-fed control rats were consistently the same as those of *ad libitum*-fed controls, confirming that the time of day dependent changes were due to daily ethanol consumption and daily withdrawal independent of the potential confounding influence of other stresses such as hunger or malnutrition. Thus, non-specific stresses due to abrupt initiation of ethanol treatment, which produces nutritional deficiencies in both the ethanol-treated rats and pair-fed controls, may explain similarities (i.e., lack of significant differences) of thyroid hormone changes in ethanol-treated versus pair-fed rats reported in other previous studies in which ethanol treatment was rapidly initiated *(10–12)*.

Determination of thyroid hormone concentrations at both the start and the end of the light period during chronic daily ethanol consumption was another important feature of the current experimental design. Chronic daily ethanol consumption blocked the morning increase of free T3 (the most bioactive form of the more potent of the thyroid hormones), but did not alter free T3 levels in the afternoon. This morning inhibition completely eliminated the diurnal changes in free T3 evident in both the *ad libitum*-fed and pair-fed controls, suggesting that the nocturnal consumption of ethanol may have disrupted circadian periodicity in regulation of the thyroid axis. This hypothesis would be consistent with evidence that chronic ethanol consumption disrupts the biological 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 *(13)*. However, because the morning plasma sample was collected when plasma ethanol levels were still elevated immediately following access to the ethanol-containing liquid throughout the night and

early morning, it is also reasonable to suggest that inhibition only in the morning may reflect relatively acute suppression by ethanol which was not present 9–10 h after daily withdrawal of the ethanol diet; we previously reported that plasma ethanol levels early in the light period using the same model were approx 135 mg/dL, but declined to undetectable at the end of the light period when rats also exhibited daily physical withdrawal *(14)*. Plasma total T3 likewise exhibited a diurnal pattern of increased morning concentrations in both the *ad libitum*-fed and pair-fed controls; in contrast to the pattern with free T3, the afternoon total T3 concentrations were slightly but significantly increased relative to both *ad libitum*-fed and pair-fed controls, again eliminating this diurnal pattern. Free and total T4 concentrations were both suppressed in the morning but not in the afternoon. Although the mechanisms of change and functional interrelationships between these thyroid hormone states cannot be determined from our data, it is nonetheless clear that (a) the circulating concentrations of the most biopotent (free) forms of both T3 and T4 were suppressed in the morning but not afternoon during chronic daily ethanol consumption and daily withdrawal, and (b) the time of day at which thyroid hormone levels were evaluated determined whether responses to the chronic ethanol treatment were detected. There has been considerable controversy regarding whether circulating thyroid hormone levels are altered in alcoholism *(8,15, 16)*; the current results suggest that determination of plasma thyroid hormone responses at one time of day may not adequately reveal changes that may be experienced repeatedly by alcohol abusers.

Three weeks after complete removal of ethanol from the liquid diet, there were no significant differences in morning free or total T3 or T4 relative to levels in *ad libitum*-fed or pair-fed controls. Free and total T3, but not T4, levels in the *ad libitum* controls at this time point were approx 25–50% higher than those in *ad libitum* controls killed 4 wk earlier (i.e., before gradual withdrawal of ethanol over 1 wk followed by 3 wk "abstinence" by the ethanol-treated rats) at the same time of day. The reason for these differences is unknown, and may simply reflect uncontrolled differences in environmental conditions or stresses in the animal colony at each end of this 1-mo interval. However, consumption of a high-fat liquid diet similar to the diet used in this study has been demonstrated to increase rat free and total T3, but not T4, levels relative to those associated with consumption of chow control diet *(9)*. Consequently, the apparent relative increase in T3 levels in the control rats over this time period may also reflect effects of an additional month (i.e., 57% longer) consumption of high fat (35% of total calories) liquid diet.

There was sufficient plasma remaining from rats killed 3 wk after cessation of ethanol consumption to also allow determination of TSH concentrations, which were likewise not significantly changed. However, as illustrated by the time of day dependence of changes during chronic daily ethanol consumption, it is plausible that changes at different times of day may have occurred but not been detected. It was not possible to assay TSH concentrations from rats killed at the end of chronic ethanol treatment due to depletion of available plasma by other prior analyses at that time point.

Mechanisms potentially contributing to chronic ethanol-induced suppression of thyroid function have recently been thoroughly reviewed by Herman et al. *(8)*, and previously by Baumgartner et al. *(17)*. The most consistent observation is blunted TSH response to thyrotropin-releasing hormone (TRH), the hypothalamic peptide that is the primary regulator of pituitary TSH secretion. A demonstration by Zoeller et al. *(12)* that chronic ethanol consumption produced an increase of TRH mRNA in rat paraventricular nucleus neurons supports the hypothesis that chronically increased release of TRH by these neurons down-regulates pituitary responsiveness to TRH. This hypothesis is consistent with evidence of an inverse relationship between cerebrospinal fluid TRH concentrations and the TSH response to TRH in abstinent alcohol-dependent patients *(18)*.

Stress and consequent activation of the HPA axis suppress HPT function *(4)*, so the current results could also be due to the hypercorticosteronemia we demonstrated in these same animals during chronic daily ethanol consumption and daily withdrawal *(1)*. We have demonstrated that rats develop physical dependence on ethanol with this model, and experience withdrawal daily *(14)*, so 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 ethanol abusers. Consequently, if stress and HPA activation did contribute to the changes in thyroid hormone levels demonstrated here, these changes are clinically relevant.

Finally, it should also be noted that changes in plasma thyroid hormone levels are dependent not only on changes in T3 and T4 synthesis, but also on degradation and clearance. Consequently, ethanol consumption may have induced changes in thyroid hormone inactivation and elimination, perhaps through modifications of liver functions. Resolving the mechanisms by which chronic daily ethanol and daily withdrawal induced time of day dependent changes in plasma thyroid hormone levels will clearly require further studies.

Because thyroid function has complex roles in regulation of, and by, the forebrain POMC, HPA, leptin, and hypothalamo–pituitary–gonadal axes *(5–7,19)*, the demonstrated disruptions in diurnal regulation of thyroid hormone levels could be responsible for chronic daily alcohol consumption and daily withdrawal-induced time of day dependent alterations which we have demonstrated in these other neuroendocrine functions in the same animals *(1–3)*. Further-

more, since HPT dysfunction has been associated with severity of withdrawal *(20,21)* and appears to have roles in negative mood states *(22–24)*, and experimental alterations of HPT function alter alcohol self-administration *(25–27)*, it is reasonable to suggest that an alcohol-induced hypothyroid state may increase risk for continued alcohol abuse. Further investigations with this rat model of daily ethanol consumption and daily withdrawal will help resolve these interactions and roles of the HPT axis in alcohol abuse.

Methods

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 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 (1985).

Liquid Diet

Bio-Serv Liquid Rat Diet L/D'82 (Bio-Serv, Frenchtown, NJ) was provided daily during the final 15 min of the light period in graduated Liquidiet Feeding Tubes (Bio-Serv, Frenchtown, NJ), and removed the next day at approx 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 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. Supplemental water was available at all times for all rats. Each rat was also provided with a non-nutritive nylon Nylabone (Bio-Serv, Frenchtown, NJ) to maintain gnawing and chewing behaviors.

Treatments

There were three treatment groups: ethanol-treated, pair-fed control, and *ad libitum*-fed control. Ethanol-treated rats were introduced gradually to 5% ethanol (w/v, prepared with 95% ethanol) in the liquid diet over a 3-wk period (i.e., 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 withdrawn gradually from ethanol over a 1-wk period (i.e., one d each at 4.3, 3.3, 2.5, 1.7, 0.8, and 0% ethanol). Control rats were individually pair-fed or fed *ad libitum* with isocaloric control liquid diet in which the calories from ethanol were replaced by maltose dextrin. We accomplished pair-feeding by daily measuring the amount of diet consumed by each ethanol-treated rat and providing that amount of control diet to the individually matched pair-fed control animal for the subsequent day. After gradual withdrawal of ethanol from the liquid diet of the ethanol-treated rats, the rats in all three treatment groups were switched to *ad libitum* chow for the final 3 wk of the study.

Sample Collection

Nine rats from each treatment group (i.e., ethanol-treated, pair-fed control, and *ad libitum*-fed control) were decapitated in the morning (3 h after lights on) and another nine per treatment group in the evening (in the last hour before lights off) at the end of the 4 wk of chronic 5% ethanol treatment. Nine more rats from each treatment group were decapitated in the morning (3 h after lights on) 3 wk after complete cessation of ethanol consumption (i.e., 3 wk after the return to chow). At decapitation, trunk blood was collected into chilled polypropylene tubes containing 100 µL of 100 mg ethylenediaminetetraacetic acid per mL of water; plasma was stored at –70°C in polypropylene tubes.

Radioimmunoassays (RIA)

Total T3 and T4 concentrations were assayed with Active™ Triiodothyronine (T3) and Active™ Thyroxine RIA kits, respectively, from Diagnostic Systems Laboratories, Inc. (Webster, TX). Free T3 and free T4 concentrations were assayed with Coat-A-Count Free T3 and Coat-A-Count Free T4 RIA kits, respectively, from Diagnostic Products Corp. (Los Angeles, CA). TSH concentrations were assayed with a Rat TSH RIA kit from ALPCO Diagnostics (Windham, NH). The detection limits for the total T3, total T4, free T3, free T4, and TSH assays were 4.3 ng/dL, 0.4 µg/dL, 0.2 pg/mL, 0.01 ng/dL, and 0.1 ng/mL, respectively. All samples were assayed in duplicate in a single assay for each hormone. The intra-assay coefficients of variation for all assays were <10%.

Statistical Analyses

Data are presented as mean ± SEM. Statistical tests are noted in the figure legends.

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