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Effect of Ethanol on 24-h Hormonal Changes in Prolactin Release Mechanisms in Growing Male Rats

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This study analyzes the effect of chronic ethanol feeding on 24-h variation of hypothalamic–pituitary mechanisms involved in prolactin regulation in growing male Wistar rats. Animals were maintained under a 12:12 h light/dark photoperiod (lights off at 2000 h), and they received a liquid diet for 4 wk, starting on d 35 of life. The ethanol-fed group received a similar diet to controls except that maltose was isocalorically replaced by ethanol. Ethanol replacement provided 36% of the total caloric content of the diet. Rats were killed at six time intervals every 4 h, beginning at 0900 h. Mean concentration of serum prolactin in ethanol-fed rats was 58.7% higher than in controls. Peak circulating prolactin levels occurred at the early phase of the activity span in both groups of rats, whereas a second peak was found late in the resting phase in ethanol-fed rats only. In control rats, median eminence dopamine (DA), serotonin (5-HT), γ-aminobutyric acid (GABA), and taurine levels exhibited two maxima, the major one preceding prolactin release and a second one during the first part of the resting phase. Median eminence DA and 5-HT turnover (as measured by 3,4-dihydroxyphenylacetic acid, DOPAC/DA, and 5-hydroxyindoleacetic acid, 5-HIAA/5-HT ratio) showed a single maximum preceding prolactin, at 0100 h. Ethanol treatment did not affect median eminence DA or 5-HT levels but it decreased significantly their turnover rate. The midday peak in DA and 5-HT levels (at 1300 h) was abolished and the night peak (at 0100 h) became spread and blunted in the ethanol-fed rats. This was accompanied with the disappearance of the 0100 h peak in DA and 5-HT turnover and the occurrence of a peak in 5-HT turnover at 1700 h. Ethanol intake suppressed the night peak in median eminence GABA and taurine (at 0100 h) as well as the midday peak of GABA. Ethanol augmented pituitary levels of DOPAC and 5-HIAA. The results indicate that chronic ethanol administration affects the mechanisms that modulate the circadian variation of prolactin release in growing male rats.

Key Words: Prolactin; median eminence; anterior pituitary; dopamine; serotonin; taurine; gamma-aminobutyric acid; circadian rhythms.

Introduction

Ethanol has multiple adverse effects on reproduction, mainly by its influence on endocrine secretion and function, disruptions being documented at the hypothalamic, pituitary, and gonadal levels *(1)*. The hypersecretion of prolactin has been implicated as one of the factors that mediate ethanol-induced hypogonadism *(2–6)*. Prolactin secretion is under tonic inhibitory control by hypothalamic dopamine (DA) *(7)*. Prolactin release is regulated by inhibition of DA release and, secondarily, by the release of prolactin releasing factors, e.g., vasoactive intestinal peptide and thyrotropin-releasing hormone (TRH) *(8)*.

It is remarkable that most studies on ethanol effects on prolactin secretion have been carried out by assessing the differences between control and treated animals at single time points, a serious drawback when dealing with a hormone that shows significant 24-h variations *(9,10)*. Chronic ethanol intake and withdrawal are associated with disruptions of circadian biological rhythms and ethanol administration produced a dampened oscillation and phase changes of the circadian oscillator *(11,12)*; thus any comparison relying only on single point assays could be misleading.

In a previous study, we reported the effect of chronic ethanol feeding on 24-h changes in prolactin, FSH, LH, TSH, and testosterone release in peripubertal male rats *(13)*. The secretion of prolactin increased and that of FSH, LH, testosterone, and TSH decreased in ethanol-fed rats. Significant changes in the 24-h secretory pattern of circulating hormones occurred in rats receiving ethanol.

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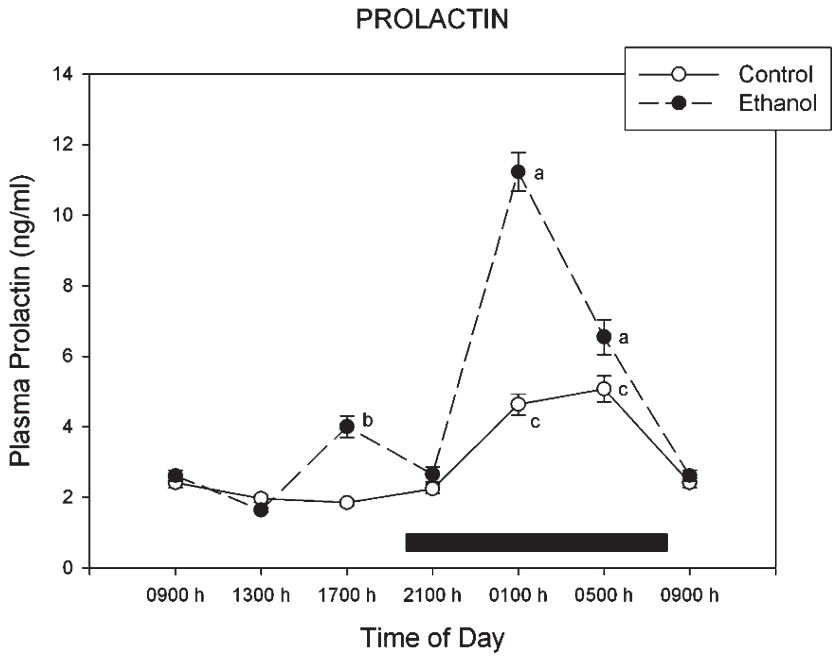

Fig. 1. Twenty-four hour changes of serum prolactin in control and ethanol-fed rats. Groups of 7–8 rats were killed by decapitation at six different time intervals throughout a 24-h cycle. Values at 0900 h are repeated on the "second" day. Bar indicates scotophase duration. Shown are the means ± SEM. Letters indicate the existence of significant differences between time points within each experimental group after a Bonferroni test, as follows: $^{a}p < 0.01$ vs all time points; $^{b}p < 0.01$ vs 1300, 0100, and 0500 h. For further statistical analysis, see text.

As a continuation of those experiments, the present study was carried out to analyze whether 24-h rhythms of several hypothalamic and pituitary modulators involved in regulation of prolactin release were affected by chronic ethanol feeding. Serum prolactin levels and median eminence and anterior pituitary levels of DA, 3,4-dihydroxyphenylacetic acid (DOPAC), serotonin (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) were measured in groups of control and ethanol-fed rats, killed at six time intervals during a 24-h period. Median eminence content of the inhibitory amino acids γ-aminobutyric acid (GABA) and taurine (TAU) were also determined.

Results

Figure 1 shows the levels of prolactin throughout the day in control and ethanol-fed rats. Mean concentration of serum prolactin was 58.7% higher in ethanol-fed rats as revealed by a factorial ANOVA taking treatment as a main factor ($F = 146.3$, $p < 0.000001$). Peaks in both groups of rats occurred at the activity span, as shown by a significant effect of time of day when analyzed as a main factor ($F = 154.1$, $p < 0.000001$). A significant interaction "treatment × time" in the factorial ANOVA ($F = 41.3$, $p < 0.000001$) was found, a second peak or prolactin at the second part of the resting phase (at 1700 h) occurring in ethanol-fed rats (Fig. 1).

Figures 2–6 depict the effect of ethanol intake on several median eminence and anterior pituitary transmitters and metabolites involved in prolactin release. In control rats, DA and 5-HT levels exhibited a hemicircadian pattern with two peaks, the major one preceding prolactin release (at 0100 h) and a second one at about midday (at 1300 h) ($F = 45.7$ and $F = 7.1$ for time effect on DA and 5-HT concentration, respectively, $p < 0.01$, factorial ANOVA, Fig. 2). As depicted in Fig. 3, a single maximum preceding prolactin release was found for median eminence DA and 5-HT turnover, as evaluated by DOPAC/DA and 5-HIAA/5-HT ratio, respectively ($F = 48.8$ and 29.8, $p < 0.01$, factorial ANOVA).

When analyzed as a main factor in the factorial ANOVA, ethanol treatment did not affect significantly median eminence DA or 5-HT levels (Fig. 1) but it decreased their turnover rate ($F = 14.4$ and 15.8, $p < 0.01$ for DOPAC/DA and 5-HIAA/5-HT ratio, respectively; Fig. 2). However, the temporal pattern in median eminence DA and 5-HT levels and turnover became significantly disrupted in ethanol-fed rats, as indicated by the significant "time × treatment" interaction in the factorial ANOVA ($p < 0.0001$) (Figs. 2 and 3). The midday peak in DA and 5-HT levels (at 1300 h) was abolished and the night peak (at 0100 h) became spread and blunted in the ethanol-fed rats (Fig. 2). This was accompanied with the disappearance of the 0100 h peak in DOPAC/DA and 5-HIAA/5-HT ratio and the occurrence of a peak in 5-HIAA/5-HT ratio at 1700 h (Fig. 3).

In correlation to monoamine content, GABA and taurine content of median eminence exhibited two peaks in control rats, at 0100 and 1300 h ($F = 127.1$ and 47.2 for time effect in the factorial ANOVA, $p < 0.000001$) (Fig. 4). In ethanol-fed rats, a significant disruption of this hemicircadian rhythm was detected, as revealed by the interaction "treat-

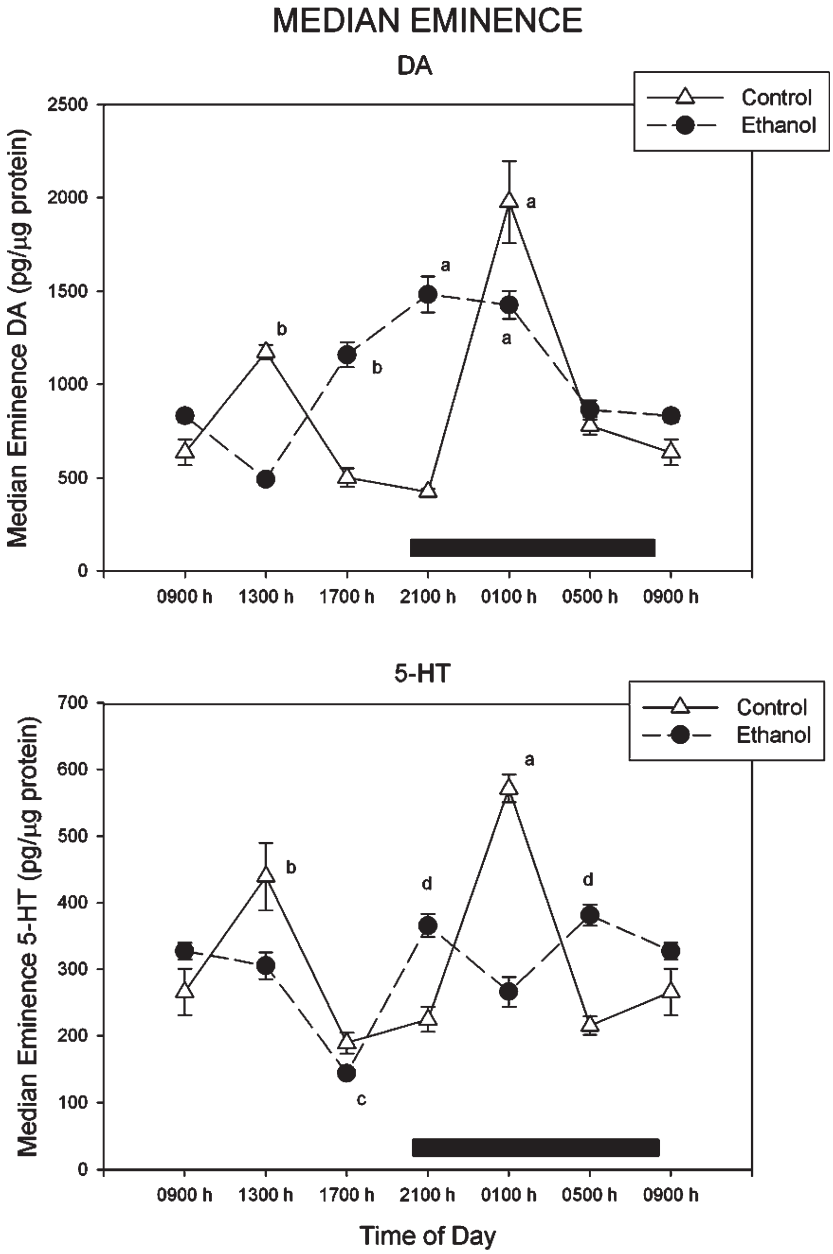

Fig. 2. Twenty-four hour changes of median eminence DA (upper panel) and 5-HT (lower panel) concentration in control and ethanol-fed rats. Groups of 7–8 rats were killed by decapitation at six different time intervals throughout a 24-h cycle. Values at 0900 h are repeated on the "second" day. Bar indicates scotophase duration. Shown are the means ± SEM. Letters indicate the existence of significant differences between time points within each experimental group after a Bonferroni test, as follows: Control: $^{a}p < 0.01$ vs 0900, 1700, 2100, and 0500 h, $p < 0.05$ vs 1300 h. $^{b}p < 0.01$ vs 0900, 1700, 2100, and 0500 h, $p < 0.05$ vs 0100 h. Ethanol: $^{a}p < 0.01$ vs 0900, 1300, and 0500 h, $p < 0.05$ vs 1700 h. $^{b}p < 0.01$ vs 0900 and 1300 h, $p < 0.05$ vs 2100, 0100, and 0500 h. $^{c}p < 0.01$ vs all time points. $^{d}p < 0.01$ vs 1700 h, $p < 0.05$ vs 0100 h. For further statistical analysis, see text.

ment × time" in the factorial ANOVA ($F = 71.2$ and 39.1 for GABA and taurine, $p < 0.000001$) (Fig. 4). Ethanol intake suppressed the night peak in median eminence GABA and taurine (at 0100 h) as well as the midday peak of GABA.

Figures 5 and 6 depict the changes in DA, 5-HT, DOPAC, and 5-HIAA concentration in the anterior pituitary. Pituitary DA levels did not change significantly as a function of time in any experimental group (Fig. 5), although time-related changes in the concentration of the DA metabolite DOPAC were detected ($p < 0.001$) (Fig. 6). Ethanol did not change pituitary DA levels but increased the levels of DOPAC, as indicated by main effect analysis in the factorial ANOVA ($p < 0.001$). Pituitary 5-HT concentration attained a maximum at light–dark transition in controls and about 4 h in advance (at 1700 h) in ethanol-fed rats ($p < 0.001$) (Fig. 5). These changes were unrelated to those of the 5-HT metabolite 5-HIAA (Fig. 6). Pituitary 5-HIAA levels augmented significantly in ethanol-fed rats ($p < 0.001$). The

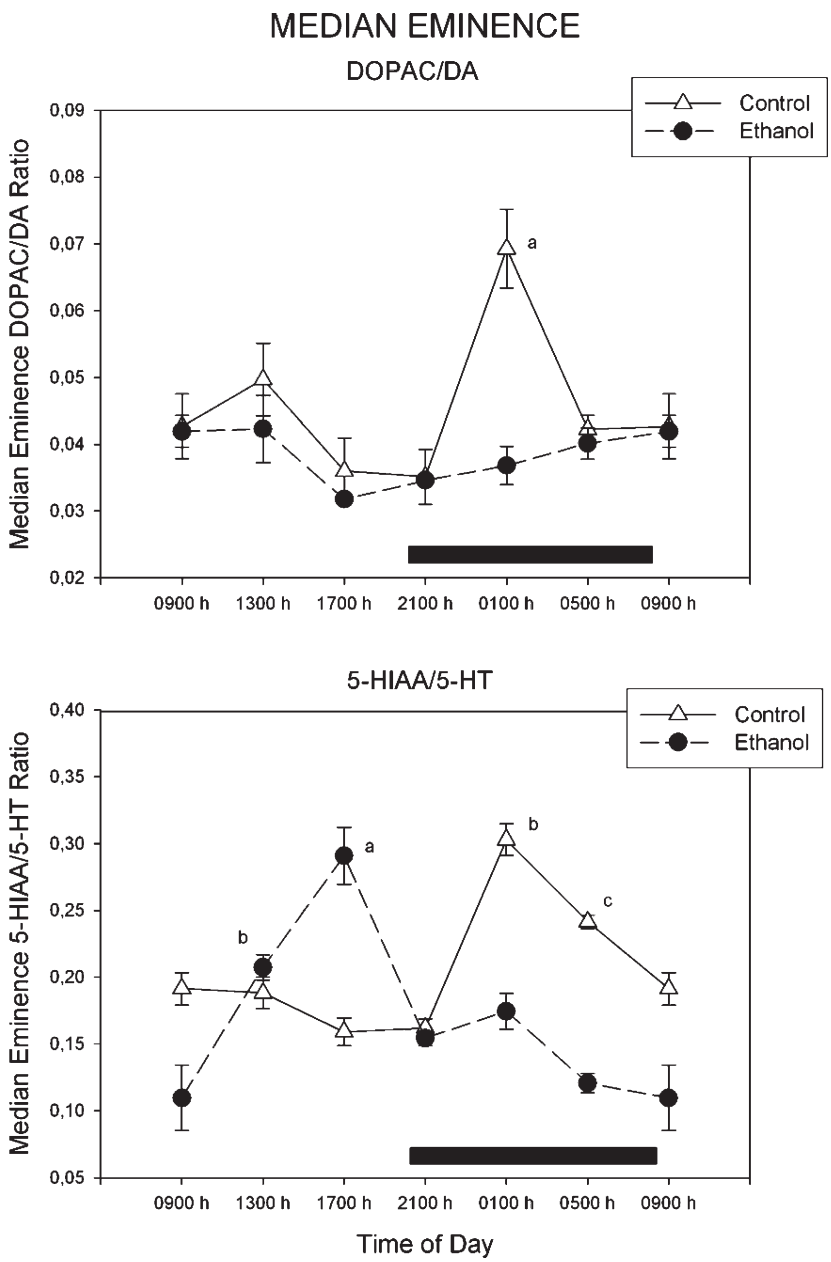

Fig. 3. Twenty-four hour changes of median eminence DA (upper panel) and 5-HT (lower panel) turnover in control and ethanol-fed rats. Groups of 7–8 rats were killed by decapitation at six different time intervals throughout a 24-h cycle. Values at 0900 h are repeated on the "second" day. Bar indicates scotophase duration. Shown are the means ± SEM. Letters indicate the existence of significant differences between time points within each experimental group after a Bonferroni test, as follows: Control: $^{a}p < 0.05$ vs 0900, 1700, 2100, and 0500 h. $^{b}p < 0.01$ vs all time points. $^{c}p < 0.01$ vs 1700, 2100, and 0100 h, $p < 0.05$ vs 0900 and 1300 h. Ethanol: $^{a}p < 0.01$ vs all time points. $^{b}p < 0.01$ vs 1700 and 0500 h, $p < 0.05$ vs 0900 h. For further statistical analysis, see text.

increases in pituitary DOPAC and 5-HIAA were observed mostly on the morning (from 05.00 to 13.00) while during the afternoon and early evening the values were similar to controls (Fig. 6).

Discussion

The foregoing results confirm that ethanol feeding significantly augments prolactin release in growing rats, an effect seen mainly during the activity phase of the 24-h cycle. This helps to explain discrepancies about the direction of prolactin changes in ethanol-fed rats *(14)*. For example, by measuring prolactin levels at single morning time points it was concluded that the administration of ethanol p.o. for 4 wk did not affect this parameter *(15)*. In addition to the major peak of plasma prolactin occurring at the early phase of the activity span, a second maximum taking place late in the resting phase was detected in ethanol-fed growing rats. The distorted prolactin rhythm found after ethanol administration correlated with a significant disruption of 24-h

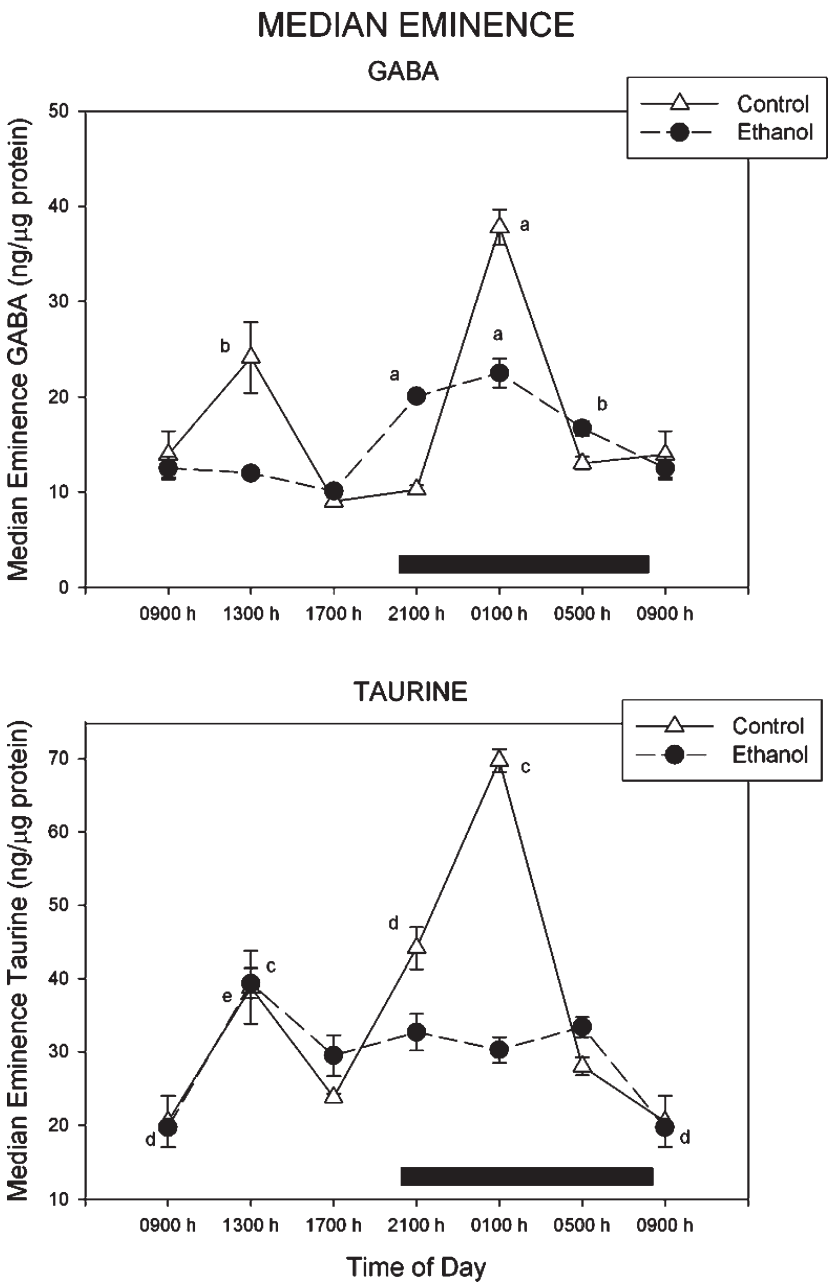

Fig. 4. Twenty-four hour changes of median eminence GABA (upper panel) and taurine (lower panel) concentration in control and ethanol-fed rats. Groups of 7–8 rats were killed by decapitation at six different time intervals throughout a 24-h cycle. Values at 0900 h are repeated on the "second" day. Bar indicates scotophase duration. Shown are the means ± SEM. Letters indicate the existence of significant differences between time points within each experimental group after a Bonferroni test, as follows: Control: $^{a}p < 0.01$ vs 0900, 1700, 2100, and 0500 h, $p < 0.05$ vs 1300 h. $^{b}p < 0.05$ vs all time points. $^{c}p < 0.01$ vs all time points. $^{d}p < 0.01$ vs 0900, 1700, 0100, and 0500 h. $^{e}p < 0.01$ vs 0100, $p < 0.05$ vs 0900, 1700, and 0500 h. Ethanol: $^{a}p < 0.01$ vs 0900, 1300, and 1700 h. $^{b}p < 0.05$ vs 0900, 1300, and 1700 h. $^{c}p < 0.01$ vs 0900 h. $^{d}p < 0.01$ vs 1300 h, $p < 0.05$ vs 2100, 0100, and 0500 h. For further statistical analysis, see text.

rhythms in median eminence DA, 5-HT, GABA, and taurine levels as well as in median eminence DA and 5-HT turnover, all suspected modulators of prolactin release. The temporal patterns of pituitary monoamine content became also disrupted in ethanol-fed rats.

DA released from tuberoinfundibular dopaminergic neurons enters the hypophysial portal blood to reach the anterior pituitary and to inhibit prolactin secretion *(7,16)*. The present study, comprising the determination of DA concentration and turnover in the median eminence at six time points during a 24-h cycle, offers support to the hypothesis of a disrupted tuberoinfundibular dopaminergic transmission after chronic ethanol ingestion in rats. DA content and turnover in the median eminence of control animals reached peak concentration at 0100 h and then diminished as prolactin levels increased. In ethanol-fed rats, this cor-

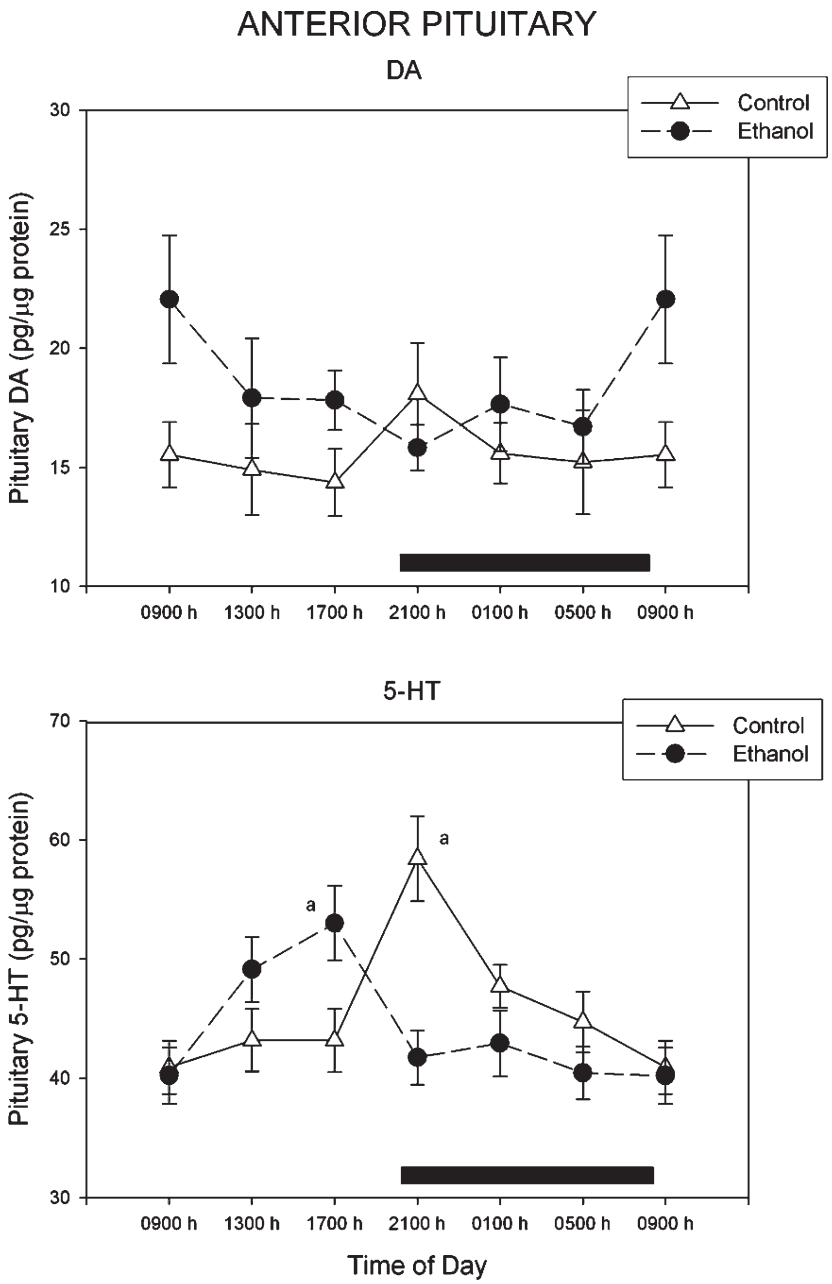

Fig. 5. Twenty-four hour changes of anterior pituitary DA (upper panel) and 5-HT (lower panel) concentration in control and ethanol-fed rats. Groups of 7–8 rats were killed by decapitation at six different time intervals throughout a 24-h cycle. Values at 0900 h are repeated on the "second" day. Bar indicates scotophase duration. Shown are the means ± SEM. Letters indicate the existence of significant differences between time points within each experimental group after a Bonferroni test, as follows: Control: $^{a}p < 0.01$ vs all time points. Ethanol: $^{a}p < 0.05$ vs 0900, 2100, 0100, and 0500 h. For further statistical analysis, see text.

relation became distorted, presumably explaining the increased prolactin levels found. The midday peak in median eminence DA was abolished, while the night (0100 h) peak became spread and blunted. This was accompanied by the disappearance of the 0100 h peak in DOPAC/DA ratio, which appeared to be directly correlated with the increased nocturnal prolactin peak seen in ethanol-fed rats.

The effect of ethanol on tuberoinfundibular DA neurons reported herein is not unique. Ethanol in low doses activates other dopaminergic pathways of the brain, e.g., nucleus accumbens *(17,18)*, whereas in high doses it suppresses dopaminergic activity *(19,20)*. In the nucleus accumbens, a high concentration of ethanol suppressed DA release, whereas low and moderate concentrations had no effect *(21)*. It is interesting that ethanol, injected daily for 7 d in rats, markedly increased the expression of galanin in the hypothalamus *(22)*. Galanin could be responsible for the changes in median eminence DA reported herein, because the neuropeptide increased the DA release in the nucleus accumbens after its hypothalamic injection *(23)*.

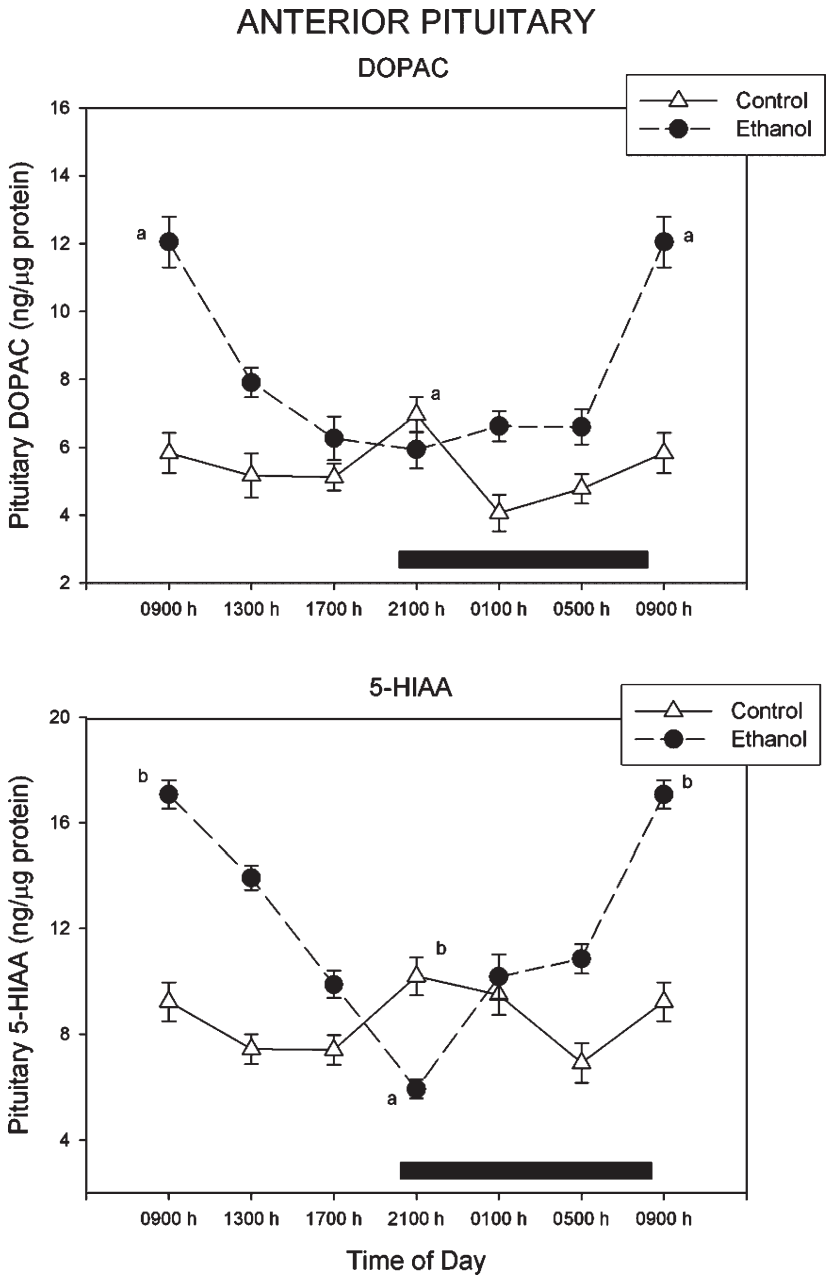

Fig. 6. Twenty-four hour changes of anterior pituitary DOPAC (upper panel) and 5-HIAA (lower panel) concentration in control and ethanol-fed rats. Groups of 7–8 rats were killed by decapitation at six different time intervals throughout a 24-h cycle. Values at 0900 h are repeated on the "second" day. Bar indicates scotophase duration. Shown are the means ± SEM. Letters indicate the existence of significant differences between time points within each experimental group after a Bonferroni test, as follows: Control: $^{a}p < 0.05$ vs 1700, 0100, and 0500 h. $^{b}p < 0.05$ vs 1300, 1700, and 0500 h. Ethanol: $^{a}p < 0.01$ vs all time points. $^{b}p < 0.01$ vs 1700, 2100, 0100, and 0500 h, $p < 0.05$ vs 1300 h. For further statistical analysis, see text.

The arcuate nucleus receives a dense serotonergic innervation consisting of a population of brainstem neurons arising mainly from the midbrain raphe nuclei *(24)*. Fibers also originate from 5-HT cell bodies located within the hypothalamus. There is a close proximity of 5-HT fibers to dopaminergic cell bodies in the arcuate nucleus *(25)*. Therefore, the stimulatory effect of 5-HT on prolactin release has been linked to the modulation of inhibitory dopaminergic inputs to the pituitary. The afternoon (1700 h) peak in prolactin reported in ethanol-fed rats in the present study was correlated with the afternoon increase in 5-HIAA/5-HT ratio at 1700 h, suggesting the possible involvement of 5-HT release. Neither 5-HT levels nor turnover at night (or DA levels or turnover in the afternoon) correlated with the changes in prolactin release seen in ethanol-fed rats.

As far as the anterior pituitary is concerned, there was no correlation between monoamine levels with those of median eminence. In ethanol-fed rats, pituitary levels of DOPAC and 5-HIAA were significantly higher than in controls, a finding that could be interpreted in terms of an increased

oxidative injury by ethanol. Indeed, ethanol-augmented free radicals altered the oxidant/antioxidant balance and caused oxidant-induced changes in cellular proteins and lipids, whereas pretreatment with antioxidants such as vitamin E, vitamin C, and agents that enhance antioxidant capacity, attenuate alcohol-induced effects *(26)*.

A relatively dense innervation of GABA terminals exists in the external layer of the median eminence *(27,28)* and the ability of median eminence neurons to release GABA in portal blood has been demonstrated *(29)*. There are GABA receptors in the anterior pituitary that suppress prolactin secretion *(30,31)*. Maximal values of median eminence GABA took place concomitantly with DA and 5-HT content, with a major peak at 0100 h and a second one at 1300 h. A similar pattern to GABA was depicted by taurine another putative amino acid transmitter implicated in the regulation of prolactin release *(32–34)*.

Considerable evidence points to a GABAergic component in the behavioral effects of ethanol *(35)*. Using electrophysiological and microdialysis techniques, it was reported that chronic ethanol administration enhances GABAergic transmission and GABA tone at synapses via vesicular release *(36)*. Acute and chronic ethanol consumption differentially affects GABAergic function in the brain *(37)*. Although there is still controversy over the mechanisms involved, available evidence suggests that acute ethanol facilitates GABAergic neurotransmission *(38)*. In the present study, the overall effect of ethanol on median eminence GABA and taurine concentration was a reduction in the peaks observed at 0100 h, coincident with the augmented nocturnal prolactin peak. Assuming that the decrease in amino acid content was ascribed to its release, and in view that the central administration of GABA either intracerebroventricularly or directly into the hypothalamic arcuate nucleus stimulates prolactin secretion *(30)*, the decrease in GABA content coinciding with the increased prolactin release at 0100 h could be instrumental in the prolactin changes observed. One possible mechanism for GABA action in the inhibition of the activity of tuberoinfundibular DA neurons *(31)*. It must be noted, however, that the physiological role of GABA as a prolactin-inhibiting factor has been questioned because of its low potency in vitro and its weak correlation with prolactin secretion in vivo *(31)*.

One limitation of the present study must be kept in mind. Rats consume most of their diet during the dark hours and so the consumption of alcohol and the blood level of alcohol are expected to be highest during the scotophase. Hence, there are two variables to be considered: (1) the chronic daily nature of alcohol consumption; (2) the acute action of elevated alcohol during the night. This needs further experiments to be understood.

Summarizing, the present study in Wistar male rats at six different time points along a 24-h cycle further supports the conclusion that prolactin secretion increases in ethanol-fed rats. A diurnal rhythm of median eminence DA and 5-HT content and turnover, and median eminence GABA and taurine content exists in growing male rats and is disrupted by ethanol. How ethanol acted to disrupt circadian organization response remains to be defined. Both ethanol intake and ethanol withdrawal alter the period and amplitude of free-running circadian activity rhythms in the rat *(11,12)*. While the effects of ethanol on circadian amplitude can potentially be mediated entirely by mechanisms downstream from the circadian pacemaker, the effects of ethanol on free-running period indicate that this drug must ultimately affect the underlying circadian pacemaker, either directly, or via action on pacemaker input (entrainment) pathways *(11)*.

Materials and Methods

Animals and Experimental Design

Five week-old, peripubertal, male Wistar rats were kept under standard conditions of controlled light (12:12 h light/dark schedule) and temperature (22 ± 2°C). A liquid diet mode of ethanol administration was used *(39,40)*. Animals received the liquid diet for 4 wk, starting on d 35 of life. The diet contained an aqueous suspension of pulverized casein, L-methionine, vitamin mixture, mineral mixture, sucrose, xanthum gum, choline bitartrate, Celufil cellulose, corn oil, and maltose. Percentage composition of the diet was 35% fat, 18% protein, and 47% carbohydrates. The ethanol-fed group received a similar diet except that maltose was replaced by 96% ethanol. Final ethanol concentration was 6.2% (w/v) and ethanol replacement was isocaloric providing about 36% of the total caloric content of the diet. To mask the ethanol taste 0.1% saccharin was added to the diet. Controls also received 0.1% saccharin. Diets were freshly made each day. Rats were caged in groups of 4 animals/cage and had access to the liquid diet *ad libitum*. Daily average consumption of diet was 40–50 mL/rat. The liquid diet without ethanol was administered 4 d before the study was begun to allow the animals to become accustomed to the new diet.

The care and use as well as all procedures involving animals were approved by the Institutional Animal Care Committee, Faculty of Medicine, Complutense University, Madrid. The study was in accordance with the guidelines of the Institutional Care and Use Committee of the National Institute on Drug Abuse, National Institutes of Health and the Guide for the Care and Use of Laboratory Animals *(41)*.

Groups of 7–8 rats were killed by decapitation at six different time intervals, every 4 h, throughout a 24-h cycle starting at 0900 h. At night intervals, animals were killed under red dim light. The brains were quickly removed, and the median eminence and the anterior pituitary were removed. Tissues were weighed and homogenized in chilled (0–1°C) 2 *M* acetic acid. After centrifugation (at 15,000*g* for 30 min, at 5°C), the samples were either analyzed for DA, DOPAC, 5-HT, and 5-HIAA or boiled for 10 min and

further centrifuged at 14,000 rpm for 20 min to measure amino acids in the supernatant (median eminence). Aliquots for protein determination were also taken. Plasma from the trunk blood was kept at –20°C after being centrifuged at 3500 rpm at 4°C.

Monoamine Analysis

Monoamine concentration was measured by high performance liquid chromatography (HPLC) using electrochemical detection (Coulochem, 5100A, ESA; USA), as described elsewhere *(43)*. A C-18 reverse phase column eluted with a mobile phase (pH 4, 0.1 *M* sodium acetate, 0.1 *M* citric acid, 0.7 m*M* sodium octysulfate, and 0.57 m*M* EDTA containing 10% methanol, v/v), was used. Flow rate was 1 mL/min, at a pressure of 2200 psi. Fixed potentials against H_2/H^+ reference electrode were: conditioning electrode: –0.4 V; preoxidation electrode: +0.10 V; working electrode: +0.35 V. DA, DOPAC, 5-HT, and 5-HIAA concentrations were calculated from the chromatographic peak heights by using external standards and were expressed as pg/µg protein. The linearity of the detector response was tested within the concentration ranges found in median eminence and adenohypophysial supernatants. The turnover of DA was assessed by the DOPAC/DA ratio, whereas that of 5-HT, by the 5-HIAA/5-HT ratio. Although DOPAC and 5-HIAA concentration depends on the balance between catabolite synthesis and clearance, its acidic nature slows substantially its clearance from tissue, so that catabolite concentration reflects an integral of past DA or 5-HT release.

Amino Acid Analysis

Amino acids were separated and analyzed by HPLC with fluorescence detection after precolumn derivatization with *O*-phthalaldehyde (OPA), as described elsewhere *(42)*. An aliquot of the tissue supernatant containing homoserine as internal standard was neutralized with NaOH (4 *M*), and was then incubated at room temperature with OPA reagent (4 m*M* OPA, 10% methanol, 2.56 m*M* 2-mercaptoethanol, in 1.6 *M* potassium borate buffer, pH 9.5) for 1 min. The reaction was stopped by the addition of acetic acid (0.5 v/v). Samples were loaded through a Reodyne Model 7125 injector system with a loop sampler of 20 µL to reach a C-18 reverse-phase column (4.6 mm ID × 150 mm, Nucleosil 5, 100 A). A mobile phase of 0.1 *M* sodium acetate buffer (pH 6.5) and containing 35% methanol was used at a flow rate of 1 mL/min and a pressure of 140 bars. The column was subsequently washed with the same buffer containing 70% methanol and reequilibrated with the elution buffer before reuse. The HPLC system included a solvent delivery system coupled to a filter fluorometer (excitation 340 nm, emission 455 nm). The procedure allows a clearcut separation and resolution of the amino acids measured. Amino acid content was calculated from the chromatographic peak heights by using standard curves and the internal standard and expressed as ng/µg protein. The linearity of the detector response for GABA and TAU was tested within the concentration ranges found in median eminence supernatants.

Prolactin Determination

Plasma prolactin levels were measured by a homologous specific double-antibody RIA, using materials kindly supplied by the NIDDK's National Hormone and Pituitary Program. The intra- and interassay coefficients of variations were 6%. Sensitivities of the RIA was 45 pg of prolactin /mL using the NIDDK rat prolactin RP-3. Results were expressed as ng/mL *(10,43)*.

Statistical Analysis

Statistical analysis of results was performed by a two-way factorial analysis of variance (ANOVA). Generally, the analysis included assessment of the group effect (i.e., the occurrence of differences in mean values between ethanol-fed and control groups), of time-of-day effects (the occurrence of daily changes), and of the interaction between the two factors (ethanol and time, from which inference about differences in timing and amplitude could be obtained). A post-hoc Bonferroni test was then employed to show which time points were significantly different within each experimental group to define existence of peaks. *p* values lower than 0.05 were considered evidence for statistical significance.

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