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Effects of Ethanol Withdrawal on β-Endorphin Levels in Rat Brain and Pituitary

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HUTCHISON, W. D., C. GIANOULAKIS AND H. KALANT. *Effects of ethanol withdrawal on β-endorphin levels in rat brain and pituitary*. PHARMACOL BIOCHEM BEHAV 30(4) 933–939, 1988.—Rats which received a liquid diet containing 6.5% (w/v) ethanol for three weeks became tolerant to the hypothermic effect of an acute dose of ethanol. Withdrawal of this diet was followed by loss of the tolerance within 3 days, and by an accompanying pattern of changes in levels of immunoreactive-ir-β-endorphin in several brain regions. An initial decrease in levels on days 1 and 3 of withdrawal was followed by recovery to control levels on days 8 and 15. This pattern was found in the arcuate nucleus, amygdala, septum, periventricular thalamus and pre-optic periventricular hypothalamus (POPH), but was statistically significant only in the POPH. A different pattern of change in ir-β-endorphin levels was found in the pituitary. The anterior lobe showed a significant depletion of ir-β-endorphin levels before alcohol withdrawal, which recovered by day 8 of withdrawal. This depletion was probably not related to the loss of tolerance to ethanol but was a response to a perturbation of the hypothalamic-pituitary axis of hormonal control.

Ethanol tolerance Withdrawal β-Endorphin Rat

THE shared pharmacological effects of alcohol and opiates [21] seem difficult to explain on the basis of a common molecular mechanism of action. Ethanol acts in a millimolar concentration range to produce a nonspecific action of membrane fluidization, while opiates interact in a nano- to micromolar concentration range with stereospecific opiate receptors. Despite the known discrepancies, considerable evidence exists, not only that alcohol and opiates have certain actions in common [4], but also that opiate receptors may be involved in some of the actions of ethanol [14]. It was, therefore, of interest to examine the possibility that endogenous opioids participate in the phenomenon of alcohol tolerance. Previous work in this laboratory [15,16] has shown an increase in synthesis and release of ir-β-endorphin by the neurointermediate lobe (NIL) of the alcohol-tolerant rat. However, changes in the hypophysis do not necessarily mirror those occurring in the brain, and the neuroadaptive processes involved in tolerance might differ. Therefore, the present study was designed to investigate possible changes in the central pools of β-endorphin associated with alcohol tolerance.

In several animal studies, brain endogenous opioids have been measured after chronic treatment with alcohol. The voluntary consumption of a 5% ethanol solution (in a 3-bottle choice paradigm) for a 12-month period was associated with a depletion of Leu-enkephalin in the basal ganglia of hamsters [5]. When rats consumed 20% ethanol in the drinking water for 30 days, there was no change in levels of ir-β-endorphin in the hypothalamus, but Met-enkephalin levels were reduced in the striatum, medulla pons and midbrain [28]. This may have been related to an initial weight loss observed in the rats receiving 20% ethanol [28]. Golden hamsters given a 10% ethanol solution for a two-week period showed no significant difference in ir-β-endorphin levels in the brain, but a group of rats treated in parallel with the hamsters showed significant weight losses after ethanol treatment and a concomitant decrease in ir-β-endorphin levels in the brain [9]. The administration of 15% ethanol in the drinking water to Sprague-Dawley rats was not associated with any alteration in hypothalamic ir-β-endorphin if no weight loss occurred in the ethanol imbibing group [13]. The present study therefore involved administration of ethanol in the form of a liquid diet which had been shown previously to maintain stable growth at the same rate as in pair-fed nonalcohol controls [23].

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ABBREVIATIONS

ΔT_{max}	maximal change in rectal temperature
ir	immunoreactive
LPH	lipotropin
NIL	neurointermediate lobe of the pituitary gland
POMC	proopiomelanocortin
RIA	radioimmunoassay

METHOD

Eighty male Sprague-Dawley rats (Charles River Breeding Labs, Canada) with initial weights of 180–200 g were fed four Purina chow pellets a day, with water ad lib, during an initial acclimatization period of 7 days. They were then paired by weight, and one rat in each pair was randomly chosen to receive a nutritionally complete liquid diet containing 6.5% (v/v) ethanol, in which 36% of the calories were provided as ethanol [23]. All rats in the ethanol treatment received 21 days of ethanol liquid diet. The pair-feeding system was employed to feed each sucrose-treatment rat a volume of sucrose diet each day that was equal to the volume of alcohol diet consumed by its paired rat the day before. During the withdrawal phase (1, 3, 8, 15 days), all rats were offered 75 ml daily.

An additional group of 18 animals (9 alcohol, 9 controls) was treated in parallel to test the development and loss of tolerance to ethanol-induced hypothermia. For this purpose, a test dose of ethanol (3.0 g/kg, as a 17.5% w/v ethanol solution in 0.9% saline) was administered IP to each rat on days 0, 7, 14, and 21 of the chronic treatment phase and on days 1, 3, 8 and 15 of the withdrawal phase. A telethermometer (model 43TA Yellow Springs Instruments Co. Inc.) was used to measure core temperature: the thermistor probe was inserted 4.5 cm into the rectum, and left to equilibrate for 30 sec or until a stable reading was attained.

For the study of β-endorphin levels, the main groups of 40 ethanol-treated and 40 control rats were subdivided into five subgroups of 8 each. One subgroup from each major treatment group was killed on the last day of chronic treatment (day 0 of withdrawal) and on days 1, 3, 8 and 15 of the withdrawal phase. The animals were decapitated and the trunk blood was collected into chilled centrifuge tubes containing EDTA (1 mg/ml final concentration), for the measurement of plasma levels of ir-β-endorphin. An aliquot of this blood was also taken from those rats in the 0 and 1 day withdrawal groups for the determination of blood ethanol levels by an alcohol dehydrogenase method [18]. As rapidly as possible after decapitation, the brain was removed from the rat cranium and placed on an aluminium pan set on crushed ice. The neurointermediate lobe (NIL) and anterior lobe(AL) of the pituitary were dissected with a fine needle. The rest of the brain was immediately placed into a prechilled aluminium cutting block with transverse channels spaced 1.5 mm apart, and single-edged razor blades were inserted into the channels to prepare serial coronal slices for dissection of selected regions [20]. The brain slices were laid out on a large rubber stopper and the following individual regions were recovered from the appropriate slices: nucleus accumbens, septum, pre-optic periventricular hypothalamus, preoptic ventromedial hypothalamus, periventricular hypothalamus, ventromedial hypothalamus, arcuate nucleus, amygdala, periventricular thalamus, periaqueductal grey matter, and cortex. Each region was combined with the corresponding one from another rat of the same group to make one sample, which was transferred immediately to a microcentrifuge tube containing 0.5 ml of an acidic extraction medium containing 1.0 M HCl, 5% formic acid (v/v), 1% NaCl (w/v), 1% trifluoroacetic acid (v/v) [6]. Samples were frozen upright in dry ice and stored at −70°C until analyzed.

On the day of analysis, samples were thawed and weights were recorded with a Cahn G-2 Electrobalance (Ventron Instruments) and homogenized with a Polytron PT10/35 fitted with a P7 generator head (Brinkman) for 15 sec at a setting of 6 or 7. After centrifugation in an Eppendorf Microcentrifuge for 10 min at 4°C, the supernatant was retained. The pellet was resuspended in another 0.5 ml of acidic extraction medium and centrifuged again and the first and second extracts were combined for purification of a Sep-Pak C18 cartridge (Waters Associates) [3]. Plasma samples were prepared by mixing 3.0 ml of plasma with 0.45 ml of acidic extraction medium, centrifuging to remove the precipitated protein, and treating the supernatants in the same manner as the tissue extracts. The radioimmunoassay (RIA) of these purified extracts of pituitary, brain and plasma was carried out by the "second antibody" method of Cahill *et al.* [7]. The antiserum (kindly donated by Dr. M. Chrétien, Clinical Research Institute, Montréal, Québec) was specific for the C-terminal portion of β-endorphin, so that it cross-reacted with ovine β-LPH and N-acetyl-β-endorphin on an equimolar basis and exhibited 70% cross-reactivity to β-endorphin$_{1-27}$. No cross-reactivity was observed with ACTH, α-MSH, β-MSH and the bovine β-endorphin fragments 1–5, 2–7 and 20–27. Standard curves for pure β-endorphin were carried out over a concentration range of 2–10,000 pg per tube; optimum results were obtained with an antibody dilution of 1:30,000. The results were fitted to a log-logistic equation by nonlinear regression analysis, for estimation of the four binding parameters; specific binding, nonspecific binding, and the y-intercept and slope of the log-logit line [19]. Unknowns (the biological samples) were quantified by interpolation in the linear range to which the equation was applicable [33]. The intra- and interassay variation were 7% and 9% respectively.

Student's t-test for paired data was used to analyse the hypothermic response to the ethanol test dose (Fig. 1) and body weights of animals at the time of testing (Table 1), and differences between the alcohol and sucrose groups were considered significant at the $p<0.05$ level.

In Table 2, Duncan's multiple range test [30,31] was used to rank the means for each brain region in a descending order and compare the differences between various regions. This test is preferred when no planned comparisons are to be made, and it provides protection against finding false significant differences among the many means given in the table.

RESULTS

Figure 1A and B shows the development and loss of tolerance to the hypothermic effect of ethanol. The maximal fall in rectal temperature after ethanol injection (ΔT_{max}) was significantly decreased in the chronic ethanol group on day 14, $t(16)=4.11$, $p<0.001$, and decreased further on day 21 (or day 0 of withdrawal), $t=4.06$, $p<0.001$, indicating the development of alcohol tolerance. Residual tolerance was still present in this group on day 1 of ethanol withdrawal, $t(16)=4.78$, $p<0.001$, but had dissipated by day 3. This tolerance to the test dose of ethanol is probably due to a decreased sensitivity of the central nervous system to

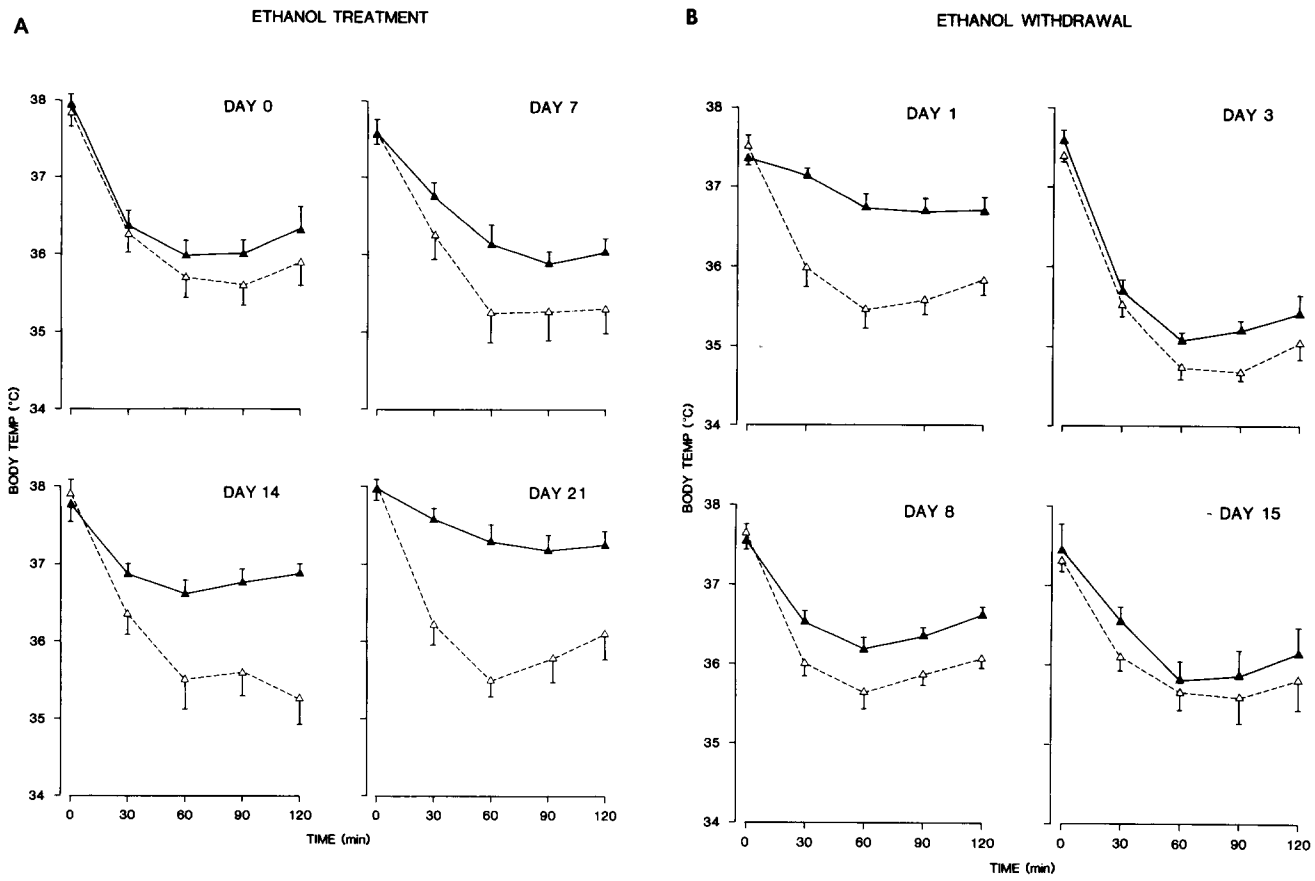


FIG. 1. (A) Development of tolerance to ethanol-induced hypothermia after various periods of treatment with ethanol liquid diet. ▲, Ethanol treatment + ethanol test dose given IP; △, sucrose treatment + ethanol test dose given IP. Plotted points are means±S.E.M. with n=9 animals per group. (B) Loss of tolerance to ethanol-induced hypothermia after various periods of withdrawal from ethanol liquid diet. ▲, Ethanol treatment + ethanol test dose given IP; △, sucrose treatment + ethanol test dose given IP. Plotted points are means±S.E.M. with n=9 animals per group.

ethanol and not entirely due to an increase in elimination of ethanol from blood (metabolic tolerance) [22].

Animals pair-fed the liquid diets gained about 20% of initial body weight over the three-week treatment period, and there were no significant differences in body weight between the two treatment groups. The chronic alcohol animals exhibited a stable daily ethanol consumption of about 14 g/kg/day. At the time of sacrifice, 2 of 8 rats had significant blood ethanol levels (93 and 43 mg/dl) in the day 0 withdrawal group. All others had undetectable levels of less than 5 mg/dl.

To estimate the efficiency of the extraction procedure known amounts of unlabeled β-endorphin were added to samples containing low endogenous β-endorphin levels and the steps for removal of the peptide were followed as described in the Methods section. The recovery of ir-β-endorphin added back to preextracted cortex samples was found to be 96.4±0.12% (n=15) for the concentration range of 10–10,000 pg of peptide. The regional heterogeneity of ir-β-endorphin distribution in the rat brain is shown in Table 2, and the concentrations of peptide in the various regions can be seen to vary over a 100-fold range. These values represent the baseline concentration for each region shown

TABLE 1

BODY WEIGHTS OF ANIMALS AT TIME OF TESTING FOR HYPERTHERMIA

Days Ethanol Treatment	Ethanol	Sucrose
0	206.3 ± 2.4	204.9 ± 1.9
7	197.6 ± 2.4	194.7 ± 1.9
14	212.9 ± 3.3	209.6 ± 2.6
21	218.3 ± 4.6	216.4 ± 3.3
Days Ethanol Withdrawal	**Sucrose**	**Sucrose**
1	223.8 ± 5.2	224.2 ± 2.2
3	227.1 ± 5.8	231.2 ± 2.2
8	237.8 ± 5.6	241.1 ± 2.9
15	253.8 ± 6.6	260.1 ± 4.6

Values are means ± S.E.M. for weight in grams with n=9 for all groups. No groups were found to have significant weight differences by Student's *t*-test for paired data.

TABLE 2

REGIONAL HETEROGENEITY IN THE DISTRIBUTION OF IMMUNOREACTIVE β-ENDORPHIN IN RAT BRAIN AND PITUITARY

Region	Mean ir-β-endorphin (fmol/mg wet wt.)	$\log_e$ Log Mean	Duncan Grouping
Neurointermediate lobe*	450,449	13.018	A
Anterior lobe*	149,342	11.914	B
Arcuate nucleus	238.4	5.474	C
Periventricular hypothalamus	167.8	5.129	C D
Ventromedial hypothalamus	120.9	4.795	E D
Periaqueductal grey	116.0	4.754	E D
Periventricular thalamus	87.8	4.475	E F
Nucleus accumbens	85.3	4.446	E F
Preoptic periventricular hypothalamus	85.1	4.444	E F
Preoptic ventromedial hypothalamus	63.4	4.149	G F
Septum	52.8	3.967	G
Amygdala	21.7	3.076	H
Cortex	3.8	1.337	I

The pituitary means had n=19. All other regions had n=20. The mean square error for Duncan's multiple range test on the $\log_e$ mean values was 0.3345 and 44 degrees of freedom at an $\alpha=0.05$. Means that share the same letter are not significantly different.

in Fig. 2A to C. The concentration in the arcuate nucleus, which contains the perikarya of the β-endorphin system, was highest in the brain (238.4 fmol/mg wet weight tissue) while the amygdala and cortex were the lowest with 21.7 and 3.8 fmol/mg wet weight tissue. A natural logarithmic transformation of these data was used for comparisons, since the variance appeared to increase with an increase in the absolute value of the mean concentration, and the RIA calibration curve, analyzed as a four-parameter log-logistic transformation, introduces a nonuniformity of variance [33].

In Fig. 2A and B, a pattern was observed of a progressive decrease in $\log_e$(ir-β-endorphin) levels over day 0, 1, and 3 of ethanol withdrawal, followed by a recovery to baseline values on days 8 and 15. The arcuate nucleus showed this pattern as well as other regions including septum, pre-optic ventromedial hypothalamus, amygdala and periventricular thalamus. This pattern was not apparent in nucleus accumbens, pre-optic periventricular hypothalamus, periventricular hypothalamus, ventromedial hypothalamus and periaqueductal grey matter. The statistical method suggested by this pattern was to fit a regression line through the first three time points alone, and to use a t-test for the null hypothesis that the slope parameter is equal to zero. If good evidence can be found to reject the null hypothesis, then this indicates that the time course of levels of ir-β-endorphin over the acute withdrawal phase differs in the two treatment groups.

In regions having no observable pattern of change by visual inspection of the graphs, there were no significant differences of the slope from zero. In the arcuate nucleus and amygdala, the changes seen did not reach statistical significance, $t(1)=1.65$, $p=0.14$; and $t(1)=1.65$, $p=0.13$, respectively, and this was likely due to the greater degree of error in the measurement of these regions. In the septum, the change was closer to significance, $t(1)=1.96$, $p=0.079$, and in the pre-optic hypothalamus, a significant change, $t(1)=3.67$, $p=0.004$, indicated a reduction of peptide levels in the acute withdrawal period.

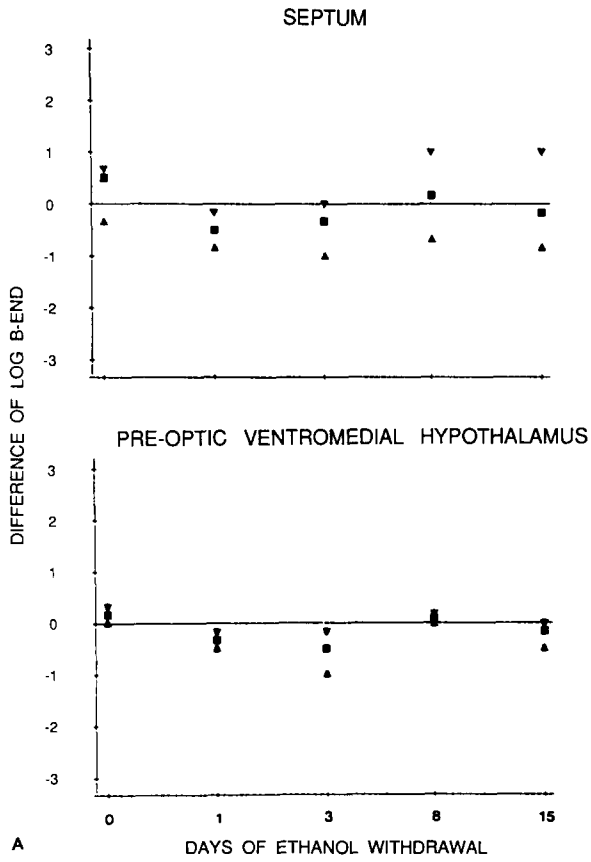
The results from the pituitary appeared to be dissociated from those of the brain. The NIL showed a nonsignificant decrease on day 0 of withdrawal and the AL showed a significant depression to roughly 20% of the control values which then recovered by day 15 of the withdrawal phase. The treatment main effect was significant, $F(1,4)=15.6$, $p=0.02$, for the comparison of ethanol versus control groups for levels of ir-β-endorphin in the anterior lobe. In a separate but identical experiment, the decrease in levels of ir-β-endorphin in the AL lobe was again observed, and was significant on days 1 and 8 ($p<0.05$ by two-tailed t-test for paired data) with recovery by day 15. In the two separate but identical experiments, there was no change detected in the levels of ir-β-endorphin in plasma.

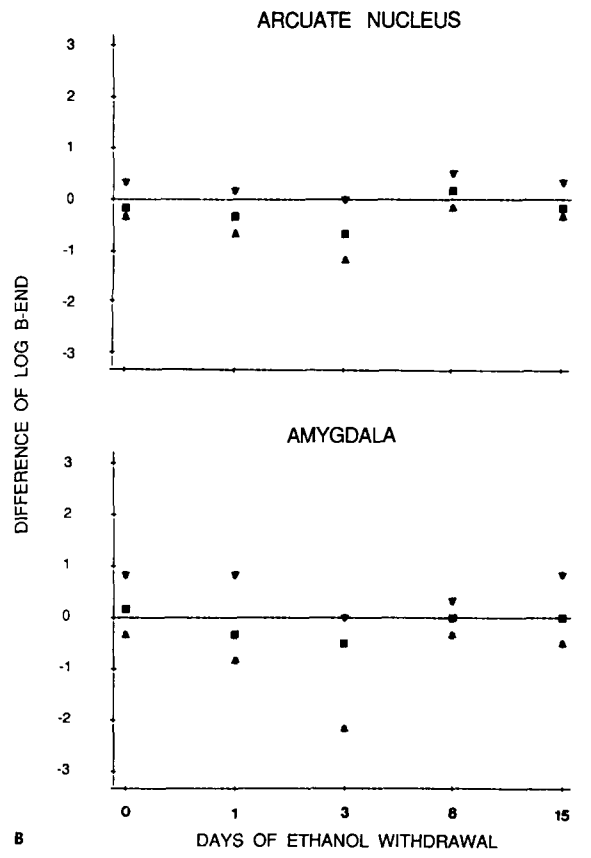
DISCUSSION

The analysis of data from only those animals receiving the control liquid diet revealed a heterogeneous distribution of ir-β-endorphin in the various brain regions examined. The rank order of regions according to the concentration of peptide was quite similar to that found by other [26, 29, 34] even though different dissections and extraction methods were used in the present study.

A similarity in the patterns of peptide depletion, with respect to both magnitude and direction, in the arcuate nucleus and in various other brain regions is consistent with studies demonstrating that the major source of β-endorphin is the arcuate nucleus. A perturbation of the source of peptide synthesis is expected to be reflected in those regions which it supplies, and conversely, the absence of an identical pattern in the pituitary tends to support the view that there is independent regulation of synthesis and release for pools of β-endorphin in this gland [8].

The question of whether it is possible to evaluate the dynamic status of the endorphinergic system by the measurement of levels of ir-β-endorphin is critical to the interpre-





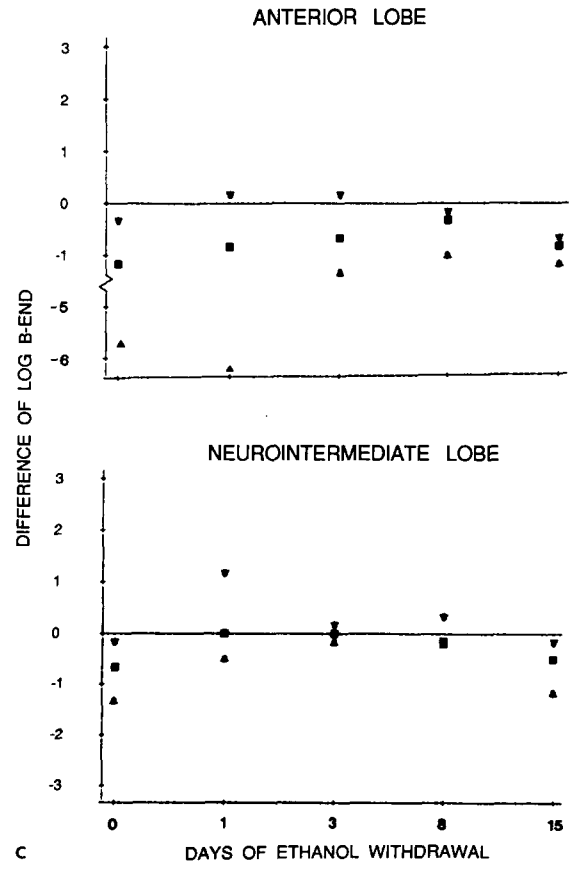


FIG. 2. Ethanol-minus-control difference in $\log_e$ levels of ir-β-endorphin in rat brain and pituitary over various periods of ethanol withdrawal. ▼, third quartile; ■, median; ▲, first quartile. (A) Septum and pre-optic periventricular hypothalamus; (B) arcuate nucleus and amygdala; (C) anterior lobe and neurointermediate lobe. N=4 samples per point, each corresponding to 2 animals.

tation of these experimental data [1,17]. In acute studies, it is probably true that a decreased level can be interpreted as an increased release and subsequent metabolism, because the induction of POMC precursor synthesis is a slow process taking several days or weeks to be realized [15,16]. On the other hand, in chronic studies such as the present work, there is more time for compensatory increases in synthesis to replace the acute deficit. However, there is some evidence that decreased peptide levels do indeed reflect increased release. The significant depression of β-endorphin levels in the AL seen in the present study is in agreement with previous observations on the whole gland or the AL [13, 15, 28], and may be related to the increased release of this peptide from the anterior lobe observed under in vitro experimental conditions [29]. At the same time that levels are decreased in the AL, studies using the ethanol liquid diet have shown an increase in synthesis of POMC as determined by the incorporation of radiolabeled amino acids [15, 16, 29], so the decreased level is most likely due to an increased release which exceeds the rate of synthesis. Some support for this suggestion is provided by the finding that the rate of production and release of ir-β-endorphin from the NIL in vitro is signifi-

cantly increased after 21 days of chronic ethanol feeding, at the time when the content in the NIL in vivo is decreased (day 0, Fig. 2), and significantly decreased on day 1 of withdrawal when the content in the NIL in vivo is normal (C. Gianoulakis, W. D. Hutchison and H. Kalant, Endocrinology, in press, 1988). However, another study has demonstrated decreased mRNA for POMC in the AL and a decrease in plasma levels of ir-β-endorphin after 14 days of exposure to ethanol vapour [10]. This is in contrast to the result with liquid diet and would suggest that the decreased level is due to decreased synthesis and release. It remains to be determined if the route of administration and nutritional factors are responsible for this discrepancy, or possibly the fact that the vapour inhalation method produces a sustained high level of alcoholemia, while the liquid diet method results in cyclic rise and fall of the blood alcohol level.

There are a number of possible reasons why no changes were found in the plasma levels of ir-β-endorphin in the present study. Firstly, the NIL may be a major contributor to circulating levels of peptide and no significant alterations were found in the tissue content. Secondly, a change in β-endorphin may have occurred which was obscured by the presence of other related immunoreactive endorphin fragments. Thirdly, a transient increase in β-endorphin levels due to increased release may have resulted in an enhanced metabolism and clearance of the peptide from the plasma under conditions of chronic ethanol exposure.

When the various forms of β-endorphin are taken into account in each brain region [11], it is apparent that the changes in levels of the immunoreactivity of these peptides in the regions represented in Fig. 2A to C actually reflect mostly β-endorphin$_{1-31}$, which is active at opiate receptors [34]. By comparison, the NIL contains chiefly the N-acetylated forms that are inactive at opiate receptors [34].

Alcohol withdrawal has been associated with a lowering of seizure threshold and it was not an unexpected finding that 2/8 rats in the day 1 withdrawal group exhibited spontaneous seizure activity. Repeated maximal electroshock in rats produces a progressive decrease in seizure severity that could be likened to CNS tolerance [32]. This neuroadaptive process can be blocked with naloxone, and is reversed if rats are first made tolerant to morphine, i.e., the adaptation of seizure threshold shows a form of cross-tolerance to morphine. It was subsequently shown in these experiments that the endogenous anticonvulsant could be identified as ir-β-endorphin [24]. This line of evidence raises the possibility that the changes seen in ir-β-endorphin levels could be due, in whole or in part, to the stress of seizures or other secondary effects of alcohol withdrawal, rather than to the withdrawal per se. Several investigators [25,27] have noted a release of brain β-endorphin with various stressors.

In summary, previous work has shown that central pools of β-endorphin do not change during chronic ethanol treatment in the absence of significant weight losses. This has been confirmed for the brain, but the AL does show a reduction even in the absence of weight loss or dehydration. Moreover, with the onset of alcohol withdrawal and the loss of tolerance to ethanol-induced hypothermia there is an associated change in the levels of ir-β-endorphin in some specific rat brain regions.

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