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# One injection of estradiol valerate induces dramatic changes in rats' intake of alcoholic beverages

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**Abstract**

A series of experiments investigated the effects of a single injection of estradiol valerate (EV) on female rats' consumption of alcoholic beverages. EV provides sustained release of estradiol. Just after an injection of EV, rats' intake of a palatable alcoholic beverage, which had been taken regularly before, is reduced dramatically. Subsequently, rats' intake of alcoholic beverage returns to baseline levels. With continued opportunity to drink, rats take more ethanol than controls. When EV was given 15 and 31 days before the first opportunity to drink an alcoholic beverage, female rats markedly enhanced their intake of ethanol. Once enhanced intakes emerged, they were observed with different kinds of alcoholic beverages and endured for months. © 2002 Elsevier Science Inc. All rights reserved.

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## 1. Introduction

This research was prompted by the extensive work of Brawer et al. (1993) and Desjardins et al. (1990, 1993). A conclusion derivable from that research is that an injection of estradiol valerate (EV), a compound producing sustained release of estradiol, produces selective damage to the β-endorphin-producing neurons of the hypothalamus. Given that conclusion, it follows that injections of EV might be used to assess the role of β-endorphin in processes related to alcohol abuse and alcoholism (AAA). There are a number of hypotheses that emphasize the role of β-endorphin in the etiology of AAA (e.g., Gianoulakis et al., 1990).

As will be reported in more detail subsequently, the staff of the Rensselaer Laboratory, using a group of female rats, arranged the circumstances for considerable intake of an alcoholic beverage. Then, some were injected with a single dose of EV. EV produced a transitory reduction in body-weight and an abrupt, dramatic reduction in intake of alcoholic beverage. Day after day, placebo controls continued to drink alcoholic beverage, whereas EV-injected females drank only small amounts.

Sandberg and Stewart (1982) and Sandberg et al. (1982) studied the effects of estradiol benzoate (EB) on intake of alcoholic beverage among ovariectomized rats. They found that daily injections of EB reduced intakes across a number of days. They also showed that the effect waned after about 2 weeks. Further, the daily injections lead to rather marked reductions in bodyweights across initial 10 days. Subsequently, rats gained weight regularly.

Sandberg and Stewart (1982) and Sandberg et al. (1982) noticed that EB-induced reductions of intake of alcoholic beverage paralleled EB-induced reductions in intake of food as observed by others (e.g., Wade, 1975). Sandberg and Stewart (1982) also assessed the effects of MER-25, an antiestrogen. MER-25 blocks many effects of estrogen, but not those salient to food intake. They reasoned that if both EB and MER-25 produced similar effects on intake of food and alcoholic beverage, the mechanism of both effects would be similar and not related to the many effects of estrogen blocked by MER-25. Given that the effects on alcohol intake of MER-25 and EB were similar to each other

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and similar with respect to food intake, they concluded that estradiol's effects were relevant to mechanisms associated with ingestion rather than estradiol's other effects.

In another experiment, Sandberg and Stewart (1982) demonstrated that neither EB nor MER-25 reliably modified rate of elimination of ethanol from blood. In yet another, they found that treatment with estradiol did not affect the extent of conditioned taste aversions induced by large doses of ethanol. They concluded that estradiol inhibits ethanol consumption by some other mechanism than producing sickness or malaise.

The initial results with EV were concordant with those of Sandberg and Stewart (1982) and Sandberg et al. (1982) with the exception that EV produced a more lasting suppression of intake. It was presumed that the prolonged effects of EV were due to the neurotoxicity that Brawer and his colleagues had observed. These findings were also concordant with hypotheses of AAA featuring β-endorphin (e.g., Gianoulakis et al., 1990).

As the staff of the Rensselaer Laboratory was observing the EV-induced reductions in intake, the abstracts of presentations for an annual meeting of the Research Society on Alcoholism became available. Marinelli and Gianoulakis (2000) of McGill University reported they injected EV 11 weeks before female rats were given an opportunity to sample alcoholic beverage. After sampling various concentrations, the rats were given an opportunity to take an alcoholic beverage (8% ethanol to 92% water, volume to volume) for a number of days. The EV-treated rats did not decrease their intakes. In fact, they took more than controls. Following behavioral observations, β-endorphin levels in hypothalamus were assessed. The β-endorphin levels among EV-treated rats were similar to controls and not those expected provided EV was toxic to hypothalamic β-endorphin cells.

Initial observations at Rensselaer indicated EV produced marked reductions in intake of alcohol while observations at McGill indicated the opposite. A major difference in procedures was the amount of time between injections of EV and opportunities to drink. The rats of the Rensselaer Laboratory were drinking daily when given EV, whereas rats of the McGill laboratory were first presented with an opportunity to drink weeks after their injections.

To see if the procedural differences could account for the different results, female rats were given EV months before the opportunity to take alcoholic beverage. The original plan was to inject EV and wait about 3 months, as was done at McGill, but we did not wait that long. We did not wait because, as we, at Rensselaer, continued to observe the first set of rats given EV, some of them began taking substantial amounts of alcohol earlier.

Following conversations among the staff of the McGill and Rensselaer Laboratories, it was decided to take the hypothalami of the rats with suppressed drinking and to measure their β-endorphin levels, thus, of course, ending the measurement of their drinking (and Experiment 1 of this report). Experiment 2 reports the effects of injections given 1 or 2 months before females had daily opportunities to drink. Subsequently, these rats were given 24-h access to alcoholic beverage and later, across days, the saccharin concentration of their alcoholic beverage was slowly reduced to zero. Another experiment followed similar procedures to those of Experiment 1, but extended the period of opportunity to drink. In addition, rats were given naloxone on 1 day as an assessment of opioid involvement in their drinking. An experiment tested the effects of EV on male rats. Another experiment gave EV 3, 15 or 31 days before initial opportunity to drink a sweetened alcoholic beverage.

Consistent findings emerged. With daily pharmacological doses of estradiol, rats' intakes of alcoholic beverage are suppressed, confirming Sandberg and Stewart (1982) and Sandberg et al.'s (1982) findings. Subsequent to pharmacological doses of estradiol, rats' intakes of alcoholic beverages are often enhanced, findings concordant with those of Marinelli and Gianoulakis (2000). The results are also compatible with those of Ford et al. (2000). The enhanced intakes can endure for a prolonged time. The conclusion is that pharmacological doses of estradiol can produce profound and enduring effects on rats' appetite for alcoholic beverages.

## 2. General methods

### 2.1. Subjects

The subjects of these experiments were female and male Sprague–Dawley rats obtained from Taconic Farms (Germantown, NY) when they weighed about 180 g. Upon arrival at the laboratory, they were housed individually in cages with food and water always available. The windowless room housing their cages was maintained at nearly 22 °C with 12 h of light a day beginning at 0700 h. Subsequent to a few days to habituate to the conditions of the laboratory, the procedures of the separate experiments began. Across experiments, different pairs of fluids were presented to the rats. One fluid was always tap water. These experiments were approved by the RPI Institutional Animal Care and Use Committee in accordance with NIH Guide for Care and Use of Laboratory Animals.

### 2.2. Drugs and beverages

EV is commercially available. EV is converted slowly into 17-β-estradiol and valeric acid. The 17-β-estradiol, produced by the conversion, behaves like the endogenous steroid in terms of both pharmacodynamics and pharmacokinetics. An intramuscular injection of EV can produce meaningful doses of estradiol for 2–4 weeks (Dusterberg and Nishino, 1982). Most of the doses of EV were 2.0 mg/rat given intramuscularly by way of a 0.2-ml injection of EV and carrier. The carrier was sesame oil. An injection of oil

served as the placebo. In one instance, doses of EV were 1.0 mg/rat given in the same volume of oil as 2.0 mg.

In one experiment, naloxone hydrochloride (10 mg per kg of bodyweight) was given. The carrier of naloxone was physiological saline. Injections of saline served as placebos.

An alcoholic beverage used initially was 12% absolute ethanol, 0.25% saccharin and tap water, i.e., 100 g of solution contained 12.00 g of ethanol, 0.25 g of saccharin and 87.75 g of tap water. In one experiment, the amount of saccharin in the solution was reduced 10% at a time.

### 2.3. Assays for β-endorphin levels

The β-endorphin assays were conducted as previously described (Gianoulakis and Gupta, 1986; Jamensky and Gianoulakis, 1999). Briefly, to prepare tissues for the radioimmunoassay, samples were thawed on ice and then extracted by sonication using a microultrasonic cell disrupter (Kontes, Vineland, NJ). An aliquot was taken from each sample for protein estimation (Bradford, 1976). The remaining samples were centrifuged at 4 °C (14,000 rpm) for 7 min and the supernatants were collected and stored at − 75 °C for estimation of the content of β-endorphin using sensitive radioimmunoassays. [$^{125}$I] β-endorphin (Amersham Pharmacia Biotech, Buckinghamshire, England) was used as the tracer. The antiserum was specific to the C-terminus of β-endorphin and recognized POMC, β-lipotropin, β-endorphin 1–31, β-endorphin 1–27 and β-endorphin 1–26 in both acetylated and nonacetylated forms. This antiserum did not recognize adrenal corticotropic hormone, α-melanotropin or the β-lipotropin fragments 1–65, 7–62 and 8–84. The intra- and interassay coefficients of variation were 5% and 10%, respectively. Results are expressed as nanogram of β-endorphin peptides per milligram of protein.

### 2.4. Procedure

Procedures enhancing rats' ethanol intakes are (a) presenting many daily opportunities to take alcoholic beverage, (b) providing a palatable alcoholic beverage and (c) presenting alcoholic beverage on a schedule when other ingestive behavior is likely (Reid, 1996). A procedure based on these generalizations, and one we used here, presents a sweetened 12% ethanol solution and water for only 2 h/day. Under such a daily regimen, rats gradually develop intakes averaging more than 2 g of ethanol/kg bodyweight in about 3 weeks (Reid, 1996). Without modification of the procedures, male rats sustain that level of intake for months.

### 2.5. Data reduction and statistics

Fluids were presented to subjects by way of bottles equipped with ballpoint sipping tubes. The bottles were weighed before and after their presentation. The differences in weights, corrected for spillage, are the raw data of these experiments. Subjects were usually weighed once a day when they had alcohol available and periodically otherwise. When a bottle was dropped or another obvious problem emerged (e.g., a pellet of food became lodged under the drinking spout), rare events, the issue of missing data was handled by taking the average of that rat's intakes across the days before and after the incidence as a substitute for the missing data.

To simplify this report, water intake data are not presented. In general, all rats take some water during every measurement period, but when intakes of alcoholic beverage are large, intakes of water are small. The total amount of fluid taken remains nearly constant across the days of measurement. It follows, therefore, that the data of interest are grams of ethanol per kilograms of bodyweight (g/kg) and that is what is presented.

The experimental designs conform to factorial analysis of variance (ANOVA) having factors of between groups (placebo versus EV) and days of repeated measures. Following the finding of statistical significance for factors associated with EV treatment, tests for simple main effects were assessed by *t* tests.

## 3. Experiment 1: EV to females taking alcoholic beverage

The first experiment was based on the idea that EV was toxic to neurons of the arcuate n. producing β-endorphin (e.g., Brawer et al., 1993). Disruption of β-endorphinergic functions, according to theory (Gianoulakis et al., 1990), should produce systematic changes in intake of alcoholic beverage.

### 3.1. Method

Shortly after their arrival at the laboratory, 24 females were placed on the daily regimen of presentation of water and a saccharin-sweetened 12% alcoholic beverage for 2 h/day. After 40 days, 12 received an injection of EV (2 mg/rat) and 12 received placebos. After the 78th daily session, six of the placebo controls (randomly selected) received an injection of EV while all others received placebos.

In addition to the injections given after the 78th daily session, 12 additional female Sprague–Dawley rats purchased from Taconic Farms were also injected. They had arrived at the laboratory only 6 days before. They were individually housed with food and water always available but no alcoholic beverage. Six of them received EV 2 mg. The other six received placebos.

After the session of the 82nd day (rats had taken their alcoholic beverage and water, had eaten and were alert), all hypothalami were taken. Assays of the 12 additional rats' hypothalami provide an estimate of β-endorphin levels among rats with no history of opportunity to take alcohol. Given these procedures, we had five groups whose hypothalami were assayed for β-endorphin (Table 1).

Table 1
β-endorphin levels

| Groups | Treatment | *n* | (ng/mg) | Percent of Group 1 |
|---|---|---|---|---|
| 1 | Alcohol daily, placebos | 6 | 3.662 (0.292) | |
| 2 | Alcohol daily, EV 42 days before | 12 | 2.244 (0.338) | 0.61 |
| 3 | Alcohol daily, EV 3 days before | 6 | 3.002 (0.322) | 0.82 |
| 4 | No alcohol, placebo | 6 | 4.643 (0.126) | 1.28 |
| 5 | No alcohol, EV 3 days before | 6 | 5.710 (0.830) | 1.56 |

Values are nanograms of β-endorphin peptides per milligram of protein (standard error of means).

### 3.2. Results

The females developed substantial intakes of ethanol. Upon receiving EV, subjects (a) lost weight across the first few days after EV (Fig. 1) and (b) dramatically reduced intakes of alcoholic beverage for a number of days.

The intake of the rats given EV dropped from a level similar to that of placebo controls before injections (i.e., 2.81 g/kg for a mean across 10 days before injections) to a mean of 0.77 g/kg across the 39 days post injections. During the time of estradiol release, all rats given EV reduced intakes. The values for comparing EV-treated intakes with those of placebo controls are, for the group effect, $F(1,22) = 68.23$, $P < .0000001$.

The comparison of intakes of the six females who received EV 3 days before the end of the procedures with the six controls indicates the same as the first injections. The placebo controls, both before and after injections, sustained intakes greater than 2.00 g/kg. The six that eventually got EV took a mean of 2.89 g/kg daily for 3 days just before injections and 0.06 g/kg after injections, $P = .0005$.

Notice an interesting feature of EV-treated bodyweights (Fig. 1). Means for Blocks 2 and 3 are somewhat lower than baseline. In addition, the rats of EV seem to recover their weight loss (see Blocks 5 and 6). During the time of Blocks 5 and 6, EV-treated rats continued to take less alcoholic beverage than baseline intakes and intakes of placebo controls. Following Blocks 5 and 6, however, there is a period of weight loss and no weight gain. Throughout this period, placebo controls are steadily gaining weight. These findings are reflected in the results of an ANOVA.

The ANOVA (Fig. 1) yields: for the factor of Groups, $F(1,22) = 5.38$, $P = .03$; for the factor of Blocks, $F(12, 264) = 6.01$, $P < .0000001$; for the interaction, $F(12,264) = 6.63$, $P < .000001$. Tests for simple main effects for assessing differences between groups at each block indicates that groups are not reliably different at Block 1, but that they did differ ($P < .05$) at Blocks 3 and 4, but not at Blocks 5, 6 and 7. Subsequent to Block 7, the groups are significantly different than one another ($P < .03$). Some within group comparisons are of interest. The mean bodyweight of EV-treated rats at Block 2 and 3 is significantly lower than that of Blocks 5 and 6. The EV-treated rats weighted reliably more at Block 6 than they did at Blocks 7, 9 and 10 ($P < .03$).

Table 1 presents the β-endorphin levels for the five groups. The rats receiving alcohol daily had reliably less β-endorphin than the rats with no history of alcohol, $P = .0004$. The two groups, however, are different along a number of variables in addition to history of alcohol intake. The rats without a history of alcohol, for example, arrived at the laboratory some days after the others. These rats had no history of a daily regimen comparable to the rats being presented alcohol. Given these limitations, it is difficult to draw strong conclusions from this apparently robust difference.

Among the subjects taking alcoholic beverage daily, EV given 3 or 42 days before did not lead to marked reductions in β-endorphin. Based on the conclusions of Brawer et al. (1993), it was expected that rats receiving EV 42 days before their hypothalami were taken would have almost no β-endorphin. Group 4 (no alcohol, placebo) and Group 5 (no alcohol, EV 3 days before) are comparable in terms of history. The β-endorphin levels associated with these sub-

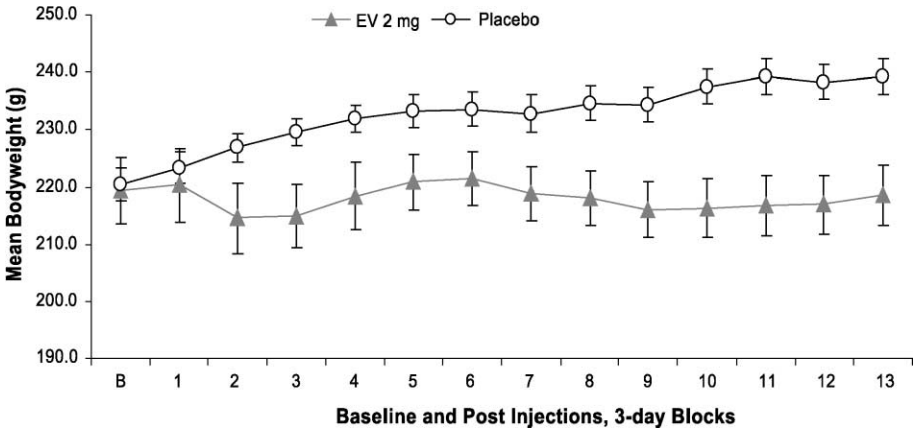


Fig. 1. The mean body weights across 39 days after injections for two groups of rats are depicted. One group received 2 mg of EV just after baseline (B). The other group received placebo.

jects provide no support for a significant difference between groups ($P$=.28). Group 1 (alcohol daily, placebo) and Group 3 (alcohol daily, EV 3 days before) are similar in terms of history, and there is no support in the statistics to indicate that there is a difference in β-endorphin levels ($P$=.53).

### 3.3. Discussion

The prediction that EV would completely lesion the β-endorphin-producing cells of the hypothalamus is not confirmed. It is not apparent why we did not see the marked toxic effect described by Brawer et al. (1993). There are potentially important differences in procedures across experiments (e.g., time after EV injections that hypothalami were taken) that could account for the differences in outcomes.

The results of these experiments can be not explained in terms of EV's ability to lesion the neurons of the arcuate n. of the hypothalamus because, apparently, the neurons are producing β-endorphin, hence not lesioned. It does not follow, however, that EV, long term, is without effects on the arcuate n. Notice the differences between Groups 4 and 2 in Table 1. Our measurements are not complete enough to detect all, or even most, relevant events associated with β-endorphinergic processing.

These results are concordant with those of Sandberg and Stewart (1982) and Sandberg et al. (1982). Rats given pharmacological doses of estradiol, by way of EV or EB, initially reduce intakes of alcohol.

There is an interesting pattern of changes in bodyweights discernable with these and other subjects of this report. There is the initial loss of weight across a few days just after injections of EV and an initial recovery of weight (with these subjects 15–21 days after injections). After the recovery, however, and at a time when EV is no longer delivering pharmacological doses of estradiol, there is again a loss of weight. Following this second loss of weight, there is a period of little or no weight gain. The working hypothesis is that adaptation to sustained estradiol (tolerance) and adaptation to the end of sustained estradiol (a "withdrawal" condition) are both significant events in determining appetite for alcoholic beverages.

The results, in some ways, were very satisfying. EV reduced alcohol intakes as predicted from hypotheses emphasizing the role of β-endorphin. The results were concordant with those of Sandberg and Stewart (1982) and Sandberg et al. (1982). The recovered weight loss seen at Blocks 5 and 6 of Fig. 1 might indicate that the reduced intakes of alcoholic beverage were not merely because the rats were sick. Although the β-endorphin levels of EV-treated rats were not as low as predicted; they were, indeed lower than those of controls. The data, across days during which estradiol was being released, support a hypothesis of a salient role for β-endorphin in the regulation of alcohol intake. Dissonance, however, emerged. Marinelli and Gianoulakis (2000) found almost the opposite. Some of these EV-treated rats, toward the end of the 39 days, on occasion took very large amounts of alcohol. Toward resolving the dissonance, Experiment 2 was engaged.

## 4. Experiment 2: EV given to rats 1 or 2 months before first opportunity to drink alcoholic beverage

Experiment 1 showed that an injection of EV produced dramatic reductions in rats' ordinary consumption of an alcoholic beverage. What happens when EV (2.0 mg/rat) is given 1 or 2 months before opportunity to take alcoholic beverage?

### 4.1. Method

Groups ($n$ = 9) received EV (2 mg/rat) either 31 or 61 days before the start of the daily regimen used in Experiment 1 (a daily chance to choose sweetened alcoholic beverage) (the first data points are 32 and 62 days after injections). A placebo control group ($n$ = 9) received carrier of EV. When a group received a dose of EV, other subjects received placebos. Consequently, all rats received an equal number of injections and at the same time before being put on the daily regimen. They were maintained on that regimen for 32 days before changes in the procedure (described in the next experiment).

### 4.2. Results and discussion

The subjects did not differ reliably, in terms of bodyweights, before injections. Fig. 2a presents mean bodyweights for the three groups of subjects and depicts the effects of EV. The presented data are of days just before and after EV treatment for the group that received alcohol about a month later. This is a period when the group that got EV earlier is probably not having estradiol delivered. During this time, bodyweights were taken every other day. The data point of Day 33 of the figure reflects the first measurement of a potential EV effect for one group 1 day after injection and the potential EV effect of another group 31 days after injection. During this time, all rats had unlimited access to food and water. Therefore, the data reflect the effects of EV on rats with no history of any other experimental manipulations.

Fig. 2b presents a summary of intake of alcoholic beverage in terms of amount of ethanol taken (g/kg). An ANOVA of only the data of the placebo controls yields for the effect of blocks an $F(7,56) = 1.62$, $P$=.15. The indication is that the placebo controls sustain a consistent level of intake when the conditions remain constant, as they did here. Consequently, any change in the experimental groups is apt to be due to EV treatments.

The groups getting EV a month before presentations of alcohol increased intakes across the blocks of daily sessions. These rats' intake scores are significantly larger than those

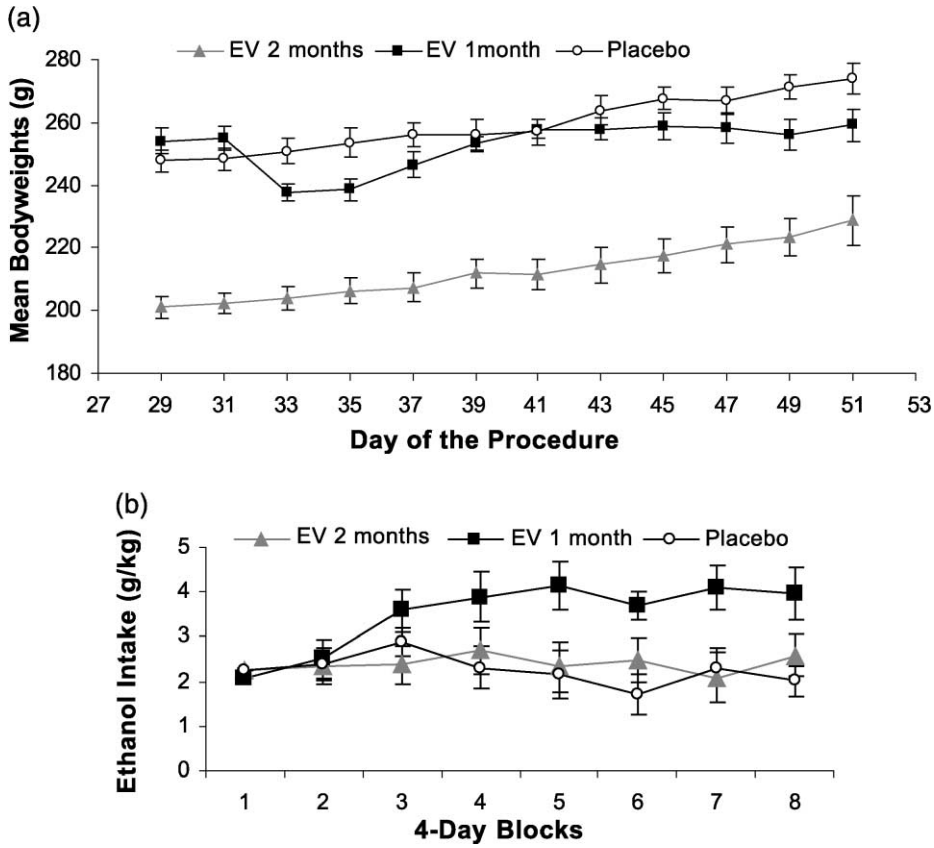


Fig. 2. (a) The bodyweights of three groups are presented. One group (designated Placebo, with open circles as data points) received only placebos. Another group (designated EV 2 months) received an injection of EV 2 months before first opportunity to take alcoholic beverage. The other group (designated EV 1 month) received an injection of EV 1 month before the opportunity. The data presented here are just before and after the EV 1 month group received their injection. Injections for EV 1 month group were given after measurements on Day 31 of the procedure. EV was given to the EV 2 months group a month before Day 31 of the procedure. An ANOVA of the data used to derive the figure yields the following values: for the effect associated with group, $F(2,24)=41.8$; for the effect of days, $F(11,264)=41.5$; for the interaction, $F(22,264)=4.34$; all $P<.0000001$. (b) Across a period of 32 days (eight blocks of 4 days each), three groups of subjects had an opportunity to take alcoholic beverage, hence, ethanol, for 2 h a day. The graph depicts mean intakes for each group (in terms of mean intake per day) for the eight blocks. An ANOVA of the data used to derive the figure (means across 4 days) yields the following values: for the effect associated with group, $F(2,24)=6.80$, $P=.004$; for the effect of blocks, $F(7,168)=2.98$, $P=.006$; for the interaction, $F(14,168)=3.22$, $P=.0002$. Notice that initial intakes were very similar. Subsequently, the subjects who received EV 1 month before these sessions took more ethanol than the other two groups.

of placebo controls from the fourth block to the end of the procedures ($P<.02$). A $t$ test yields, for the comparison of values of the last block for EV 1 month and placebo controls, a $P$ value=.0003. There is no support in these initial analyses to indicate a reliable difference between scores of placebo controls and of those getting EV 2 months before having a chance to drink.

With respect to intake of alcoholic beverages, the results of this experiment and Experiment 1 indicate that EV can induce a variety of outcomes. If observations are made shortly after injections of EV, intakes of alcoholic beverages are reduced. If observations are made sometime after the injections and if rats have at least a short history of opportunity to drink alcoholic beverage, intakes can be similar to or greater than controls. Specifying the time after pharmacological doses of estradiol resolves apparently discrepant conclusions that could have been drawn from extant observations.

## 5. Experiment 3: EV-treated rats given 24 h/day to take alcoholic beverages

The results of Experiments 1 and 2 indicate that a single injection of EV produces remarkable changes in rats' consumption of alcoholic beverages using a daily regimen involving only 2 h/day to take sweetened alcoholic beverage. An obvious question is whether the effects of EV are limited to the procedures of Experiments 1 and 2.

### *5.1. Method*

This experiment used the three groups of rats of Experiment 2 that had either placebo or EV injections 61 or 31 days before being put on the 2-h daily regimen for 32 days. These procedures began with all subjects being given the sweetened alcoholic beverage and water for 24 h/day for 8 days (food always available). Subsequently, the concentration of

saccharin was reduced by 10% every 2–4 days until the alcoholic beverage contained no saccharin (12% ethanol in tap water). After reaching zero saccharin, the rats continued with that alcoholic beverage for 6 days.

Subsequent to the data collection associated with alcoholic beverage without saccharin flavoring, the rats were returned to the limited access regimen used initially for 3 days. After a session with access to a flavored alcoholic beverage and water, the rats' hypothalami were taken and eventually assayed as described above. The data for two subjects of the group EV 2 months were lost. Consequently, for consideration of β-endorphin, the *n* for that group is 7 rather than 9.

### 5.2. Results

These females consumed, on average, very large amounts of the beverage when it was presented 24 h/day and continued to do that day after day. Females of group EV 1 month consumed extraordinary amounts of alcoholic beverage (Fig. 3a), particularly across the first days of unlimited access. Mean intakes across the days of 24-h availability for rats of placebos = 4.81 g/kg/day, rats given EV 61 days before first alcoholic beverage (EV 2 months) = 5.39 g/kg, rats given EV 31 days before (EV 1 month) = 9.04 g/kg, $F(2,24) = 8.82$, $P = .001$. Further analyses provide no support for the conclusion that the group EV 2 months drinks amounts different than the placebo controls. An ANOVA comparing only the scores of EV 1 with those of placebo yields the following: $F(1,7) = 17.5$, $P = .0007$, indicating that the group given EV 1 month before their first opportunity to take alcoholic beverage drank significantly more than controls. An ANOVA comparing all subjects getting EV to those of placebo yields, for the group effect, the following: $F(1.16) = 12.6$, $P = .003$.

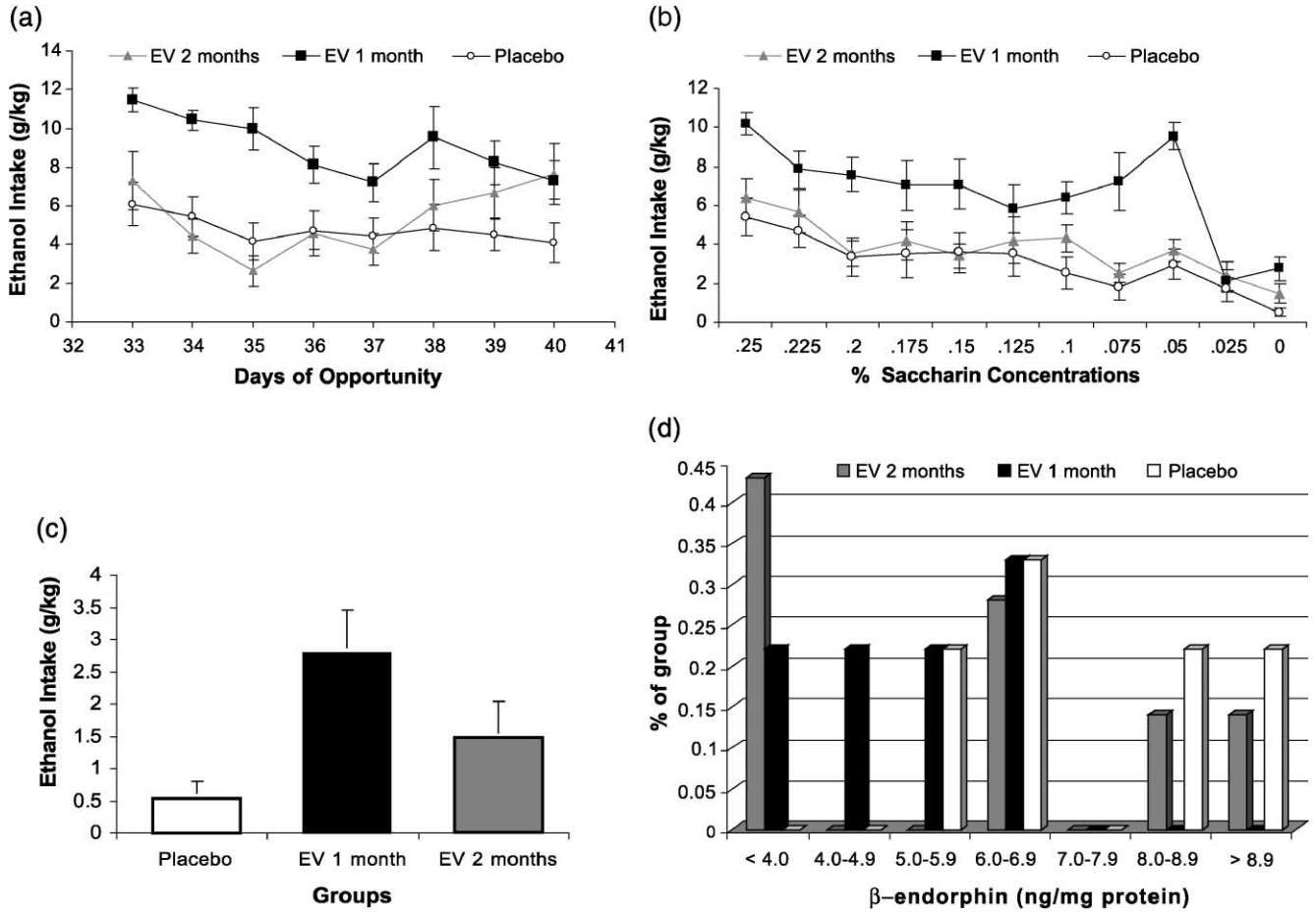


Fig. 3. (a) Intervening between the rats' injections and just before opportunities to take alcoholic beverage 24 h a day, there was 1 month of limited access to alcoholic beverage (Experiment 2). The data of this figure summarize the daily intakes when the same alcoholic beverage was available 24 h a day. (b) The data are means across days for each concentration of saccharin in a 12% ethanol solution. The left-most data points are the means across days of (a). The right-most data points are the means across 6 days with zero saccharin concentration and represent data concerning how rats respond to otherwise unflavored alcoholic beverage (see c). (c) Mean intakes of the groups when the alcoholic beverage was 12% ethanol and zero saccharin. This is the same data as the left-most data point of (b). (d) A frequency distribution of β-endorphin scores for each group is depicted as a proportion of the *n* showing an amount of β-endorphin. Notice that subjects of placebo are grouped toward the larger amounts and that the subjects of EV 1 month are grouped toward the smaller amounts. In addition, the group EV 2 months seems to consist of two separate groups. Some subjects' scores are lower and clearly fall within the range of EV 1 month group. There are, however, other subjects that clearly fall within the range of the placebo controls.

Fig. 3b presents the means across all days with a particular concentration. The left-most data points are the means across the days of Fig. 3a. With reductions of saccharin concentration, all rats reduced intakes. The rats given EV, however, reduced intakes less. An ANOVA of the means used to derive Fig. 3b yields the following: for the group effect, $F(2,24) = 8.49$, $P = .002$; for the block effect (decreasing concentration of saccharin), $F(10,240) = 19.03$, $P < .0000001$; for the interaction, $F(20,240) = 2.41$, $P = .0009$. Further analyses provide no support for the idea that subjects of EV 2 months took more ethanol than placebo controls. Generally, however, subjects of EV 1 month took significantly more ethanol at each concentration, except at concentration 0.025.

The means for the last 6 days of the experiment, when the concentration of saccharin was zero (ethanol concentration remained 12%) are presented in Fig. 3c. An ANOVA of the daily scores of the three groups yields, for the group effect, an $F(1,24) = 6.08$, $P = .007$. The $P$ values, derived from $t$ tests using the subjects' mean consumption across the 6 days of zero saccharin concentration are: (a) for the placebo controls compared to group EV 1 month, $P = .003$; (b) for the placebo controls compared to group EV 2 months, $P = .09$; (c) for placebo controls compared to intakes of all EV subjects, $P = .01$.

Fig. 3d presents the data of β-endorphin as frequency distributions for each group. As might be discerned from a look at the distributions, a comparison of mean β-endorphin levels between subjects of EV 2 months and placebo controls does not yield evidence supporting a conclusion of reliable differences between the two. On the other hand, a $t$ test comparing values of EV 1 month and placebo controls yields $P = .02$. Means of the groups are presented in Table 2.

An inspection of the data of Fig. 3d along with intakes of ethanol for each subject yields some interesting observations. Two indices of a rat's intakes are (a) a mean of intakes across all days of opportunity to drink during limited access (data germane to Fig. 2b) and (b) mean of intakes across all days of unlimited access (data germane to Fig. 3a). The correlation coefficient for the two sets of data is .55.

Using all subjects regardless of EV treatment, the correlation between mean 2-h intakes and β-endorphin levels is −.51 and between mean 24-h intakes and β-endorphin levels is −.25. Given the bimodal distribution of scores of the β-endorphin assays for the group EV 2 months, it is of interest to see how this group of subjects took ethanol. The correlation between their mean 2-h intakes and β-endorphin levels is −.90. The correlation between their 24-h intakes and β-endorphin levels is −.44. The mean rank of scores across the 2- and 24-h intakes is a single index of these rats' drinking. The highest mean ranks (i.e., the three largest drinkers) are those with the lowest β-endorphin levels and identify the three subjects of Fig. 3d that clearly distribute themselves among the EV 1 month group.

Table 2
β-endorphin levels

| Groups | Treatment | $n$ | (ng/mg) | Percent of Placebo |
|---|---|---|---|---|
| 1 | Alcohol daily, placebos | 9 | 7.682 (0.849) | |
| 2 | Alcohol daily, EV 158 days before | 7 | 5.971 (0.899) | 0.77 |
| 3 | Alcohol daily, EV 127 days before | 9 | 5.355(0.376) | 0.70 |

Values are nanograms of β-endorphin peptides per milligram of protein (standard error of means).

### 5.3. Discussion

A remarkable feature of these data is the amount of alcoholic beverage, hence ethanol, taken by the placebo controls. Upon informal observations, they frequently showed signs of behavioral toxicity such as slowed righting reflexes. Given these large intakes, the findings with the subjects administered EV, some months before the opportunities to drink, are even more striking.

The findings with the rats given EV 2 months before first opportunity to drink alcoholic beverage (Experiments 2 and 3) are not, on the surface, concordant with the findings of Marinelli and Gianoulakis (2000). Their rats were given EV about 3 months before opportunity to drink. The conclusion that EV treatment enhances rats' intakes when given 1 and 3 months after the injection, but not at 2 months (which these findings apparently indicate), may not be reasonable on the grounds that nothing would predict such dynamic shifts without further experimental manipulations. Marinelli et al. (2001) also observed increases in intakes when EV was given about 2 months before the beginning of opportunities to take alcoholic beverage. There are considerations, however, that reduce the apparent dissonance produced by the apparent lack of concordance in the findings.

The conclusion of no statistically significant difference from placebo controls for the rats given EV 2 months before may merely be because of the relatively small $n$. Some of the rats of EV 2 took very large amounts of ethanol at various times across their history. While inspecting an individual rat's daily intakes, it was not unusual to observe large to moderate intakes for a few days and then observe huge intakes during a day. Occasionally, after an incidence of huge intakes, a rat would stop taking the beverage (or reduced intakes to nearly none) across a period of days. It is as if they developed a conditioned aversion, by way of toxic overdose, to the alcoholic beverage. This apparent effect, along with some placebo control's development of large intakes, probably demands somewhat larger $n$ per group than used here to avoid making the error of concluding no difference when, indeed, a difference exists.

The most comparable circumstances of these subjects to those of Marinelli and Gianoulakis (2000) are when these subjects were taking 12% ethanol with zero saccharin concentration. At this point, all subjects had EV over 2 months before and, in general, the rats of EV were taking

more than the controls. In addition, under these circumstances, the rats given EV 2 months before their first opportunity to drink did take over two times the amount of ethanol as controls. The *P* value associated with that intake is .09, a two-tailed value. A one-tailed value (which is probably more appropriate because it assesses previous results specifying the direction of potential change) does meet conventional standards for statistical significance. Therefore, an apparent lack of agreement in results across experiments does not emerge as being discordant upon closer inspection.

Although there are no large discrepancies in results across the various experiments, there are still some differences that might be of interest. The rats given EV 1 month before opportunities to drink did develop very large intakes. Perhaps, when opportunities to drink are provided at about 1 month after administration (thereby arranging for the more recent EV-induced changes to interact with the reinforcing effects of ethanol) that a larger enhancement of consumption of alcoholic beverages emerges compared to when the alcoholic beverages are presented later. The dynamics of weight changes seen with Fig. 1 might, also, be relevant.

As with every fading procedure (procedures of decreasing concentrations of saccharin), the results are a product of confounded variables. The subjects are becoming inured to the raw taste of ethanol and learning to respond to the new flavor while, at the same time, the palatability of the beverage is shifting. Therefore, we do not know, for example, whether the relatively high intakes associated with concentrations of 0.075% and 0.05% saccharin are reactivity toward a beverage that is more palatable than some others or whether the subjects are learning to accept the less palatable beverage. Furthermore, there are apt to be interactions with the state induced by EV. Therefore, our conclusions are limited: EV treatment can enhance intakes of a variety of alcoholic beverages, but we can not specify the flavors that are more apt to be enhanced.

The relationships discerned with the assays of β-endorphin (subjects of Experiments 1–3) open the possibility that further research will be able to specify conditions that lead to reduced β-endorphin levels and enhanced intakes of alcoholic beverages. It should be noted that the levels measured here occurred after subjects had their daily opportunity to take alcoholic beverage. As determined by way of assays of hypothalami of a few subjects of Experiments 4 and 5, levels measured before opportunity to drink may not show the same relationship between controls and EV-treated subjects as shown after a session of drinking.

In summary, EV given months before first opportunity to take alcoholic beverage produces a change in the subjects that is manifest by enhanced consumption of unflavored alcoholic beverage. EV, given 1 month before first opportunity to take flavored alcoholic beverage, produces a change manifest in large intakes of saccharin sweetened alcoholic beverage. The enhanced intakes are observable when alcoholic beverage is presented for only a limited amount of time daily or when beverages are available continuously. Furthermore, propensity for enhanced intakes, once observed, seems to endure across months of opportunity to take alcoholic beverage.

## 6. Experiment 4: the effects of naloxone on EV-treated rats

The procedures of this experiment were very similar to those of Experiment 1, except that an additional group received a 1.0 mg/female dose of EV. At one point in the daily regimen, all rats were given placebos, naloxone and then placebos again. Naloxone is the antagonist at the opioid receptors and, in ordinary rats, reduces intakes of alcoholic beverages (Hubbell and Reid, 1990). If neurons producing β-endorphin are disrupted by EV, it is presumed that endogenous opioid systems will be disrupted. Given this presumption, one might hypothesize that naloxone would have a different effect on placebo- and EV-treated rats.

### *6.1. Method*

The subjects were 20 female Sprague–Dawley rats. Each had 70 days on the daily regimen of Experiment 1. After the 70th daily session, rats received injections of EV (1 or 2 mg/rat, $n=7$ for each dose) or placebo ($n=6$).

After the 140th day, the procedures for assessing the effect of naloxone began. The design involved giving the carrier of naloxone (physiological saline) on first day, naloxone (10 mg/kg, subcutaneously, 20 min before opportunity to take alcoholic beverage) on second day and then carrier again on third day while the daily regimen continued.

A few days subsequent to the assessment for naloxone's effects, the rats were allowed access to alcoholic beverage and water for 24 h/day for 16 days.

### *6.2. Results*

Results are summarized in the accompanying figures. The results are similar to those of Experiment 1. Before injections, females developed rather large daily intakes of alcoholic beverage, hence large intakes of ethanol. With injections of EV, body weights were reduced (Fig. 4a). The placebo-treated rats, although gradually decreasing their intakes, maintained high levels of intakes of ethanol. The rats of both doses of EV initially dramatically reduced intakes of alcoholic beverage. Subsequently, intakes of EV-treated rats increased eventually matching their previous intakes (Fig. 4b).

An AVOVA of the data of Fig. 4a (bodyweights) indicates that the main effect of groups was not a reliable source of variance ($P=.25$). In general, the rats grew. Consequently, the variable of blocks was a reliable source of variance ($P<.0000001$). From the prospective of previous analyses, the variable of interest is the interaction term between groups

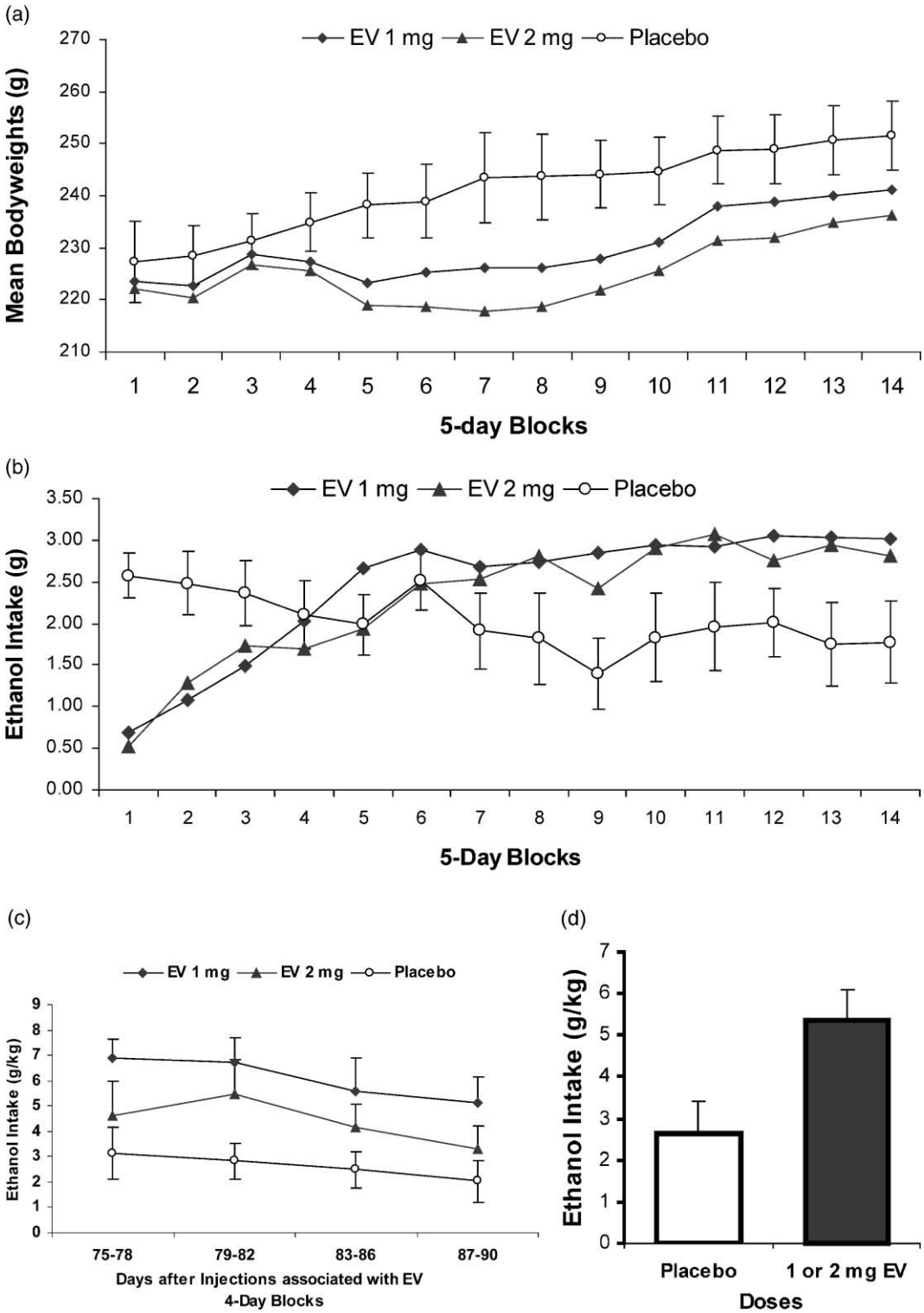


Fig. 4. (a) From the time of EV and placebo injections to a change in procedures, there was a span of 70 days. Bodyweights of the rats during that 70-day period are depicted here as mean weights across each 5-day block. The bars of the data points of placebo controls are standard errors of the mean. Notice the loss of weight from Blocks 3 and 4 to Block 5 and the prolonged period of little or no gain in weight from Blocks 5 to 10. (b) The figure presents mean daily intakes (averaged across 5 days within a block). The data summarize intakes for 70 days following either EV or placebo injections. (c) Mean daily intakes of ethanol (in terms of g/kg) are presented across 4-day blocks for the 16 days that the subjects had access to alcoholic beverage and water 24 h a day. The bars are standard errors of the means. (d) This figure is a summary of the data of (c). The value labeled placebo is the mean daily intake of rats receiving placebo injections across the 16 days of opportunity to take alcoholic beverage. The other value is the mean for all subjects that received a dose of EV. The bars are standard errors of the mean for the data points.

and blocks, which yields an $F(26,221) = 2.34$, $P = .0005$. As before, EV seems to induce a transitory weight loss followed by recovery (groups are not reliably different at Block 3 (16–20 days after injections). Subsequent to this recovery, there is again loss of weight and a period of little or no gain. Mean weight on Day 16 was the peak weight for the two EV groups across the first month after injections. Mean weights on Day 16 for placebo-treated subjects = 231 g compared to rats treated with 1 or 2 mg of EV = 229 and 226 g, respectively. Mean weights 10 days later for placebo-treated subjects was 238 g (a 3% gain over the 10 days). Mean weights for rats treated with EV = 224 and 219 g for EV 1 and EV 2 mg, respectively (a 2% and 3% loss over the 10 days). The reductions according to *t* tests for within subject analyses indicate that these reductions are reliable ($P < .03$).

The intakes of alcoholic beverage for the 70 days following injections are summarized in Fig. 4b. EV 1 or 2 mg a rat clearly reduced intakes for the first 10 days after injections (all *P* values comparing an EV score to the comparable placebo control score $< .05$). Further, the reduction occurred with every subject. Mean intakes of EV-treated subjects are larger than the placebo controls from Blocks 8 through 14 (40–70 days after EV injections). The interaction term of the ANOVA reflects the changes in intakes of the EV-treated subjects, $F(26.221) = 4.08$, $P < .0000001$. Further analyses do not provide support for the conclusion that adaptation to EV leads to greater intakes. The *P* values for comparing 1 mg of EV to placebo for Blocks 13 and 14 are .03 and .08, respectively.

Table 3 summarizes data associated with assaying the effects of naloxone. All subjects' intakes under the influence of naloxone were less than those under placebos which, of course, indicates statistical significance (a paired *t* test yields $P < .00005$). For the placebo controls, a reduction in intakes, due to this dose of naloxone, was certainly expected based on previous work. Naloxone also reduced intakes of EV-treated rats.

The rats that previously received EV took more ethanol under placebos associated with naloxone injections than placebo controls. This difference makes it difficult to determine if naloxone had a greater effect on EV-treated rats because the meaning of, say, a 50% reduction may be very different for a small intake compared to a large intake.

Table 3
Naloxone's effects on intake

| Group | *n* | Placebo | Naloxone | *P* value | Nal/Placebo |
|---|---|---|---|---|---|
| Placebo | 6 | 1.89 | 1.04 | .015 | 0.55 |
| EV 1 mg | 7 | 3.03 | 1.58 | .01 | 0.52 |
| EV 2 mg | 7 | 2.72 | 0.92 | .007 | 0.34 |
| EV 1 mg + 2 mg | 14 | 2.88 | 1.25 | .0003 | 0.43 |

Values for placebo and naloxone are gram per kilogram of ethanol. Placebo refers to mean intakes under the influence of placebos given before and after a naloxone injection. Naloxone (Nal) refers to intakes obtained under the influence of naloxone (10 mg/kg). The *P* values are from paired *t* tests for subjects' placebo scores compared to those under the influence of naloxone. The last column is the ratio of naloxone scores to the placebo scores.

Nevertheless, an index of the extent of the reduction is presented in the table. As can be observed, the extent of the reduction in intakes for all groups, and for a grouping of all rats receiving EV, is substantial.

The data of 24-h access to alcoholic beverage and water are presented in Fig. 4c. Fig. 4d is the mean of all daily scores for placebo controls and all rats that received EV and is a summary of the data of Fig. 4c. Notice that, in general, these females take large amounts of ethanol daily and that the females given EV take very large amounts. Statistical analyses provide no support for the conclusion that scores of EV 2 mg are reliably different from those of placebo controls. The *P* values for comparison of each of the scores of EV 1 mg to those of placebo controls for the four data points (Days 75–78, 79–82, 83–86 and 87–90) of Fig. 4c are .01, .005, .08 and .03, respectively. The *P* value associated with the difference depicted in Fig. 4d is .04.

### 6.3. Discussion

The placebo controls showed gradual reduction in intakes of ethanol across the period of assays for EV's effects (Fig. 4b and c). Notice, however, that these rats began by taking large amounts during 2 h. Despite this trend toward lower intakes, the placebo controls maintained large intakes.

The changes in bodyweights are similar to what was observed previously (Fig. 2a). EV induces an initial reduction in bodyweight, a recovery of bodyweight, followed by another period of weight loss. Subsequently, the subjects of EV gain little or no weight for a period of weeks before they gain weight at the rate of placebo controls. It appears as if the subjects lose weight as they adapt to the release of estradiol and again lose weight as they adapt to the end of the release. Throughout, the subjects' general appearance is not noticeably different from placebo controls.

The small numbers of subjects of this experiment limit its value. Although the trends all support the idea that adaptations associated with pharmacological doses of estradiol can set the conditions for an enhanced appetite for an alcoholic beverage, these results and those of Experiments 2 and 3 do not produce unequivocal support for that idea. Despite the limitations, some strong conclusions can be drawn. The dose of 1 mg EV per rat seems sufficient to induce the enhanced intake of alcoholic beverage. Once the effect of enhanced intake of alcoholic beverage is manifest, the effect seems to persist for many days. The enhanced intake of EV-treated rats is sensitive to opioid antagonism.

## 7. Experiment 5: EV given to male rats

A number of male rats were available who had an extensive history of daily intake of alcoholic beverage. To address the question of whether injections of EV would also dramatically reduce their intakes, half of them were injected with EV and half with placebo.

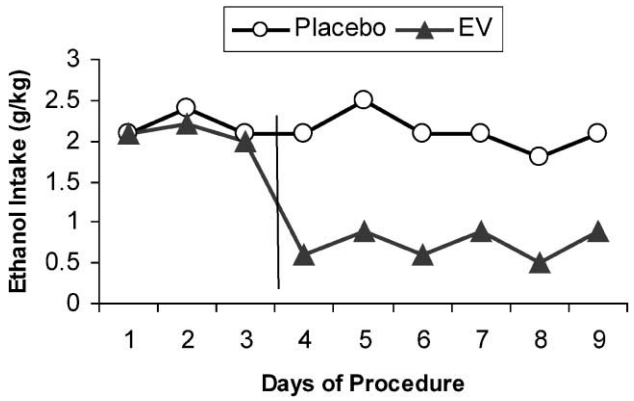


Fig. 5. Mean intakes of ethanol by males are depicted before and after injections. Injections of either placebos or EV occurred the third day of the procedure. The first data point depicting the injections' effects are the fourth day.

### 7.1. Method

The male Sprague–Dawley rats of this experiment had an extensive opportunity to take sweetened 12% alcoholic beverage on the daily regimen involving presenting fluids only 2 h/day. Thirty-two rats ($n = 16$) were maintained on the regimen used in Experiment 1 for 69 days. The scores of the last 3 days of the 69 days were used as a baseline. There were no reliable differences between groups at baseline (mean intakes = 2.2 and 2.1 g/kg, a 2-h period). One group was selected randomly to receive 2 mg of EV, the other placebo. Intakes were measured for 5 days post injections.

### 7.2. Results and discussion

EV reduced intakes of alcoholic beverage (Fig. 5). Daily intake across 5-days post injections: placebo-treated rats' mean = 2.1 g/kg; EV-treated rats' mean = 0.8 g/kg, $F(1,30) = 187.8$, $P < .000001$. EV clearly leads to a reduction in intake of this alcoholic beverage among males. The unanswered question is whether the males adapt to the doses of estradiol and that produces further changes in their alcohol consumption.

## 8. Experiment 6: EV given at various times before opportunity to take an alcoholic beverage

The results of Experiments 1–4 are derived from experiments involving relatively small numbers of subjects observed over many weeks. Conclusions derivable from those experiments are (a) EV initially leads to reduced intakes of alcoholic beverage and (b) adaptations inherent to EV treatment change female rats so that they have an enhanced appetite for palatable alcoholic beverage. This experiment tests those conclusions.

### 8.1. Method

Shortly after arrival at the laboratory, these Sprague–Dawley females began a schedule of injections. During this period, the subjects were individually housed with food and water always available (no alcoholic beverage). All subjects received the same number of injections at the same time: three sets of injections spaced so that the injections were either 3, 15 or 31 days before their first opportunity to take alcoholic beverages. One group received placebos on each occasion. One group received EV, 2 mg, 31 days before the opportunity and placebos on the other occasions. The other two groups received their EV, 2 mg, either 3 or 15 days before and placebos at other times. There were 10 subjects in each group.

In addition to the subjects mentioned, another group of 20 subjects experienced the same regimen of injections as the group receiving injections 15 days before the first opportunity to drink alcoholic beverage. They were treated differently during the period between EV injections and first opportunity to take alcoholic beverage. They were exposed to sweet solutions for 2 h/day. Their intakes of sweet solutions, although interesting, are not summarized here. An ANOVA comparing this group's scores with respect to alcohol intake with those of the other group that received EV 15 days before a regimen of presentation of alcoholic beverage indicated that the groups were not significantly different. Consequently, we treated the two groups receiving EV 15 days before the regimen as a single group.

Three days after the end of the last injections, rats were put on the daily regimen used in Experiment 1 and their intakes measured for 35 days.

### 8.2. Results

These subjects' bodyweights varied nearly the same as those depicted in Fig. 2a and 4a. As with other subjects under this daily regimen, all subjects on all occasions take some water. Their intakes of water varied with their intakes of alcoholic beverage: larger intakes of water when intake of alcoholic beverage was small and small intakes when alcoholic beverage was large.

The results, in terms of mean intakes across 5-day blocks, are presented in the accompanying figure. As can be seen from the figure, and the accompanying analyses, the timing of injections made a significant difference.

The placebo controls behaved, in general, as expected. They rapidly stabilized their intakes at about 2.0–2.5 g/kg per session and took about the same amount for 35 days. Notice, however, that at Block 6 and 7 they took over 2.5 g/kg, larger amounts than we usually see with unselected males on the same daily regimen.

The rats given EV 3 days before their first opportunity to drink did not consume significant amounts of alcoholic beverage during the initial 10 days. Subsequently, they

gradually increased their intakes until they were taking about the same as the placebo controls.

The rats given EV 15 and 31 days before their first opportunity to drink did not take significantly more alcoholic beverage during the 1st 10 days. Subsequently, the rats given EV 2 weeks before the sessions took reliably more ethanol than placebo controls: for Blocks 3–7, $P < .02$.

The rats given EV 31 days before drinking sessions took reliably more ethanol at Blocks 3, 4 and 5, but not at other blocks. The comparison of scores of placebo and EV 31 days before at Block 7 yields $P = .12$ (Fig. 6).

### 8.3. Discussion

These data confirm results from the previous experiments. Female rats given only placebos readily develop moderate to large levels of intake of alcoholic beverage, hence ethanol. The placebo controls of this experiment took less alcoholic beverage initially than those of Experiment 4 and sustained that level of intake throughout the observations. With subjects of both experiments, mean intakes across 2 h of opportunity to drink lead to substantial intakes of ethanol. Once again, given the relatively large intakes of placebo controls, increases above that of placebo controls are notable.

These data confirm the results from the previous experiments: While the injection of EV is providing pharmacological doses of estradiol, intakes of sweetened alcoholic beverage are decreased. After subjects have adapted to prolonged release of estradiol, the termination of release and experienced the effects of alcohol, their intakes of alcoholic beverage are enhanced.

The variability of placebo controls daily intake of alcohol and the variability of effects of EV (e.g., the variability

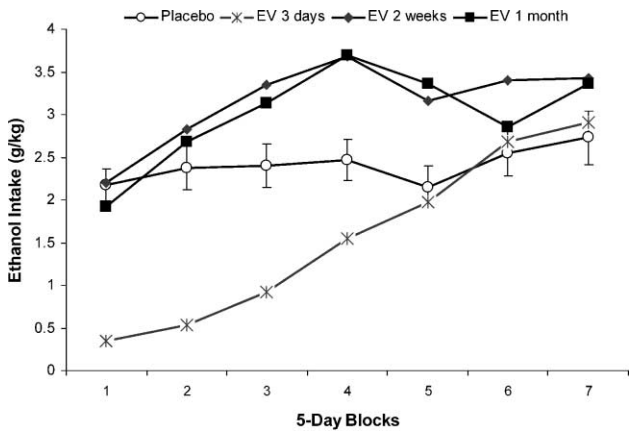


Fig. 6. The rats of placebo received carrier of EV when the other rats received EV. EV injections were either 3, 15 or 31 days before the opportunity to take alcoholic beverage. The values are means for each group across 5-day blocks. An ANOVA of the scores of this figure yields: for the effect of Groups, an $F(3,56) = 18.4$, $P < .0000001$; for the effect of Blocks, $F(6,336) = 20.7$, $P < .0000001$; for the interaction, $F(18,336) = 4.0$, $P = 0.0000001$.

associated with different levels of β-endorphin associated with the same injection of EV, Fig. 3d) and related statistics all indicate that statistically significant results will uniformly emerge from experiments with slightly larger numbers of subjects than used in these experiments. A test of reliability of the major observations is complete, however, on the basis that similar results are obtained from a number of different experiments.

These data, plus those of the previous experiments, support the conclusion that adaptation to EV and opportunities to drink alcoholic beverage can produce a female rat that has an enduring propensity to take larger amounts of alcoholic beverage than rats that have not received EV.

## 9. General discussion

These data confirm what has been known for a very long time: For rats, palatability of an alcoholic beverage is a major determinant of how much ethanol will be taken during a measurement period. When concentration of ethanol is held constant (e.g., as here, 12%) and concentration of a sweetener (e.g., saccharin) is varied, intakes of ethanol change with the palatability of the beverage. Palatability can be enhanced by adding sweeteners or the flavor of beer (Lancaster and Spiegel, 1992). After some minimal masking of the apparently harsh taste of ethanol, however, intakes of alcoholic beverages are influenced more by other factors.

Another important determinant of how much, on average, rats will drink is the number of consecutive daily opportunities they have had to choose to drink alcoholic beverages. When first presented a palatable alcoholic beverage, rats take little ethanol. But after some daily opportunities, they take sufficient amounts to produce behavioral signs of toxicity. After some minimal number of days of opportunity (usually in less than 3 weeks), however, rats' intakes stabilize. With stabilization of intakes, and without further manipulation, average daily intakes of ethanol will remain constant for months.

After average daily intakes stabilize, individual rats' intakes do vary some across sessions. This within-subject variability, however, is usually minimal under the standard, rather constant, laboratory conditions. For a group of unselected laboratory rats, there is considerable among-subjects variability with some rats taking considerable amounts of beverage (hence, ethanol) and some taking very little. It is, of course, this among-subject variability that is of great interest to those refining theories of AAA.

The just stated generalizations were derived following observations of males (for reviews, see Reid, 1996; Hubbell and Reid, 1990; Reid and Hubbell, 1992). These experiments involved over a hundred females observed under a variety of conditions. The observations confirm what others (e.g., Ford et al., 2000) have observed: Female rats' intakes of alcoholic beverages, although showing some interesting differences, are not markedly different than males'. There is

within-subject variability, but not as much as one might infer from knowing that females sexual cycle is 3.5–5 days long. Roberts et al. (1998) had to hormonally synchronize female rats' cycles to see reliable effects of phases of the cycle on operant responding for an alcoholic beverage. With free-cycling females, no differences across days were apparent. These Sprague–Dawley females took more daily ethanol, under comparable conditions, than we have come to expect of males. The females' greater intakes correspond to what others have observed (e.g., Lancaster and Spiegel, 1992; Lancaster et al., 1996; Li and Lemung, 1984). The greater intakes were particularly apparent shortly after initial presentations of the sweetened alcoholic beverage. Despite some moderation of intake with continued opportunity to drink alcoholic beverages, the females sustained high levels of intake across many days.

There is apparently nothing inherent to being female that is a deterrent to sustaining high levels of intake of alcoholic beverages. The differences seen between women and men's rates of AAA, therefore, are likely to vary according to social norms. It follows, if societies sanction women and men's drinking equally, nearly equal rates of AAA will develop. The data with rats, and some data with people (e.g., Harper and Krill, 1990), in fact, lead to the warning that with equal social sanctions with respect to drinking, women may incur more problems than men.

The hypothesis of equal potential for AAA to develop for men and women holds, of course, provided no extraordinary social or biological factors are extant. The data presented here, and elsewhere (Ford et al., 2000; Marinelli and Gianoulakis, 2000; Marinelli et al., 2001), indicate estrogenic factors may be extraordinary events modifying an individual's propensity to take alcoholic beverages.

Large doses of estradiol, by way of EV or EB, reliably (a) reduce bodyweights and (b) reduce intakes of alcoholic beverages that are usually taken in moderate to large amounts (these experiments; Ford et al., 2000; Sandberg and Stewart, 1982; Sandberg et al., 1982). The reductions are seen in both males and females. The reductions occur with both females that are ovariectomized (Sandberg and Stewart, 1982; Sandberg et al., 1982) and those with intact ovaries (these experiments). The possibility for enhanced intakes of alcoholic beverages seen post pharmacological doses of estradiol have only been assessed with intact females.

There are marked changes in bodyweights of EV-treated rats in comparison to the rather steady increases in bodyweights of placebo controls. The initial reductions seen shortly after EV injections are temporary, even though estradiol is being delivered in pharmacological doses, and the rats regain a considerable proportion of this lost weight. When the EV injection is no longer delivering estradiol in sustained amounts, there is a further reduction of bodyweight and a prolonged period of little or no weight gain. These dynamic changes in bodyweights occur among rats with and without opportunity to take alcoholic beverages (Fig. 2a and 4a). These changes in bodyweights are a correlate of adaptations that eventually manifest themselves as an enhanced propensity to take ethanol.

The reduction in intake of ethanol, seen shortly after initiation of regimens of estradiol, could be a function of a wide variety of variables indirectly affecting appetite for alcoholic beverages rather than a more salient modification of appetite for alcoholic beverages. The rats, for examples, may merely be ill, suffering a malaise, or have liver or other organ modifications affecting pharmacokinetics of ethanol. These possibilities were checked by Sandberg and Stewart (1982) and Sandberg et al. (1982). They concluded that estradiol did not affect appetite for ethanol by producing a malaise or by affecting pharmacokinetics. They concluded that the estradiol-reduced intake of ethanol was a subset of more general effects on appetite for other ingesta. Sanchis-Segura et al. (2000) asked whether EV modified the pharmacokinetics of ethanol in mice and came to the conclusion that any effects EV might have were small and inconsequential.

Experimental manipulations leading to reductions in intake of alcoholic beverages are common. Aside from certain experimental manipulations, such as using a flavored alcoholic beverage, manipulations leading to changes in the rat which, in turn, increase intakes are rare. A manipulation that persistently sustains an increase in intake, while that manipulation is continuing, is even more uncommon. Daily administrations of small doses of morphine are an example of such a manipulation (Hubbell et al., 1986). When morphine is given daily, rats take more alcoholic beverage than controls. With termination of injections of morphine, however, average daily intakes of ethanol return to baseline levels. An experimental manipulation that has an enduring effect, extending beyond the duration of the initial manipulation, and one that apparently changes the state of the subject so that it has an apparent increase in appetite for alcoholic beverages is singular. Adaptation to pharmacological doses of estradiol produces changes in female rats which are manifest by increased intake and that manifestation seems to persist for months (these experiments; Reid et al., 2001).

EV itself does not produce increases in intake of alcoholic beverages. It is adaptation to EV that induces the increase. Further, the induction, of a state manifest as increased intakes, is not specific to a strain of rats. It has been shown in four strains: Lewis, Long–Evans, Sprague–Dawley and Wistar strains (these experiments; Ford and Samson, 2001; Marinelli and Gianoulakis, 2000; Marinelli et al., 2001). That induction is not specific to a particular flavor of alcoholic beverage having been seen with saccharin sweetened beverages using a wide range of concentrations of saccharin and having been seen with alcoholic beverages with no flavoring other than that of ethanol and water (these experiments; Ford et al., 2000; Marinelli and Gianoulakis, 2000; Marinelli et al., 2001). The greater intakes are seen with experimental procedures involving limited daily access to an alcoholic beverage as

well as continuous access (e.g., these experiments). The propensity to take more alcoholic beverage is manifest months after the initial single injection of EV and is sustained, once observed, for months (these experiments; Reid et al., 2001).

Although it is reasonable to conclude that adaptations to EV treatment can enhance propensity to take ethanol, there is still considerable work to do to specify the optimal conditions for producing enhanced intakes. The optimal time after the beginning of EV treatment to initially expose subjects to alcoholic beverage may not be, for example, 2 months after EV injections, but rather 2 weeks (Experiments 2 and 6) The optimal dose, for example, may not be 2 mg of EV a rat, but rather 1 mg or less (Experiment 4). Further, a serious limitation on the conclusions to be drawn from this study is the absence of a more complete study of doses and responses. Relatedly, we did not measure blood estradiol levels which would have added considerably to the value of the study. The optimal beverage to present to the rats may not be 0.25% saccharin-flavored 12% ethanol solution, but rather a 0.05% saccharin-flavored 12% ethanol solution (Experiment 3). Procedures limiting access to alcoholic beverage may not be optimal and unlimited opportunity may allow the effect to emerge more consistently (Experiment 2–4). In addition, as pointed out above, the variability in intakes across subjects and within a subjects' scores lead to the suggestion that a larger number of subjects per group is warranted.

The conclusion that pharmacological doses of estradiol can enhance appetite for alcoholic beverages makes other issues come into focus. The combination of EV treatment and presentation of a sweetened alcoholic beverage for 24 h/day (Experiments 3 and 4) can lead to extraordinarily high intakes of ethanol that are sustained for a number of days. We (Reid et al., 2001) have subsequently replicated that finding. The implications of these findings are particularly problematic, because they may be particularly relevant to conditions and circumstances of many women. Women of many societies are being treated with estrogens and have plenty of opportunities to take flavored alcoholic beverages.

Although these experiments are among the first studies to show that treatment with EV can induce a robust, persisting appetite for alcoholic beverages, they are not the first to show a correlation between estrogen levels and alcohol consumption. Muti et al. (1998), for example, showed that women with high serum estradiol levels drank, as indicated by personal report, about 93 g of ethanol a week in comparison to women with low estradiol who drank about 32 g of ethanol a week.

A condition that afflicts a relatively large number of women is polycystic ovarian syndrome (PCOS). Hyperestrogenism prevails in PCOS (Lobo, 1988). A question that emerges from this research and the findings of Muti et al. (1998) is whether the estrogen status of women of PCOS mimics the hyperestrogenism that initially follows the injection of EV or whether their status is more like the state that emerges from adaptation to pharmacological doses of estrogen.

Yin et al. (2000) have implicated estrogen in early alcohol-induced liver injury. This leads us to speculate that nearly daily, moderate intakes of alcohol could lead to liver injury producing estrogen imbalances which, in turn, lead to increased appetite for alcoholic beverages which, in turn, lead to further estrogen imbalances that increase intake of alcohol. The usual generalization is that excessive intake of ethanol leads to high levels of estrogen (a symptom of AAA) without taking into account the possibility that estrogen imbalances themselves may be a salient event in the development of an excessive appetite for alcoholic beverages. This condition might also be germane to males who show high levels of estrogens with processing of ethanol (Gordon et al., 1979).

These data, although strongly suggestive, do not fully confirm that the state created by adaptation to EV treatment reflects an enhanced appetite for alcoholic beverages and, relatedly, whether the state is characterized by a motivational state that might be termed propensity toward AAA. The finding, however, that we can induce enhanced intake of alcoholic beverage has both theoretical and practical implications. In summary, it seems that we can induce, at will, a propensity for enhanced intakes of alcoholic beverages: Inject 1 or 2 mg of EV to a female rat, wait a few weeks and, then, provide an opportunity to take alcoholic beverage.

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