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EtOH Disrupts Female Mammalian Puberty

Age and Opiate Dependence

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The major drug of abuse among teenagers in the United States continues to be ethanol (EtOH), but use is seen in children as young as nine. In the studies reported here, the impact of EtOH on biologic and hormonal parameters of puberty was assessed in female rats. Rats were fed a liquid diet containing EtOH, pair fed an identical liquid diet containing dextrimaltose instead of EtOH, or fed a liquid diet not containing EtOH ad libitum. Feeding was started at 21, 25, or 28 d of age. EtOH markedly delayed the age at vaginal opening (34.5 ± 0.5 d in controls vs 48.5 ± 2.4 d in EtOH animals; $p < 0.001$), delayed the age at first estrous (40.9 ± 0.6 d in controls vs 61.2 ± 2.6 d in EtOH animals; $p < 0.001$), increased the length of the estrous cycle, and decreased the number of proestrous days. EtOH, concomitant with reduced ovarian and uterine weight, decreased serum estradiol and progesterone. Associated with these changes in ovarian hormones there was a selective increase in follicle-stimulating hormone, but not luteinizing hormone. EtOH consistently reduced insulin-like growth factor-1. In general, EtOH-induced disruption was more severe the younger the animals were at the start of feeding. Opiate receptor blockade with naltrexone completely prevented the EtOH-induced delay in vaginal opening. The impact of EtOH on female puberty is dramatic, is an emerging public health problem, and deserves more study.

Key Words: Ethanol; puberty; female rat; opiates.

Introduction

Adolescence may be defined as the period within the lifespan when most of a person's biologic, cognitive, psychologic, and social characteristics are changing from those of a child to those of an adult *(1)*. Confronting today's adolescent in the United States are contemporary sets of problems and risks with profound implications for normal development and long-range functioning of the individual. A major problem is substance abuse, with ethanol (EtOH) being the primary drug of abuse in the United States among teenagers *(2–7)*. In 1999, 52% of eighth graders and 89% of high school seniors reported using EtOH, with 3.7% of high school students reporting daily drinking *(8)*. Approximately 4 of 10 high school students reported that they were binge drinkers, defined as having consumed five or more drinks on only one occasion *(9)*. The average age when 12- to 17-yr-olds state they first used EtOH is 13.1, but use is increasingly seen in children as young as 9 *(2,9)*.

The idea that EtOH disrupts mammalian female puberty is well established; however, the mechanisms of this perturbation have not been extensively investigated *(10–13)*. The epidemic of EtOH drinking in youth provides the rationale for this study. The very disturbing reports of drinking in the preteen, prepubertal years prompted us to examine whether or not there were differences in pubertal and reproductive parameters depending on the age at onset of EtOH ingestion. Finally, since little is known about the mechanism of EtOH disruption of puberty, we investigated the possibility that EtOH's disruptive effect was mediated through opiatergic pathways. The reason for choosing this particular mechanism are the data that (1) implicate changing brain opiatergic tone in the pubertal process *(14–16)* and (2) that show that EtOH has an important effect on brain opiates *(17)*.

Results

As has been our past experience, the EtOH-fed animals weighed less at sacrifice than their pair-fed mates, despite receiving the same number of calories as assured in the pair-feeding paradigm (Table 1). However, the EtOH group did gain weight, was well groomed and playful, and appeared healthy. The weights of the ad libitum group were not significantly different from the pair-fed group.

Administration of EtOH dramatically delayed the age of vaginal opening (VO) from 34.5 ± 0.5 d of age in control animals to 48.5 ± 2.4 d of age in the EtOH-fed group ($p < 0.001$), as shown in Fig.1A. The length of delay of VO

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Table 1
Biological Parameters

	Total Group		21 Days Old[a]		25 Days Old[a]		28 Days Old[a]	
	Pair Fed	EtOH	Pair Fed	EtOH	Pair Fed	EtOH	Pair Fed	EtOH
Number of Rats	40	40	15	15	7	7	18	18
Weight (Grams)	204 ± 4.7	165 ± 4.3[c]	205 ± 3.6	154 ± 3.2[c]	148 ± 3.5	135 ± 7.9	224 ± 2.5	187 ± 4.4[c]
Normal Cyclicity (%)	97.5	75.0[b]	100	60.0[c]	100	71.4	94.4	88.8

[a]Age at beginning of feeding paradigm. Rats were fed for 2 mo except for the 25-d-old group, which was fed for 1 mo.
[b]$p < 0.05$, pair fed versus EtOH.
[b]$p < 0.01$, pair fed versus EtOH.
[c]$p < 0.001$, pair fed versus EtOH.

in the animals started at 21 d of age and was significantly longer than the delay in either the 25-d old ($p < 0.01$) or the 28-d-old rats ($p < 0.001$). The delay in VO of the 25-d-old animals was, in turn, greater than that in the 28-d-old rats ($p < 0.01$). The animals fed ad libitum had VO by 32.2 ± 0.4 d of age, slightly but significantly earlier than the pair-fed control group ($p < 0.01$) and much earlier than the EtOH-fed group ($p < 0.001$).

First estrous in the pair-fed control group, as a whole, was 40.9 ± 0.6 d (Fig. 1B). This was delayed by about 20 d to 61.2 ± 2.6 d of age by EtOH. The delay to first estrous in EtOH-fed compared with pair-fed animals was significantly greater in animals started at 21 d than in either 25 d ($p < 0.02$) or 28 d ($p < 0.001$). The interval in the 25-d-old animals was, similarly, greater than in the 28-d-old animals ($p < 0.01$). In each case, rats fed ad libitum had first estrous at a time not significantly different than the pair-fed controls, averaging 38.3 ± 0.5 d.

The delay of first estrous was not simply a function of delay in VO. The length of time between VO and first estrous was 6.4 ± 0.3 d in pair-fed animals and was significantly prolonged to 12.7 ± 1.1 d by EtOH ($p < 0.001$) in the entire group. This difference was approximately the same in each of the three age groups and was not significantly different in the pair-fed compared with the ad libitum–fed rats.

In the experiment as a whole, EtOH caused a marked disruption of estrous cyclicity. EtOH caused 25% (10 of 40) of the rats to be completely anestrous in contrast to only 2.5% (1 of 40) in the pair-fed control group ($p < 0.01$). Virtually all of this was owing to disturbance in the 21-d old rats since EtOH did not alter cyclicity in a statistically significant manner when EtOH was started at the older ages (Table 1). None of the animals fed ad libitum were anestrous in any of the three studies.

Another measure of EtOH's troublesome effect was seen in length of estrous cycle (Fig. 2A). EtOH prolonged the average cycle from 5.3 ± 0.1 d in the pair-fed control to 11.0 ± 1.1 d ($p < 0.001$). During the experimental period, the number of proestrous days was reduced from 11.3 ± 0.3 d per animal in the pair-fed group to 5.0 ± 0.6 d per animal with EtOH ($p < 0.001$). Again, this effect on cycle length and proestrous days was more dramatic when feeding was started at a younger age, as illustrated in Fig. 2. The animals fed ad libitum had an average number of proestrous days of 10.7 ± 0.5, not significantly different from the pair-fed controls.

EtOH had substantial effects on serum ovarian hormone levels as well. Overall, as shown in Fig. 3A, there was a significant reduction in serum estradiol from 27.5 ± 2.6 pg/mL in pair-fed to 19.6 ± 1.6 pg/mL in EtOH-fed rats ($p < 0.01$). Data from animals started at 25 d of age and fed for 1 mo were not used in the calculation, because there is a time-dependent effect of EtOH on estradiol (discussed subsequently). Younger animals were more seriously affected (Fig. 3A). In the 21-d old rats, EtOH reduced estradiol from 27.7 ± 4.4 to 16.2 ± 2.7 pg/mL ($p < 0.05$). By contrast, in the 28-d old animals, EtOH did not cause any significant decline in estradiol. The 25-d-old animals, which were fed EtOH for a shorter period of time than the other animals (1 mo in contrast to 2 mo), actually demonstrated that the shorter period of feeding led to an almost significant increase in estradiol ($p = 0.08$), consistent with our earlier demonstration of time-related differences in response to EtOH in adult female rats *(10)*. Estradiol levels in the animals fed ad libitum were not significantly different from pair-fed controls.

EtOH sharply reduced progesterone in the total group of animals from 49.2 ± 5.7 to 22.9 ± 4.0 ng/mL (Fig. 3B; $p < 0.001$). The EtOH-induced decline was greater in the younger (21- and 25-d-old) rats, averaging approx 40 ng/mL, than in the oldest rats, in which progesterone decreased about 16 ng/mL. The 25-d-old group, fed for only 1 mo, also demonstrated an EtOH-induced fall in progesterone, from 54.1 ± 10 to 12.5 ± 6.0 ng/mL ($p < 0.05$; data not shown). Progesterone levels in animals fed ad libitum were similar to those seen in pair-fed controls.

Reflecting the decreased hormonal function, EtOH decreased ovarian weight corrected for body weight significantly in the group as a whole (Table 2), but more profoundly in younger rats: a 37% decrease in the 21-d-old rats compared with an 8% decrease in the 28-d-old rats. Although fed for only 1 mo, the ovarian/body weight also fell signifi-

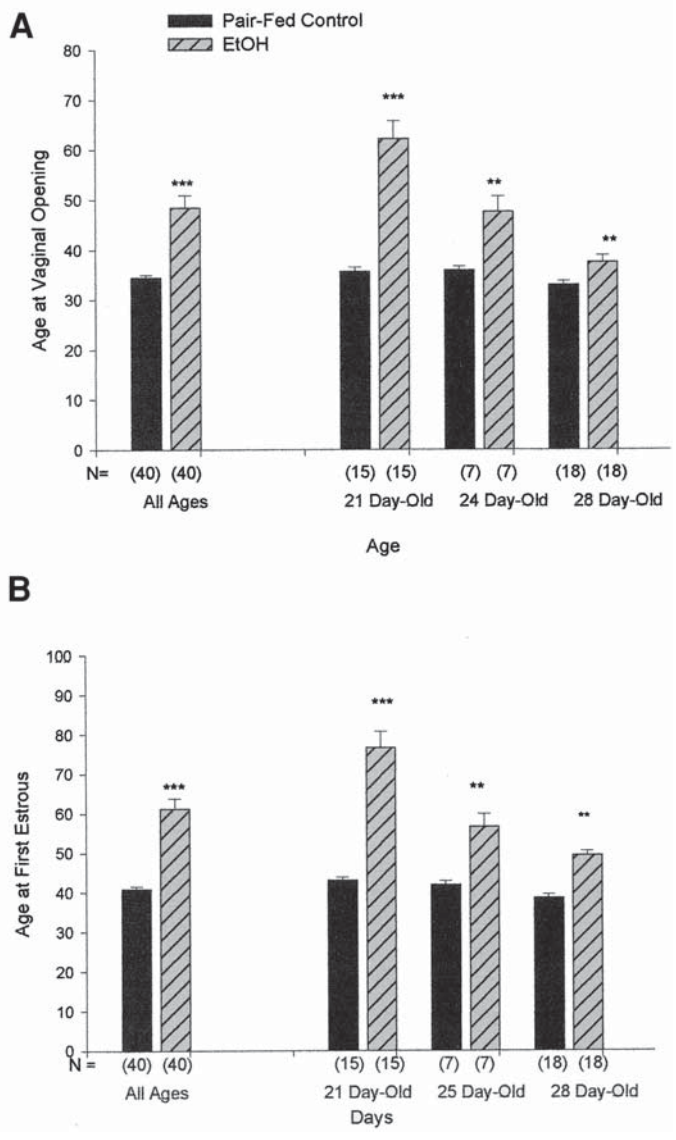

Fig. 1. Effect of EtOH feeding on age at VO (**A**) and age at first estrous (**B**). The first pair of bars on the left depicts data from all the experiments. The other age designations indicate the age at which the experimental paradigm was started. The animals were EtOH fed or pair fed for 2 mo except for the 25-d-old group, which was fed for 1 mo. $^{**}p < 0.01$; $^{***}p < 0.001$.

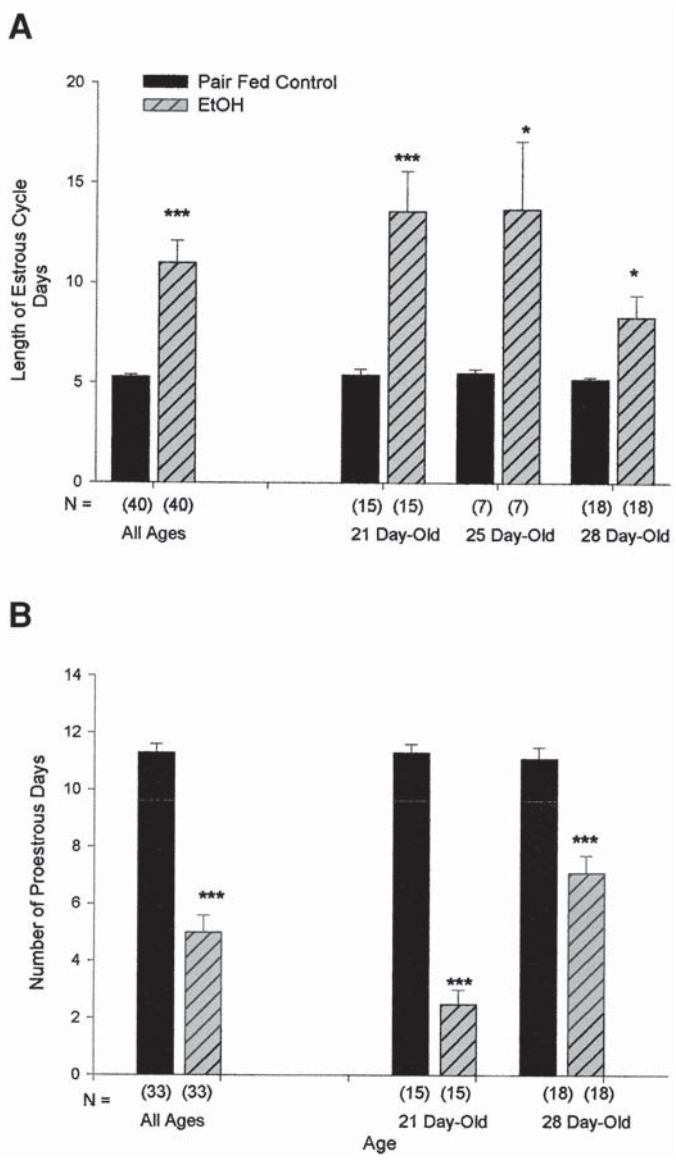

Fig. 2. Effect of EtOH feeding on length of estrous cycle (**A**) and number of proestrous days (**B**). The first pair of bars on the left depicts data from all the experiments. The other age designations indicate the age at which the experimental paradigm was started. The animals were EtOH fed or pair fed for 2 mo except for the 25-d-old group, which was fed for 1 mo. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

cantly after EtOH by 39% in the 25-d-old rats ($p < 0.01$; data not shown). Ovarian/body weight in rats fed ad libitum was almost identical to that of pair-fed controls.

The uterine weight/body weight also fell with EtOH, concomitant with decreased estradiol and progesterone stimulation (Table 2). However, the decline reached statistical significance only in the 21-d-old rats. In all cases, weight ratios in the rats fed ad libitum were highly comparable with those of pair-fed controls.

In the group as a whole, as well as in the individual age subgroups, luteinizing hormone (LH) tended to fall in EtOH-exposed animals, but this decrement never reached statistical significance (see Table 3). LH concentrations in animals fed ad libitum were not significantly different from those of pair-fed controls. In sharp contrast to LH, EtOH feeding produced a large, almost threefold increase in follicle-stimulating hormone (FSH) ($p < 0.01$). Although the FSH was higher in EtOH-fed rats at each of the three ages, the difference was statistically significant only in the oldest rats (28-d-old animals). The FSH concentration in the animals fed ad libitum was not significantly different from that of pair-fed control rats.

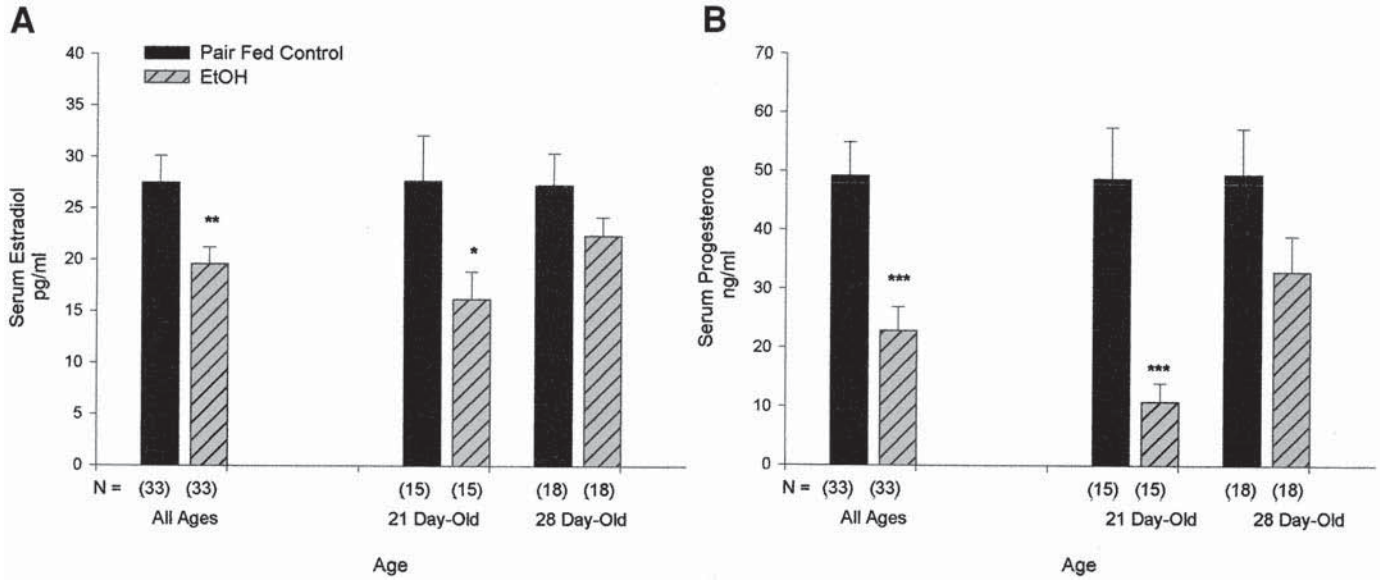

Fig. 3. Effect of EtOH feeding on serum estradiol (**A**) and progesterone (**B**). The first pair of bars on the left depicts data from all the experiments. The other age designations indicate the age at which the experimental paradigm was started. The animals were EtOH fed or pair fed for 2 mo. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Table 2
Reproductive Tract Parameters

	Total Group		21-Day Old[a]		28-Day Old[a]	
	Pair Fed	EtOH	Pair Fed	EtOH	Pair Fed	EtOH
Number of Rats	33	33	15	15	18	18
Ovarian/Body Wt. (mg/gm)	0.60 ± 0.03	0.46 ± 0.02[d]	0.68 ± 0.06	0.43 ± 0.03[d]	0.53 ± 0.02	0.49 ± 0.01
Uterine/Body Wt. (mg/gm)	3.8 ± 0.2	2.8 ± 0.2[c]	4.0 ± 0.4	2.2 ± 0.3[d]	3.5 ± 0.2	3.4 ± 0.2

[a]Age at beginning of feeding paradigm. Rats were fed for 2 mo.
[b]$p < 0.05$, pair fed versus EtOH.
[c]$p < 0.01$, pair fed versus EtOH.
[d]$p < 0.001$, pair fed versus EtOH.

Table 3
Hormonal Parameters: Neuroendocrine Hormones & IGF-1

Hormonal parameters	Total Group		21-d Old		25-d Old		28-d Old	
	Pair Fed	EtOH	Pair Fed	EtOH	Pair Fed	EtOH	Pair Fed	EtOH
Number of Rats	40	40	15	15	7	7	18	18
LH Ng/mL	14.9 ± 2.6	11.0 ± 2.7	9.68 ± 3.0	4.22 ± 2.6	20.2 ± 7.2	10.7 ± 7.0	17.2 ± 4.2	16.7 ± 4.4
FSH Ng/mL	7.7 ± 0.9	20.3 ± 3.6[c]	5.5 ± 0.7	16.7 ± 6.0	12.4 ± 3.6	30.3 ± 16.7	8.0 ± 1.0	19.5 ± 3.5[c]
IGF-1 Ng/mL	1239 ± 49	799 ± 32[d]	1126 ± 36	722 ± 31[d]	1753 ± 131	1048 ± 80[d]	1133 ± 40	765 ± 59[d]

[a]Age at beginning of feeding paradigm. Rats were fed for 2 mo except for the 25-d-old group, which was fed for 1 mo.
[b]$p < 0.05$, pair fed versus EtOH.
[c]$p < 0.01$, pair fed versus EtOH.
[d]$p < 0.001$, pair fed versus EtOH.

Insulin-like growth factor-1 (IGF-1) was EtOH responsive without any age differential. EtOH decreased IGF-1 by 36% in the entire group ($p < 0.001$), and by similar percentages in each of the age subgroups ($p < 0.001$). In most cases, serum IGF-1 concentration in animals fed ad libitum was virtually identical to that of the pair-fed control animals.

The ability of opiate receptor blockade to prevent the deleterious effects of EtOH was tested by implanting rats with naltrexone or vehicle pellets in the EtOH-feeding/pair-feeding regime (Fig. 4). In this experiment, EtOH again delayed VO. Naltrexone itself had no effect on the age at VO since VO was essentially the same in implanted ani-

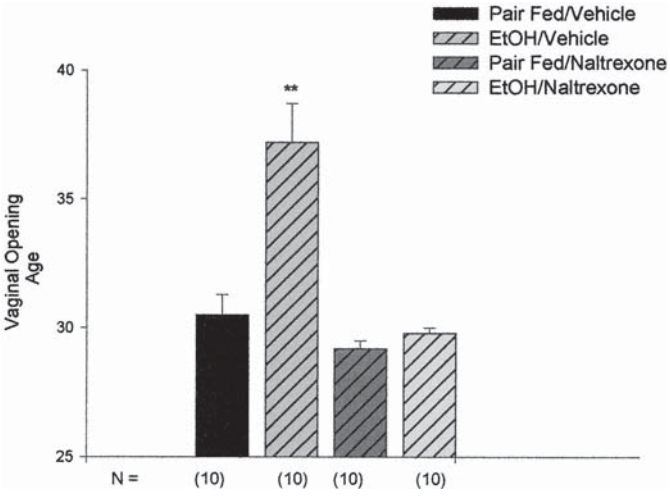

Fig. 4. Effect of opiate receptor blockade on EtOH-induced delay in VO. Animals were 28 d old at the start of the feeding paradigm, which was continued for 2 mo. $^{**}p < 0.01$.

mals pair fed naltrexone and pair fed vehicle pellets. However, naltrexone completely prevented the EtOH effect on VO. It had no effect, however, on other parameters such as estrous cyclicity or other hormonal data except that the EtOH-associated rise in FSH was partially blocked (data not shown).

Discussion

We have confirmed the disruptive effects of EtOH exposure on the onset of female puberty and early estrous cyclicity. The delay in VO and age at first estrous, as well as prolonged cycle length, in the EtOH-fed animals cannot be totally accounted for by attenuated weight gain, since it was also seen in the 25-d-old group. This group, EtOH fed or pair fed for 1 mo (rather than 2 mo, as in all the other aged animals) showed no difference in weight gain among experimental groups. We have shown, for the first time, that the impact of EtOH is more profound if exposure is started earlier. This is highly relevant to the human situation since drinking EtOH is no longer seen first in high school age individuals, but in the preteen years as well (see Introduction). We have also shown, for the first time, that part, though not all, of EtOH's effects are opiate dependent since the EtOH-induced delay in VO, a clear marker of puberty, can be prevented by naltrexone treatment.

Two decades ago, Van Thiel et al. *(10)* showed that prepubertal rats fed EtOH for 7 wk manifested marked ovarian failure (based on structural and functional evaluation) compared with pair-fed controls. Subsequently, Bo et al. *(12)* reported that VO, a well-characterized marker of puberty in the female rat, was delayed by administration of EtOH. In a series of articles, Dees and colleagues *(12,13,18)* defined the neuroendocrine concomitants of these profound biologic alterations. Notably, EtOH caused a rise in the hypothalamic content of luteinizing hormone–releasing hormone (LHRH) coupled with a fall in serum LH *(12,13,18)*. Taken together, these in vivo data suggested that there was an EtOH-induced decrease in hypothalamic LHRH secretion (leading to the increased hypothalamic content) accounting for the fall in LH. Indeed, Hiney and Dees *(19)* demonstrated in vitro that EtOH was able to reduce LHRH secretion from hypothalamic slices from prepubertal female rats.

Our in vivo design does not allow for determination of the locus of EtOH action. Since estradiol and perhaps progesterone as well exert a negative feedback on LH during at least part of the estrous cycle, the EtOH-induced fall in these ovarian hormones would be expected to elicit an increase in LH. However, the lack of rise in LH in the context of decreased estradiol and progesterone supports a hypothalamic and/or pituitary site of action for EtOH, consistent with the discussed findings. This does not, of course, rule out a direct ovarian action, as well.

By contrast, FSH did rise in the EtOH group. This may have been owing to a fall in ovarian inhibin, which exerts a specific inhibitory effect on FSH, but not LH *(20)*. Little is known about the effects of EtOH on inhibin, and it was not measured in our studies. Alternatively, the lower concentrations of estradiol noted in the EtOH-exposed animals could also explain the higher concentrations of FSH, resulting from reduced negative feedback.

We have previously shown that acute and chronic EtOH alters male rat puberty as well, so this is true of both genders *(21,22)*. Additionally, both acute and chronic EtOH alter adult female rat estrous cyclicity *(23,24)*, and adult male reproduction *(25)*; thus, the effect is consistent between genders and at different times of reproductive development.

The more profound effects of EtOH, when given earlier, are rather dramatic. The delay of pubertal onset when EtOH was started at 21 d of age was about 27 d compared with 4.5 d when feeding was started at 28 d. Estrous cyclicity was abnormal in 40% of younger EtOH-fed rats compared with only 11% of the older rats. In younger rats, EtOH increased estrous cycle length by approx 2.5-fold compared to 60% in older rats, reducing the number of proestrous days (and presumably ovulation) by almost 80% in 21-d-old compared with about 40% in 28-d-old rats.

Opiate receptor blockade with naltrexone effectively prevented the EtOH delay in VO. There are three major endogenous opioid peptides (EOPs), products of three separate genes: β-endorphin, derived from the proopiomelanocortin gene; dynorphin, from the prodynorphin gene; and met- and leu-enkephalins, from the proenkephalin A gene *(26)*. β-Endor-phin is the most important in reproduction and is made in the medial basal hypothalamus as well as widely in the brain and in the anterior pituitary. Hypothalamic β-endorphin restrains the secretion of hypothalamic LHRH and thus is inhibitory to the hypothalamic-pituitary-gonadal (HPG) axis. Opiate receptor antagonists, such as naloxone and naltrexone, have been widely used to explore the mechanisms

of opioid inhibition of the HPG axis. In early puberty, administration of naloxone does not change LH levels, indicating that normally during this time there must be little opioid inhibition of the HPG *(14,26)*. However, the situation changes in late puberty, when naloxone does normally evoke an LH response, indicating that opioid inhibition of the HPG axis increases during puberty but is low early on, allowing for or permitting the activation of the HPG axis that is the neuroendocrine hallmark of puberty. A variety of data indicate that opioid inhibition of LHRH release is dependent on the presence of gonadal steroids so that the activation of HPG during puberty leads to increased gonadal steroid levels leading to increased opioid inhibition of LHRH release in a classic negative feedback loop *(15,16)*.

Data mainly from adult animals have shown that EtOH increases opioid activity in the brain *(17)*. If this is true in the pubertal animal as well, it may represent one of the mechanisms by which EtOH disrupts puberty. Two pieces of data are relevant here. First, in a study by Creighton-Taylor and Rudeen *(27)*, the offspring of mothers fed a liquid diet containing EtOH during pregnancy were compared with those offspring whose mothers were either pair fed a diet without EtOH or fed laboratory chow ad libitum. The offspring being studied were exposed to EtOH only *in utero*. It is known that fetal alcohol exposure leads to increased β-endorphin levels and to delayed onset of puberty, assessed by the standard technique of the time of VO. In that particular study, pubertal female rats were injected with naltrexone, an opiate antagonist, over d 26–29 of life. This treatment led to an acceleration of VO in the animals that had been exposed to EtOH *in utero* but not in either of the control groups *(27)*. This suggests that at least part of the EtOH-induced pubertal delay seen after *in utero* exposure is owing to increased opioid tone. In our experiments, female prepubertal animals were given EtOH directly and not via the *in utero* exposure model, but, again, naltrexone was effective. Second, in our own laboratory, in male pubertal rats, naltrexone was able to block the ability of acute or chronic EtOH to reduce serum testosterone *(28,29)*. The mechanism of EtOH disruption of puberty is likely to go beyond opiates since naltrexone did not normalize the time of first estrous or estrous cyclicity. It is also possible that a different dose of naltrexone might have corrected these other abnormalities.

In rats and primates, including humans, IGF-1 increases with the onset of puberty *(30–33)*. As already indicated, gonadal steroids (in the case of the female, mainly estradiol) stimulate growth hormone (GH) secretion, which, in turn, increases the synthesis and secretion of IGF-1 in many tissues, especially in the liver. IGF-1 mediates many of the growth-promoting effects of GH, stimulating linear growth, and muscle and bone development, among other things. IGF-1, in addition, further amplifies the emerging activity of the HPG axis; it has been shown to evoke LHRH release from the hypothalami of prepubertal female rats, and this has been demonstrated elegantly by Hiney and colleagues *(31,32)* both in vivo and in vitro. EtOH feeding of prepubertal female rats from d 29 to 34 of life has been shown to decrease the synthesis and secretion of IGF-1 *(34)*. This may be owing to an EtOH effect on the hypothalamic/pituitary unit since EtOH has been shown to induce a rise in the content of GH-releasing factor coupled with a fall in GH *(13,18)*. Analogous to the interpretation of the LHRH/LH data we have discussed, these data suggested that EtOH led to a decreased GH secretion by a decreased release of GH-releasing hormone factor from the hypothalamus. This is relevant since GH is a major stimulator of IGF-1 synthesis and secretion. There may, of course, be a direct inhibitory effect of EtOH on IGF-1 as well. Furthermore, in acute studies, the ability of IGF-1 to increase LH was blocked by EtOH *(35)*.

We have confirmed these findings regarding EtOH's effect. These data implicate IGF-1 as a major participant in the pubertal process and suggest that EtOH's disruption of puberty may result in part by EtOH interfering with the synthesis and secretion of IGF-1. The delay in VO seems not to be IGF-1 dependent since naltrexone overcame EtOH's effect on VO without blocking the inhibitory effect of EtOH on IGF-1. This effect on IGF-1 could also account, at least in part, for the impaired growth in animals given EtOH despite pair-feeding procedures, which ensure that animals given EtOH get the same number of calories as controls.

In summary, we have confirmed and extended the findings of others that EtOH is highly disruptive to female puberty. We have shown, for the first time, that the earlier EtOH exposure is started the more profound is the effect. We have also shown, for the first time, that EtOH's effect is partially, though not totally, dependent on endogenous opiates since the EtOH-induced delay in VO is completely abrogated by naltrexone. Further studies on the longer-term impact of EtOH, its mechanisms, and the question of whether there is permanent damage after EtOH is discontinued seem warranted.

Materials and Methods

Animals

Female Sprague-Dawley rats, ages 16–23 d at the time of arrival to our animal facility, were purchased from Harlan (Indianapolis, IN). Animals were allowed to acclimate to their environment, which included a 12-h light/dark cycle, for 5 d. Lights were on from 6:00 AM to 6:00 PM.

Diet and Feeding Procedures

There were five groups of animals: EtOH/vehicle, pair-fed control/vehicle, EtOH/naltrexone, pair-fed control/naltrexone, and ad libitum/vehicle. The Joint Institutional Animal Care and Use Committee, Loyola University Stritch School of Medicine, and Edward Hines Veterans Administration Hospital approved all animal protocols.

The well-established Lieber DeCarli liquid diet without ethanol was administered. Thereafter, the ethanol group

($n = 40$) received 36%, or 6% by volume, of its calories as ethanol, as previously reported *(36)*. The pair-fed group ($n = 40$) received a number of calories equal to their EtOH-fed mates, with dextrimaltose substituted for ethanol. A third ad libitum group ($n = 20$) was given as much of the liquid diet as the animals could consume, in order to observe whether there was any difference between the control pair-fed and ad libitum groups. Feeding was started at 21, 25, or 28 d of age. In the 21- and 28-d-old animals, the experimental feeding paradigm was continued for 2 mo, and for the 25-d-old animals, 1 mo. The reason for the difference in the experimental length was the previous observation in adult female rats that the estradiol response to EtOH was time dependent, with a transient increase in estradiol after shorter periods of feeding, a phenomenon that abated with longer EtOH exposure *(23)*. To determine whether or not this was true in pubertal animals, an intermediate aged rat (i.e., 25 d old) was chosen for a shorter period of EtOH treatment. In general, all the biologic and hormonal parameters in the ad libitum animals were similar to those seen in pair-fed controls. Therefore, for clarity of presentation, data from the ad libitum animals are not shown.

Assessment of Estrous Cycle

Animals were housed singly, weighed on a weekly basis, and daily food consumption was measured. VO was visually assessed on a daily basis, and thereafter, vaginal smears were obtained utilizing a thin-tipped plastic pipet filled with s terile saline. Smears were immediately analyzed for characteristics of the estrous cycle as described by Everett *(37)*. After 1 or 2 mo of feeding, rats from each group were sacrificed by decapitation at 5:00 PM on the first day of proestrous after completing the required feeding period. Animals that remained anestrous were sacrificed on the last day of the study. Trunk blood was collected and serum separated and stored at –20ºC for subsequent radioimmunoassay (RIA). Similarly, anterior pituitaries and hypothalami were quickly removed and stored at –70ºC for further study *(38)*.

Administration of Naltrexone

Naltrexone, purchased from Innovative Research (Saratoga, FL), was administered by time release pellet implant delivering 2.5 mg/d for a 60-d period. All animals were implanted with either the naltrexone or placebo (vehicle) pellet 2 d after starting the feeding period.

Radioimmunoassays

Luteinizing Hormone

The LH RIA was conducted utilizing the materials generously contributed by the National Hormone and Pituitary Program and by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK). Assay sensitivity was 155 pg/mL and 31 pg/tube and the inter- and intraassay coefficients of variation (CVs) were 5.2 and 4.0%, respectively *(39)*.

Follicle-Stimulating Hormone

The FSH RIA was done utilizing the materials supplied by the NIDDK and the National Hormone and Pituitary Program. The assay sensitivity was 312 pg/mL and the inter- and intraassay CVs were 5 and 2%, respectively *(40)*.

Estradiol

The estradiol RIA was done by commercially available kit (Diagnostic Systems, Webster, TX). The assay sensitivity was 1.5 pg/mL and the inter- and intraassay CVs were 3 and 2%, respectively.

Progesterone

The progesterone RIA was done by commercially available kit (Diagnostic Systems). The assay sensitivity was 0.3 ng/mL and the inter- and intraassay CVs were 4 and 2.5%, respectively.

Insulin-like Growth Factor-1

The IGF-1 RIA was done by commercially available kit (Diagnostic Systems). The assay sensitivity was 150 ng/mL and the inter- and intraassay CVs were 3 and 2%, respectively.

Statistical Methods

The results of these experiments were analyzed by two-way analysis of variance (ANOVA) with Tukey follow-up for multiple comparisons. Statistical significance was determined by a value of $p < 0.05$.

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