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Effect of Repeated Exposure to Alcohol on the Response of the Hypothalamic-Pituitary-Adrenal Axis of the Rat: II. Role of the Length and Regimen of Alcohol Treatment

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Background: Prior exposure to alcohol alters the adrenocorticotropin hormone (ACTH) response to a second drug challenge administered several days later. We used three models of alcohol treatment to investigate the mechanisms that may be involved in this phenomenon.

Methods: Adult male rats were exposed to alcohol vapors daily for 3 days (4–4.5 hr/day) and then were exposed to shocks or an intragastric injection of alcohol 7 days later (group A); were injected daily with alcohol (4.5 g/kg intragastrically) for 3 days and then exposed to shocks or an intragastric injection of alcohol 7 days later (group B); or were exposed to alcohol vapors for 6 days and exposed to shocks or an intragastric injection 24 hr later (group C). Control animals were not exposed to the vapors or received the appropriate vehicle.

Results: Compared with animals administered the vehicle, rats of groups A and B that had been exposed to alcohol all exhibited a significantly decreased ACTH response to a second drug challenge. In contrast, their ACTH response to footshocks was statistically comparable to that of vehicle-pretreated animals. Rats of group C that had been exposed to alcohol for 6 days also showed decreased ACTH release when injected with alcohol 7 days later while responding normally to shocks. Measurement of anterior pituitary pro-opiomelanocortin indicated that alcohol pretreatment had produced a 54% increase of these transcripts in group C and a 27% decrease in group A. There were no changes in pituitary receptors type 1 for corticotropin-releasing factor (CRFR1) in any of the groups.

Conclusion: Regardless of whether they are delivered shortly before an acute alcohol injection or several days earlier, alcohol vapors or injections interfere with the ACTH response to the drug but not to shocks. Our results also suggest that changes in ACTH responses may not be correlated directly with small changes in pituitary pro-opiomelanocortin or CRFR1 mRNA levels.

Key Words: Adrenocorticotropin Hormone, Pro-Opiomelanocortin, Alcohol, Rat, Corticotropin-Releasing Factor.

THE ABILITY OF alcohol exposure to activate the hypothalamic-pituitary-adrenal (HPA) axis in rodents is well recognized (Rivier, 1996), and much work by many laboratories has documented the significant release of adrenocorticotropin hormone (ACTH) and the concomitant activation of hypothalamic corticotropin-releasing factor (CRF) and vasopressin (VP) neurons caused by injection of alcohol (whether intraperitoneal or intragastric) or exposure to vapors. However, in the course of our work, we have observed significant differences between the magnitude of the HPA axis response and the pattern of hypothalamic activation of rats administered alcohol through different routes (Ogilvie and Rivier, 1997; Ogilvie et al., 1998), which suggest the participation of different brain mechanisms. We decided to further explore this question by comparing the long-term consequences of exposing rats to either repeated intragastric alcohol injections or multiple sessions of alcohol vapors. We reported earlier that an initial regimen of daily intragastric injections, delivered for 3 days, blunted the ACTH response to a second, acute intragastric treatment given 7 days later but not to shocks (Lee and Rivier, 1997). In the present work, we determined whether this effect was mimicked by an initial daily session of alcohol vapors, also administered for 3 days. We then compared the effects of these two regimens to the effect of continuous exposure to vapors for 6 days, in animals tested on the 7th day. To provide information about mechanisms that might be involved in differential influences of the initial treatment, we measured pituitary pro-opiomelanocortin

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(POMC) and CRF receptors type 1 (CRFR1) transcripts at the end of these various modalities.

MATERIALS AND METHODS

Animals

Adult male Sprague Dawley® rats (180–220 g) were maintained under standard lighting (12:12 hr light/dark, lights on 0630 hr) and feeding (rat chow and water ad libitum) conditions. All rats were group-housed initially but were single-housed after surgical procedures. All procedures were approved by the Salk Institute Animal Care and Use Committee.

Surgeries and Cannulae

All procedures were carried out as described in part I of this work (Lee et al., 2001).

Alcohol Injections

For the daily alcohol injections, the intragastric cannulae were extended with a polyethylene-50 tubing connected to a syringe. Alcohol was diluted with saline to < 20% v/v and was injected slowly at 4.5 g/kg. An equal volume of saline was administered to control rats. During treatment, the rats were awake and moved freely in their home cage. Because the animals were not fasted, alcohol was injected 4 to 5 hr after lights on, a time when most of the food consumed during the previous night had left the stomach. Seven days later, rats previously injected with the vehicle or alcohol were injected again with the vehicle or alcohol (4.5 g/kg) through the intragastric cannulae. In addition, one group of rats did not undergo intragastric cannulation and were used as "absolute controls" for the experiments focused on the measurement of POMC transcripts in the anterior pituitary. These animals were killed within 10 sec of removal from their home cage.

Alcohol Vapors

We recently developed a new system of alcohol delivery to boxes that house individual rats (Lee et al., 2000) in which four pumps are used to deliver alcohol in a preprogrammed fashion that is controlled by an electronic timer (Intermatic model DT7C, Spring Grove, IL). Alcohol flows from a large reservoir to a peristaltic pump (model QG-6, FMI Laboratory, Fluid Metering Inc., Syosset, NY), from which it is carried to a side-arm flask at a flow rate that can be regulated. This flask is placed on a heater so that the drops of alcohol hitting the bottom are vaporized. Air flow controlled by a pressure gauge is delivered to the flask and carries the alcohol vapors to the individual boxes. It then leaves the box through an outlet flow tube connected to a vacuum and reaches 1 of 16 cages (8.5″ high × 10.25″ wide × 18.5″ long), each of which is divided into two compartments such that we can accommodate 32 rats per experiment. To accomplish this, a Y splitter is attached to the pump, and flow is diverted to two different side-arm flasks. A splitter is connected to each side-arm flask, which in turn is connected to two inhalation chambers. The lids of the boxes contain a 2 mm porthole to accommodate intravenous cannulae used for blood sampling and/or delivery of treatments, when warranted, and a swivel allows free movement of the animals. The portholes can be sealed if they are not needed, and the integrity of the system is maintained. The animals were kept in these chambers for 4 hr the first day (0800–1200 hr) and for 4.5 hr on subsequent days (0800–1230 hr).

Footshock

Mild electrofootshocks (0.5 mA, 1 sec duration) were delivered for 30 min to the rat's paws according to a computer-driven schedule that generated randomly distributed shocks at an average rate of two shocks per minute (see, e.g., Rivier and Vale, 1988).

Blood Alcohol Levels (BALs)

BALs were measured in separate groups of animals by using 5 μl plasma obtained through the intravenous cannulae as previously described (Lee et al., 2000; Ogilvie and Rivier, 1997). The sensitivity of the assay is 0.1 mg/dl, the precision is 1% to 2%, and the curve is linear up to 400 mg%.

ACTH

Plasma ACTH levels were determined by a commercially available two-site immunoradiometric assay (Allegro kit, Nichols Institute, San Juan Capistrano, CA). The lower detection limit and the intra-assay coefficient of variation were 15 pg/ml and <10%, respectively. Data were expressed in picograms per milliliter of plasma. We have validated this assay for use in the rat (Rivier and Shen, 1994).

Pituitary POMC and CRFR1 Transcripts

Levels of POMC and CRFR1, which are those of importance for ACTH release, were measured by Northern blot analysis. Pituitaries in each group were obtained by decapitation and were stored at −70°C until RNA extraction. Total RNA was isolated from anterior pituitaries by RNeasy Mini Kit (Qiagen Inc., Valencia, CA). Ten micrograms of RNA were denatured in 2.2 M formaldehyde and were subjected to electrophoresis on a 1.2% agarose formaldehyde gel in 20 mM morpholinopropanesulfonic acid (pH 7.0), 5 mM sodium acetate, and 1 mM ethylenediaminetetraacetic acid buffer. After electrophoresis, RNA was transferred to a Zeta-Probe blotting membrane (BioRad, Hercules, CA) and was cross-linked by ultraviolet irradiation. The RNA blot was incubated at 65°C for 4 hr in prehybridization buffer that contained 5 × SSPE (containing 0.75 M NaCl, 0.05 M NaH_2PO_4, and 5 mM EDTA at pH 7.4), 10 × Denhart's solution (0.02%, w/v, each of bovine serum albumin, Ficoll 400, and polyvinylpyrrolide), 50% formamide, 0.5% sodium dodecyl sulfate (SDS), and 500 mg/ml denatured salmon sperm DNA. Hybridization was carried out with a cRNA probe, labeled with [α-^{32}P]-uridine 5′ triphosphate at 65°C in the same buffer overnight. Filters were washed successively in 1 × SSPE/0.1% SDS, 0.1 × SSPE/0.1% SDS, and 0.1 × SSPE at 65°C. After posthybridization washes, membranes were air dried and exposed. Quantitative analysis was performed by using PhosphorImager system (Molecular Dynamics, Sunnyvale, CA) and the ImageQuant 4.0 software packages. To obtain accurate measurements, hybridization with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was performed to normalize RNA loading between lanes. The ratios of specific signal to GAPDH were calculated to estimate the levels of these signals. This procedure has been described previously by our laboratory (Lee et al., 2000).

Probe Preparation for Pituitary Signals

The pGEM-4z vector (Promega, Madison, WI) that contained a 0.9 kb fragment of rat POMC cDNA (provided by Dr. S. Watson) was linearized with *Eco*RI. Rat CRFR1 cDNA (provided by Dr. W. Vale, Salk Institute, La Jolla, CA), subcloned into pBluescript SK-1 vector, was linearized with *Bam*HI. Radioactive cRNA copies were synthesized by incubation of 250 ng linearized plasmid in 6 mM $MgCl_2$, 36 mM Tris (pH 7.5), 2 mM spermidine, 8 mM dithiothreitol, 25 mM adenosine 5′ triphosphate/guanosine 5′ triphosphate/cytidine 5′ triphosphate, [α-^{32}P]-uridine 5′ triphosphate, 1 U RNasin (Promega), and 10 U of T7 (for POMC, CRFR1, and GAPDH) RNA polymerase for 60 min at 37°C. Unincorporated nucleotides were removed by using MicroSpin G-50 columns (Amersham, Piscataway, NJ).

Statistical Analysis

Statistical analyses were performed with appropriate one-, two-, or three-way ANOVA followed by the least squares means post hoc text. *P* values are reported only when statistical significance was achieved ($p < 0.05$, two-tailed).

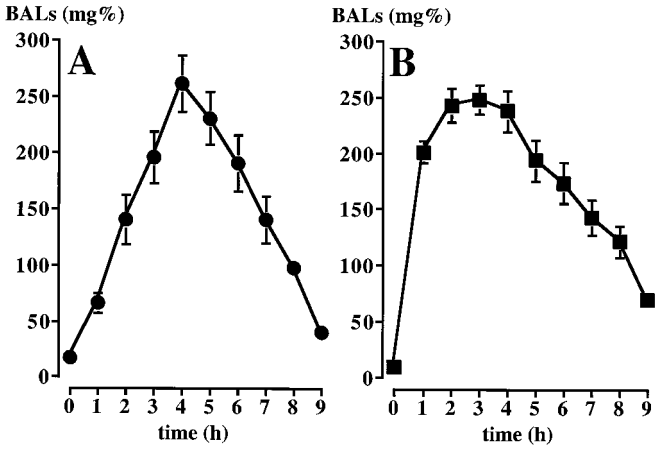

Fig. 1. BALs after exposure to alcohol vapors for 4 hr (A) or the intragastric injection of 4.5 g ethanol/kg (B). Each point represents the mean ± SEM of five to seven rats.

RESULTS

BALs and Body Weights

BALs. Because we compared two different modes of alcohol delivery, we thought it was important to provide information about BALs achieved in both models. BALs gradually increased during exposure to vapors for 4 hr, then decreased once the animals were moved back to their home cage (Fig. 1A). BALs had returned to near baseline 9 hr after the beginning of the treatment and had become undetectable when the animals started feeding (not shown). The intragastric injection of 4.5 g alcohol/kg caused an abrupt increase in BALs that peaked 2–4 hr later (Fig. 1B). BALs were still detectable at the 9 hr time point but could not be detected when the animals started feeding (not shown). The amount of alcohol injected intragastrically in the present work was chosen so that peak BALs would be comparable in both models. Indeed, they were, and both models also remained elevated for approximately the same length of time. We thought this was important for a valid comparison between the neuroendocrine effects of these two modes of alcohol delivery. On the other hand, the slope of the increase to peak level was significantly steeper in rats injected intragastrically compared with rats exposed to vapors.

Weights. Weights were monitored throughout the experiments, but here we only illustrate data obtained on the day when plasma ACTH levels were measured. Body weights of all three control groups were comparable (Table 1). Alcohol decreased body weights by approximately 10% in all animals regardless of whether the animals had recovered for 7 days or had been exposed to the vapors continuously. These results suggest that the influence of alcohol on body weight is long lasting because 7 days of recovery did not allow the animals to regain their weight. At present, the reason for this long-term effect is not known.

Table 1. Body Weights

Pretreatment	Body weight (g)
7 days of recovery	
Control	249 ± 4
Vapors	225 ± 5**
Intragastric vehicle	252 ± 4
Intragastric ethanol	235 ± 4**
Continuous treatment	
Control	244 ± 4
Vapors	226 ± 7**

In the 7-day recovery groups, rats were exposed to vapors or intragastric treatments daily for 3 days and then were tested 7 days after the last exposure. In the continuous treatment groups, animals were exposed to vapors or were kept in chambers without vapors for 6 days (4–4.5 hr/day) and were tested on day 7. Weights were taken on the day of the acute experiment (i.e., exposure to shocks or intragastric treatment for measurement of plasma ACTH levels). Data are presented as mean ± SEM (n = 5–7). ** $p < 0.01$ vs. corresponding control.

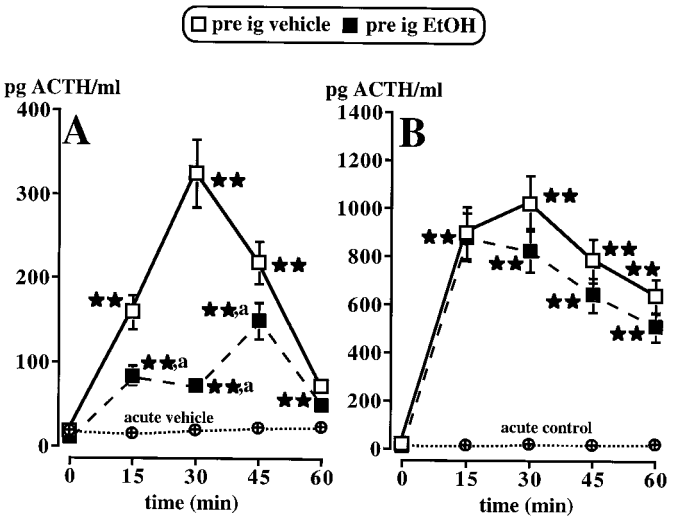

Fig. 2. Effect of prior exposure to alcohol (one daily intragastric injection for three consecutive days) on the ACTH response to an acute intragastric injection (4.5 g ethanol/kg; A) or exposure to shocks (B) 7 days later. Each point represents the mean ± SEM of five to seven rats. **$p < 0.01$ vs. vehicle; $^{a}p < 0.01$ vs. prevehicle.

Effect of Prior Exposure to Daily Intragastric Injections of Alcohol on the ACTH Response to Shocks or Acute Alcohol Administration, Measured After 7 Days Recovery

The acute intragastric injection of alcohol (4.5 g/kg), but not vehicle, significantly elevated plasma ACTH levels in all animals (Fig. 2A). However, as previously reported (Lee and Rivier, 1997), this response was significantly ($p < 0.01$) blunted in animals that had been exposed to daily intragastric injections of alcohol for three consecutive days several days earlier. In contrast, the ACTH response to shocks was decreased only slightly in this model, and this difference did not reach statistical significance (Fig. 2B). In both cases, ACTH levels of control animals (injected with the vehicle acutely or not exposed to the shocks) remained below 50 pg/ml. To facilitate visualization of the data, we also present the results as a function of cumulative ACTH levels measured over the entire time course of this response (Table 2).

Table 2. Cumulative ACTH Release Calculated From Data of Figs. 2, 3, and 4

Pretreatment	Recovery (days)	Acute treatment	pg ACTH/ml
Control	7	Shocks	2262 ± 265
3 days of vapors	7	Shocks	2304 ± 278
Control	7	ig EtOH	1366 ± 155
3 days of vapors	7	ig EtOH	901 ± 103**
ig vehicle	7	Shocks	3048 ± 320
3 days of ig EtOH	7	Shocks	2967 ± 329
ig vehicle	7	ig EtOH	956 ± 108
3 days of ig EtOH	7	ig EtOH	583 ± 66**
Control	None	Shocks	2490 ± 270
6 days of vapors	None	Shocks	2020 ± 223
Control	None	ig EtOH	1060 ± 121
6 days of vapors	None	ig EtOH	539 ± 64**

Data are presented as mean ± SEM of cumulative ACTH measured over the time course of the ACTH response to shocks (0, 15, 30, 45, and 60 min) or to intragastric ethanol (ig EtOH; 4.5 g/kg; 0, 15, 30, 60, and 120 min). n = 5–6 rats/point. ** $p < .01$ vs. corresponding control.

Effect of Prior Exposure to Repeated Exposure to Alcohol Vapors on the ACTH Response to Shocks or Acute Alcohol Administration, Measured After 7 Days Recovery

Like the intragastric treatment, exposure to alcohol vapors for three consecutive days significantly ($p < 0.01$) decreased the ACTH response to a second intragastric challenge, delivered 7 days later (Fig. 3A and Table 2) but not to shocks (Fig. 3B and Table 2). In both cases, ACTH levels of control animals (injected with the vehicle acutely or not exposed to the shocks) remained below 50 pg/ml.

Effect of Long-Term Exposure to Alcohol Vapors on the ACTH Response to Shocks or Acute Alcohol Administration, Measured at the End of the Initial Treatment

Rats exposed to acute alcohol intragastrically 24 hr after the last day of a 6 day daily vapor treatment showed a significant ($p < 0.01$) blunting of their ACTH response (Fig. 4A and Table 2) but a modest and overall not statistically significant decrease in the ACTH response to shocks (Fig. 4B and Table 2).

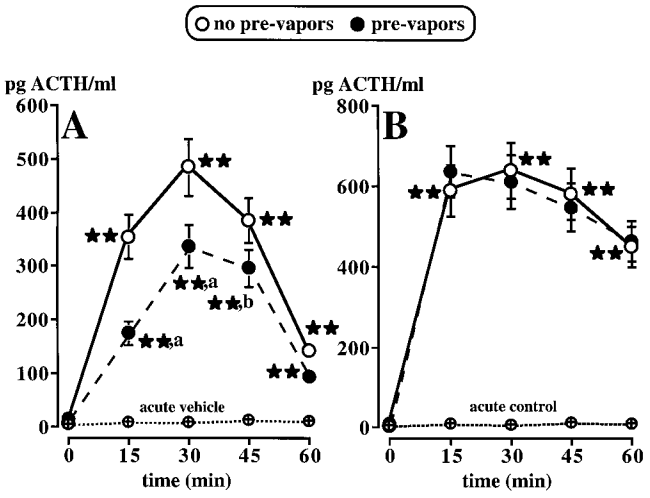

Fig. 3. Effect of prior exposure to alcohol (daily vapor exposure for three consecutive days, 4 hr/day) on the ACTH response to an acute intragastric injection (4.5 g ethanol/kg; A) or exposure to shocks (B) 7 days later. Each point represents the mean ± SEM of five to seven rats. **$p < 0.01$ vs. vehicle; [a]$p < 0.01$; [b]$p < 0.05$ vs. prevehicle.

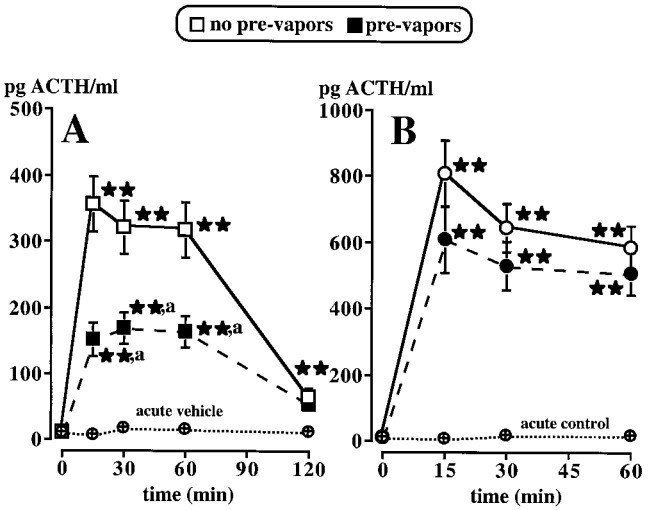

Fig. 4. Effect of exposure to alcohol vapors for six consecutive days (4 hr/day) on the ACTH response to an acute intragastric injection (4.5 g ethanol/kg; A) or exposure to shocks (B) the following day. Each point represents the mean ± SEM of five to seven rats. **$p < 0.01$ vs. vehicle; [a]$p < 0.01$ vs. prevehicle.

Measurement of Pituitary POMC and CRFR1 Transcripts

Compared with control animals, rats exposed to alcohol vapors 7 days earlier had anterior pituitary POMC mRNA levels that were significantly ($p < 0.01$) lower (Fig. 5). On the other hand, these transcripts were increased, but not significantly, by continuous exposure to the vapors for 6 days and were not altered significantly by prior exposure to repeated intragastric alcohol injections 7 days earlier. No regimen of alcohol treatment significantly altered pituitary CRFR1 mRNA levels (not shown).

DISCUSSION

Four main findings emerged from the studies presented here. First, prior exposure to alcohol blunted the ACTH response to an acute intragastric alcohol injection, administered 7 days later, and this phenomenon was observed regardless of whether the drug originally was delivered via vapors or intragastric injections. Second, these long-term effects of alcohol were not exerted on the pathways that mediate the ACTH response to footshocks. Third, these differential consequences of alcohol treatment were observed both in animals tested at the end of 6 days of drug exposure and in animals allowed to recover for 7 days. And fourth, there do not appear to be direct and obvious correlations between changes in ACTH responses and altered pituitary POMC and/or CRF receptor transcripts.

An immediate conclusion drawn from the present study is that the regimen of alcohol delivery is important for the

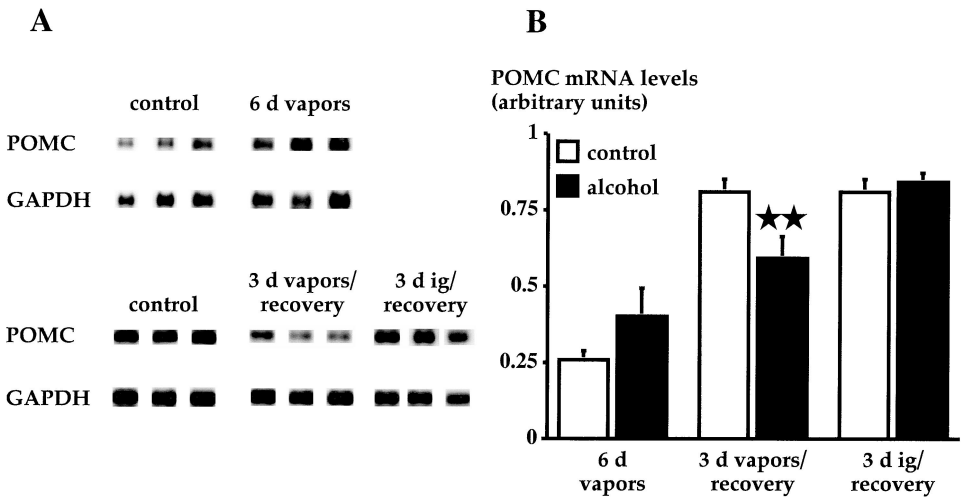

Fig. 5. Pituitary POMC mRNA levels measured 24 hr after the end of a 6-day regimen of alcohol vapors, 7 days after exposure to vapors for three consecutive days, or 7 days after intragastric injections for 3 days (4.5 g/kg) by Northern blot analysis. (A) Representative autoradiograms with 10 μg of RNA from anterior pituitary hybridized with POMC or GAPDH (control) cRNA. (B) Histogram of the data. Each bar represents the mean ± SEM of four to eight rats. $^{**}p < 0.01$ vs. control.

influence that this drug has on the HPA axis. Indeed, prior work from our laboratory indicated that exposure to alcohol vapors either throughout the day for 7 days or 6 hr daily for at least 8 days significantly decreased the HPA axis response to both neurogenic and systemic stressors (Lee et al., 2000; Rivier et al., 1984, 1990). These results indicate that differences in the length of daily alcohol treatment as well as in the overall duration of this treatment have significant consequences for the HPA axis. On the other hand, the finding that two different regimens of alcohol delivery, vapors or intragastric injections, exerted a comparable influence on the HPA axis supports the concept that the route through which the drug is delivered may not be crucial. Furthermore, earlier work carried out in our laboratory indicated that pre-exposure to alcohol via the intraperitoneal route led to similar findings (C. Rivier, unpublished data). This is an important point, because many of the experimental protocols used in the laboratory rely on modes of alcohol treatment that have little to do with human consumption, a basis for criticism of these protocols. Nevertheless, an important question is whether the type of forced alcohol treatment we used induces changes in hypothalamic activity that could be duplicated by self-administered alcohol. A comparison of investigator-induced versus self-induced alcohol treatment indicated significant differences in the reinforcing effects or cerebral glucose utilization of alcohol delivered by an investigator or after voluntary consumption (Moolten and Kornetsky, 1990; Porrino et al., 1998). Similarly, comparison of passive versus active cocaine administration indicated higher plasma corticosterone levels in rats that self-administered the drug (Galici et al., 2000). Therefore, it will be important to develop models in which rats control their alcohol intake, to determine whether this paradigm also is associated with long-term neuroendocrine consequences.

It was of interest to find that alterations in ACTH responses did not appear to parallel changes in pituitary POMC and/or CRFR1 transcripts. For example, 6 days of exposure to alcohol vapors resulted in somewhat increased POMC mRNA levels as well as both decreased ACTH release due to alcohol and unaltered response to shocks. One possibility is that stores of pituitary POMC transcripts are large enough that relatively small changes in these levels do not affect ACTH release. The inability of any of the alcohol regimens to alter CRFR1 mRNA levels was more surprising. Indeed, we had shown that the type of daily exposure to alcohol vapors we used here repeatedly increases plasma ACTH levels (Rivier et al., 1990), a response thought to be due to increased CRF delivery to the pituitary (Rivier et al., 1984). Studies conducted in isolated cells have indicated that CRF usually down-regulates its receptors type 1 (Pozzoli et al., 1996), and manipulations believed to be associated with elevated CRF secretion, such as stress, adrenalectomy, or endotoxemia, produce a similar effect (Aubry et al., 1997; Luo et al., 1995; Makino et al., 1995). The present results therefore suggest that alcohol might induce the release of factors that compensate the effect of CRF on its type 1 receptors. Corticosterone is an unlikely candidate, because adrenal steroids usually are thought to also reduce CRFR1 mRNA levels (Pozzoli et al., 1996; Sakai et al., 1996; Zhou et al., 1996). At present, therefore, the identity of this factor remains unknown.

The fact that the same alcohol regimen produced different effects on ACTH responses to shocks or acute drug injection suggests that alcohol-induced changes in ACTH responses are unlikely to be modulated by altered pituitary activity but rather are modulated by an influence of the drug on the pathways that control the activity of hypothalamic CRF and/or VP neurons. In a joint article (Lee et al., 2001), we show that the ability of prior alcohol exposure to blunt subsequent response of the HPA axis to the drug was accompanied by decreased paraventricular nucleus (PVN) CRF transcripts. Stress-related sensory information is conveyed to PVN neurosecretory neurons, and activation of these neurons triggers the release of CRF and VP to the pituitary (see Kovács, 1998; Watts, 1996). These PVN cell bodies therefore represent the final output component of a neuronal network that regulates ACTH release. As such, it

is not surprising that they would exhibit changes in activity that parallel those observed in plasma ACTH levels. This tells us little, however, about the extended circuitry that integrates and relates the multiple environmental and internal factors that regulate the response of the HPA axis to homeostatic threats. At present, the afferent pathways that impinge on the PVN and convey to it the occurrence of neurogenic stressors such as footshocks are starting to be unraveled, and these pathways are known to include regions normally associated with somatosensory/nociceptive information, as well as limbic regions of the telencephalon and associated hypothalamic structures. In contrast, ascending aminergic projections that originate in the caudal medulla, which are critical for the PVN neuronal response to immune stressors, do not appear to be essential for its response to footshocks (Li et al., 1996, Sawchenko et al., 1996). On the other hand, to our knowledge, the neurocircuitry associated with the HPA axis response to alcohol remains to be identified. Obviously, this information is necessary before we can fully understand why alcohol pretreatment does not modify the ability of shocks to release ACTH, while it blunts HPA responses to the drug itself.

Whatever the site at which this influence is exerted, it is very long-lasting. We previously reported that the ACTH response to an acute alcohol challenge remained blunted 13 days after the end of the original drug exposure (Lee and Rivier, 1997), and since then we have extended this time frame to 21 days (C. Rivier, unpublished data). Were this phenomenon to be observed in humans, this time frame would correspond to more than 1 year of human life. Therefore, uncovering the mechanisms responsible for this phenomenon is an important endeavor.

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