

Prolonged Exposure to Intermittent Alcohol Vapors Blunts Hypothalamic Responsiveness to Immune and Non-Immune Signals

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Background: We have previously shown that long-term alcohol treatment blunts the ACTH response to alcohol itself, as well as to other stresses, and is accompanied by decreased pituitary responsiveness to vasopressin (VP), but not corticotropin-releasing factor (CRF). The present work aims to determine the relevance of changes in CRF and VP receptors in the pituitary gland and/or peptide stores of CRF neurons in the paraventricular nucleus (PVN) of the hypothalamus, the areas that are most directly involved in ACTH release.

Methods: Intact male rats were exposed to alcohol using a new vapor delivery system which enables individual rat housing in boxes. Alcohol treatment was delivered for 6 hr once daily (0700–1300), after which the rats were returned to their home cages where they had free access to food and water. Control rats were kept in similar boxes, but not exposed to alcohol. Total treatment time was 8 days. All animals were equipped with indwelling jugular cannulae that were used to monitor blood alcohol levels (BALs) as well as ACTH and corticosterone release throughout drug exposure. Due to the presence of a swivel, the animals' movements were not restricted or hindered by the presence of these cannulae. On the morning of day 9, the animals were decapitated under basal conditions or exposed to a neurogenic (mild electrofootshocks) or systemic [iv lipopolysaccharide (LPS)] stimulus. PVN neuronal responses, indicated by changes in mRNA concentrations of the immediate early genes (IEGs) *c-fos* and NGFI-B, and plasma ACTH levels were measured before and during endotoxemia or electrofootshocks.

Results: In the absence of alcohol, plasma ACTH and corticosterone remained at basal levels, indicating the absence of environment-induced stress. In rats exposed to alcohol, BALs were consistent and predictable, and we targeted peak values of about 200 mg%. At the end of the drug treatment period, there were no significant differences between CRF and VP receptor mRNA levels in the anterior pituitary of control and alcohol-treated rats. In contrast, alcohol treatment respectively decreased CRF and increased VP stores in the external zone of the median eminence. It also increased NGFI-B and *c-fos* transcripts in the magnocellular (m) portion of the PVN, but not the parvocellular (p) division of this nucleus under basal conditions (i.e., in the absence of shocks or LPS). After exposure to these stressors, on the other hand, all groups of rats showed significant increases in plasma ACTH levels as well as up-regulation of their PVN neuronal response, as indicated by changes in pPVN IEGs transcripts. However, these hormonal and neuronal responses were significantly blunted in animals pretreated with alcohol.

Conclusions: Collectively, our results suggest that decreased PVN neuronal activation represents an important mechanism of the ability of long-term alcohol treatment to blunt the ACTH response to shocks or endotoxemia. In addition, the new system of alcohol delivery that we developed is practical and reliable, and has the significant advantage that it enables measurement of circulating hormone levels during drug exposure of the animals.

Key Words: Hypothalamic-Pituitary-Adrenal (HPA) Axis, Paraventricular Nucleus (PVN), Corticotropin-Releasing Factor, Vasopressin.

ACUTE ALCOHOL EXPOSURE, at least at doses that lead to blood alcohol levels (BALs) above 100 mg%, is known to up-regulate the activity of the hypothalamic-pituitary-adrenal (HPA) axis of the rat, resulting in increases in circulating ACTH and corticosterone levels and in the neuronal activity of hypothalamic cells that

express corticotropin-releasing factor (CRF) or vasopressin (VP) [ref. in (Madeira and Paula-Barbosa, 1999; Rivier, 1996; Wand and Schumann, 1998)]. Prolonged exposure to the drug is also associated with signs of HPA axis hyperstimulation, including adrenal hypertrophy and thymus involution (Spencer and McEwen, 1990) as well as increased hypothalamic function (Rivier et al., 1984, 1990). However, adaptation takes place in that the acute endocrine response to alcohol becomes blunted (Spencer and McEwen, 1990). This loss of HPA axis response to a chronically applied stress represents a well known phenomenon called homologous adaptation. However, controversy exists with regard to heterologous adaptation, that is, the response of the HPA axis to a stress that is different from the one that was previously delivered. In some cases, animals exposed to one type of stress showed an unaltered or even augmented response of the HPA axis to other stresses [see for example (Dallman, 1993; Dijken et al., 1993b; Hauger et al., 1990; Liberzon et al., 1997; Marti et al., 1994)]. This phenomenon

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is thought to be due to the facilitation of stimulatory input to CRF-releasing neurons in the hypothalamus, itself induced by a reduction of the tonic inhibitory control that is normally exerted on the PVN, and/or into altered peptide make-up of the CRF neurons which leads to an enhanced signal-to-potency ratio (deGoeij et al., 1992a, 1993). We, on the other hand, observed blunted ACTH release in rats exposed to shocks or immune challenges at the end of a week-long alcohol treatment (Lee and Rivier, 1993, 1994a, Rivier, 1995). Although differences in protocols may at least in part explain this apparent controversy, it should be noted that the rapidly saturated adrenal response to ACTH means that changes in pituitary function cannot be detected unless ACTH levels fall quite low. It is therefore feasible that the investigators who failed to observe adaptation to alcohol may have missed a significant loss in ACTH secretory rate that was not translated into a decreased corticosterone response. It is also possible that the use of different models of alcohol exposure may have yielded different results. In the study quoted above (Spencer and McEwen, 1990), the animals received daily injections of alcohol for 7–22 days. This procedure may have superimposed adaptation to the injections and to alcohol, which may have obscured the presence of cross adaptation between alcohol and other stresses. In support for the presence of cross adaptation after long-term alcohol exposure in humans, it was shown that a mixture of emotional and physical stresses was unable to stimulate ACTH release in recently abstinent alcoholics, while it was able to do so several weeks after cessation of drinking (Ehrenreich et al., 1997).

In the present work, we investigated the neuronal mechanisms that may underlie the loss of HPA axis activity during prolonged alcohol treatment. Putative mechanisms could be operative at four levels: the pituitary; the paraventricular nucleus (PVN) of the hypothalamus, which is the principal site of the CRF and VP neurons involved in the regulation of ACTH secretion; the external zone of the median eminence (ZEME) that contains nerve terminals of the CRF and VP neurons originating in the PVN; and hypothalamic or extrahypothalamic higher centers that influence PVN activity, such as the hippocampus or the amygdala (Pacak et al., 1995; Sawchenko et al., 1996; Swanson, 1986; Watts, 1996; Whitnall, 1993). We have previously shown that male rats that were fed an alcohol diet for 7–10 days exhibited a blunted ACTH response to exogenous VP injection (Lee and Rivier, 1995). In addition, preliminary results indicated that the PVN activity of rats exposed to alcohol for 7–8 days was blunted under both basal and stimulated conditions. The present work expands on these observations and provides evidence for significant alcohol-induced changes in the basal activity of the HPA axis as well as in its response to neurogenic (exposure to mild electrofootshocks) and systemic stress [intravenous (iv) injection of LPS] to stimulate the endocrine hypothalamus. In addition, we provide detailed information regarding a

new system of alcohol delivery that was used in the present study.

MATERIALS AND METHODS

Animals

Adult male Sprague Dawley® (Harlan Sprague Dawley, Inc., Indianapolis, IN) rats (about 70 days old) were kept under a standard light regimen (12-hr light/12-hr dark, lights on at 0630) and fed rat chow and water ad libitum. Alcohol (E)-exposed animals were treated as described below. For the i.v. injection of LPS and/or BALs measurement, the rats underwent aseptic insertion of a right jugular venous cannulae 48–72 hr before the experiment (Lee and Rivier, 1995). All procedures were approved by the Salk Institute IACUC.

Alcohol Boxes

Until recently, we had delivered alcohol vapors to large chambers in which cages containing 4–6 animals were placed. Although this method has been successfully used in the past, it suffers from several inherent problems. A major problem of former models is that the large chambers must be opened to access the rat-holding cages, resulting in leakage of potentially large amounts of alcohol into the environment. Significant time is then necessary to re-equilibrate the system. This usually interferes with serial measurement of either BALs and/or other biological parameters. A related problem, in particular for endocrine studies, is that this method does not allow the use of cannulae (iv or otherwise) for either injection or collection of samples because there is no direct access to the animal cages. A third problem is that all animals are exposed to the same concentration of alcohol vapors, which prevents manipulation of the system to adjust individual BALs. Consequently, all animals in the same chamber must be exposed to the same alcohol regimen. In view of these problems, we developed a new paradigm in which small cages containing 1–2 rats are individually controlled with regard to drug delivery, which were developed by La Jolla Alcohol Research, Inc. (La Jolla, CA). This allows the animals to carry cannulae for the collection of biological samples and the delivery of treatments without compromising the integrity of the system and without handling the animals. It also allows for individual manipulations of BALs, if desired.

In this new system, 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 delivered 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. Airflow controlled by a pressure gauge is delivered to the flask and serves to carry the alcohol vapors to the individual boxes. It then leaves the box through an outlet flow tube connected to a vacuum and reaches one of 16 cages (8.5" H × 10.25" W × 18.5" L), each of which is divided into two compartments such that we can accommodate 32 rats/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 is in turn connected to two inhalation chambers. The lids of the boxes contain a 2 mm porthole in order to accommodate i.v. 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. The integrity of the system (i.e., the absence of leakage of vapors to the outside environment) was assessed by monitoring alcohol concentrations during drug delivery. The system was found to be air-tight, and the variability in the alcohol concentration between similarly controlled boxes was <10%. Finally, because previous systems were plagued by significant problems of condensation, our goal was to determine the maximum airflow rate that could be delivered to the chamber with minimal condensation. The overall parameters were found to be: air pressure = 10 psi, airflow rate = 10 liter/min and alcohol flow rate = 10–75

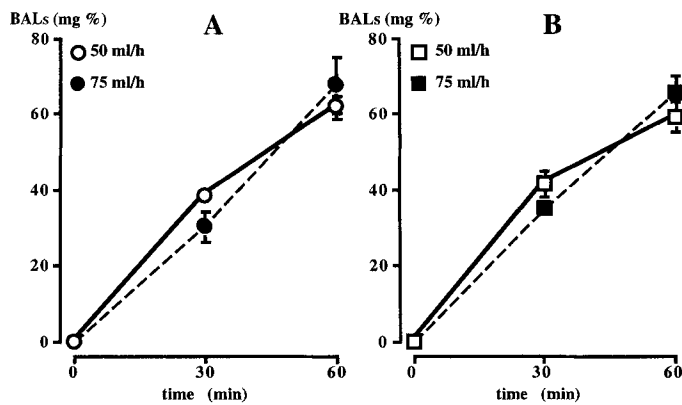


Fig. 1. Effect of two alcohol flow rates (50 and 75 ml/h) on BALs measured over a 60-min time frame. Each point represents the mean \pm SEM of 8 rats (A group, 150–170 g body weight; B group, 270–290 g body weight). mg%, mg%.

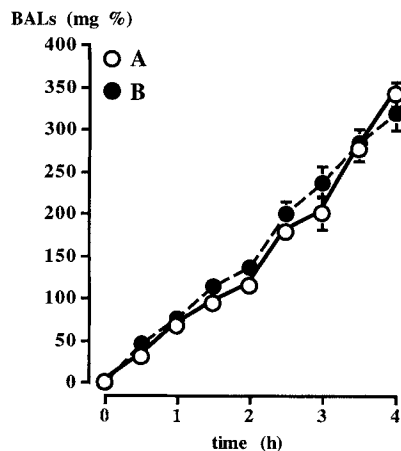


Fig. 2. BALs of group A (150–170 g body weight) and group B (270–290 g body weight) measured during 4 hr of alcohol vapors, delivered at 50 ml/hr. Each point represents the mean \pm SEM of 8 rats. mg%, mg%.

ml/hr. To further minimize condensation, we added a dryer in line with the air source and monitored the accumulation of moisture on the walls of the chamber. In 7 days of continuous operation, there was no condensation on the walls of the boxes whether animals were present or not.

Alcohol Treatment

For the experiments illustrated in Figs. 1–4, rat weights, flow rates of alcohol delivery and times over which the drug was given, were varied in order to provide general information regarding the new system we used. For all other experiments, rats in the 270–290 gm weight range were used. Animals were exposed to alcohol for 8 days. Our goal was to reach BALs comprised between 180 and 220 mg% because in our experience, these levels are the lowest that lead to consistent and homogeneous changes in HPA axis activity. Previous work has shown that brain alcohol concentrations are closely correlated to BALs (Smith et al., unpublished data, 1999). Under conditions that were not stressful and did not induce condensation, it took 4 hr to reach these BALs. We then aimed at maintaining them at about 200 mg% range for an additional 2 hr, a time chosen because we wanted no residual alcohol in the rat circulation by the time lights went off so that the animals would feed normally. Indeed, measurement of BALs indicated that daily maximum values, measured at the 6 hr time point, were 198 ± 20.4 mg%, and nondetectable 6–7 hr later. It may be useful to mention, however, that significantly higher BALs can be reached if the animals are exposed to alcohol continuously, rather than with the intermittent delivery system that we used for the present studies. Control (C)

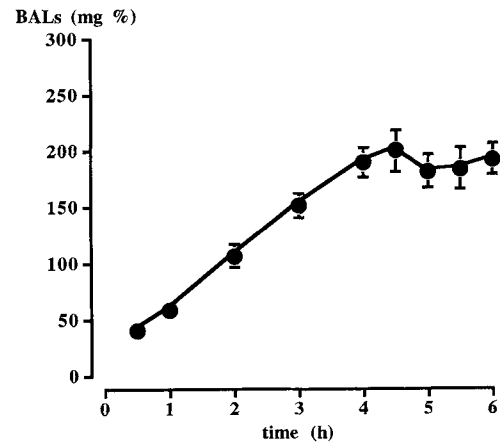


Fig. 3. BALs in group B rats (270–290 g body weight) exposed to alcohol delivered at 50 ml/hr for 4 hr, then switched off and on every 30 min for an additional 2 hr. Each point represents the mean \pm SEM of 8 animals. mg%, mg%.

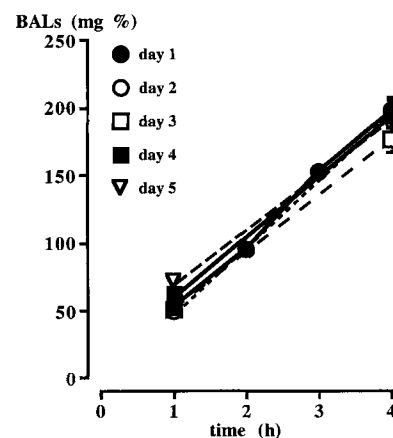


Fig. 4. Effect of repeated daily exposure to alcohol (50 ml/hr for 4 hr) on BALs in group B rats (270–290 gm) for 5 days. Each point represents the mean \pm SEM of 8 animals. mg%, mg%.

animals were placed in similar boxes, but only exposed to normal air (delivered at the same rate as alcohol, 10 psi). All animals were returned to their home cages at the end of each daily 6 hr treatment, where they had free access to food (Purina chow, Purina, St. Louis, MO) and water.

BALs Measurement

BALs were measured in 5 μ l plasma using an Analox AM 1 analyzer available from Analox Instruments LTD (Lunenburg, MA). The reaction is based on the oxidation of alcohol by alcohol oxidase in the presence of molecular oxygen (alcohol + O₂ \rightarrow acetaldehyde + H₂O₂). Under the conditions of the assay, the rate of oxygen consumption is directly proportional to the alcohol concentration. Single point calibration is done for each set of samples with reagents, provided by Analox Instruments, that read 100 mg/dl (21.7 mmol/liter). The sensitivity of the assay is 0.1 mg/dl, the precision is 1–2% and the curve is linear up to 400 mg/dl. Values for plasma are obtained within 10 sec, which allows for precise and rapid adjustments of alcohol delivery, if needed.

Procedures

All assays were carried out on the morning of day 9, with the last alcohol session having ended at 1300 the previous day. The animals were sacrificed under basal conditions (i.e. within 10 sec of removal from their

home cage), exposed to shocks [0.5 mA, 1 sec, 2 shocks/min for 30 min as previously described (Rivier and Vale, 1988)], or injected with LPS (#3755, serotype 026:B6, purchased from Sigma Corp. St. Louis, MO) diluted in apyrogenic saline. Control animals were either not exposed to the shocks or injected with the vehicle. Brains or blood samples (0.3 ml) were obtained at the times described for each experiment (see Results).

Plasma ACTH and Corticosterone Levels

ACTH levels were measured with a commercially available two-site immunoradiometric assay (Allegro kit, Nichols Institute, San Juan Capistrano, CA) which we have validated for use in the rat (Rivier and Shen, 1994). Assay sensitivity was 5 pg/ml, and the intra- and interassay coefficients of variation were 3.2 and 6.8%, respectively. Corticosterone levels were measured with a rabbit anticorticosterone-3-BSA antiserum obtained from Dr. G. Niswender (Boulder, CO). Assay sensitivity was 5 ng/ml, and the intra- and interassay coefficients of variation were 7.3 and 13.2%, respectively.

CRF and VP Receptors

Levels of CRF receptors type 1 (CRFR1) and VP receptors type 1 beta (VPR1 β) mRNA, which are involved in the control of ACTH release (Aubry et al., 1997; Chalmers et al., 1996; Ostrowski et al., 1992), were measured by Northern blot analysis. Pituitaries of 10 rats in each group were obtained by decapitation, pooled in groups of two, and stored at -70°C until RNA extraction. Total RNA was isolated from whole pituitaries by Tri-Reagent (MRC, Cincinnati, OH). Twenty-to-sixty μg of RNA was denatured in 2.2 M formaldehyde and 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 EDTA buffer. After electrophoresis, RNA was transferred to a zeta-probe blotting membrane (Bio-Rad, Hercules, CA), UV cross-linked, and baked at 80°C for 2 hr under vacuum. The RNA blot was incubated at 65°C for 4 hr in prehybridization buffer containing $5 \times$ SSPE ($1 \times$ SSPE: 0.01 M phosphate buffer, pH 7.0/0.15 M NaCl), $10 \times$ Denhart's solution (0.02%, w/v, each of bovine serum albumin, Ficoll 400, and polyvinylpyrrolidone), 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]-UTP at 65°C in the same buffer overnight. Filters were washed successively in $1 \times$ SSPE/0.1% SDS, $0.1 \times$ SSPE/0.1% SDS, and $0.1 \times$ SSPE at 65°C . After posthybridization washes, membranes were air dried. Quantitative analysis was performed using PhosphorImager system (Molecular Dynamics, Sunnyvale, CA) and the ImageQuant (Molecular Dynamics, Sunnyvale, CA) 4.0 software packages. To obtain accurate measurements, hybridization with GAPDH probe was performed as an internal standard. The ratios of specific signal to GAPDH were calculated to estimate the levels of these signals. This procedure has been previously described by our laboratory (Aubry et al., 1997).

Quantitative Immunofluorescence Microscopy of CRF and VP in the External Zone of the Median Eminence (ZEME)

Immediately after decapitation, the brains were removed from the skull and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.6) for 2 hr at 4°C . Then they were incubated in 5% sucrose/0.1% NaN_3 in 0.1 M phosphate buffer and shipped on regular ice to the Netherlands, where they arrived, unfrozen, within 48 hr. This procedure ensures that the median eminence (ME), but not necessarily the inner brain (which is not analyzed in this method), is fixed. Furthermore, as routine procedures in the Netherlands' laboratory involve a 48–72 hr immersion of the brains in PBS-buffered sucrose, there is no impact of travelling time on brain fixation. Immediately upon arrival, the mediobasal hypothalami were dissected and up to 30 specimen were embedded together in a cryomold containing Tissue-Tek OCT compound. Cryomolds were frozen in isopentane cooled with liquid nitrogen and serial frontal sections (10 μm) were cut at -20°C by using a motor driven cryostat (Micron HM 500 M,

Walldorf, Germany). Adjacent sections were incubated with rabbit anti-rat/human CRF antiserum (5Bo, 1:500) or with rabbit anti-VP antiserum (Truus, 1:1000) for 2 days at 4°C , followed by incubation with goat anti-rabbit antiserum conjugated to FITC (TAGO, Burlingame, CA) for 2 hr at room temperature (21°C). Thereafter, the sections were mounted in Vectashield anti-fading medium (Vector, Burlingame, CA). The antisera were diluted in an incubation buffer which consisted of 0.1 M Tris buffered saline (TBS, pH 7.6) containing 0.5% Triton X-100, 0.2% BSA, 1% normal goat serum (NGS, Dakopatts, Glostrup, Denmark) and 0.01% sodium azide.

Quantification of the immunofluorescence staining intensity was performed as described previously (Berkenbosch and Tilders, 1988; Schmidt et al., 1995; Tilders et al., 1989) by using a computer-controlled microfluorimeter (Leitz-Combi, Leitz, Wetzlar, Germany). Briefly, from each median eminence, three sections were analyzed between the levels A 4100 and A 4500 according to Koenig and Klippel, (1963). In each section, 10 measurements (spot diameter 12.5 μm) were made in the ZEME. In the same sections, 6 background measurements were collected in the arcuate nucleus, which does not show CRF or VP immunostaining. After background subtraction, the mean fluorescence intensity was calculated for each ZEME. Previous validation studies in animals that were manipulated to achieve increases or decreases of the CRH content showed good correlation between quantitative immunocytochemical data and CRF concentrations as measured by radioimmunoassay in median eminence extracts ($r = 0.86$ – 0.99) (Berkenbosch and Tilders, 1988; Suis et al., 1988). For specificity of the VP-antiserum "Truus" (provided by Dr R.M. Buijs) see Suis et al. (1988). For specificity of the CRF antiserum "5Bo", see Schmidt et al. (1995).

Probe Preparation for Pituitary and PVN Signals

The pBluescript SK-1 vector (Stratagene, La Jolla, CA) containing rat NGFI-B cDNA (provided by Dr. J. Milbrandt) or *c-fos* cDNA (provided by Dr. I. Verma) was linearized with *Bam*HI and *Sma*I, respectively. Rat CRFR1 cDNA (Provided by Dr. W. Vale, Salk Institute, La Jolla, CA), subcloned into pBluescript SK-1 vector, and rat VPR1 β cDNA (Provided by Dr. S. Lolait, National Institute of Mental Health, Bethesda, MD), subcloned into pGEM 4z, were linearized with *Bam*HI and *Eco*RI, respectively. 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 ATP/GTP/CTP, [α - ^{35}S]-UTP (in situ) or [α - ^{32}P]-UTP (Northern), 1 U RNasin (Promega, Madison, WI) and 10 U T3 (for NGFI-B) or T7 (for *c-fos*, CRFR1 and VPR1 β) for 60 min at 37°C . Unincorporated nucleotides were removed using Quick-Spin columns (Boehringer Mannheim, Indianapolis, IN). A sense probe was used as a control for nonspecific signal in some adjacent sections for in situ hybridization.

In Situ Hybridization Histochemistry of PVN Signals

Rats were deeply anesthetized with chloral hydrate, a drug that does not increase IEGs/peptides mRNA levels. They were then perfused transcardially with saline followed by 4% paraformaldehyde/0.1 M borate buffer, pH 9.5. The brains were removed and postfixed in 4% paraformaldehyde for 4–5 days, then placed overnight in 10% sucrose/4% paraformaldehyde/0.1 M borate buffer. They were cut into 30 μm coronal slices obtained at 120 μm intervals throughout the hypothalamus, and stored at -20°C in a cryoprotectant solution (50% 0.1 M phosphate-buffered saline, 30% ethylene glycol and 20% glycerol) until histochemical analysis. Brains from control and experimental animals belonging to the same experiment were always analyzed in the same hybridization experiment. Hybridization histochemical localization of each transcript was carried out using ^{35}S -labeled cRNA probes. Protocols and autoradiographic localization of mRNA signals were adapted from Simmons et al. (1989). All solutions were treated with diethylpyrocarbonate (DEPC) and autoclaved in order to prevent RNA degradation. Sections mounted onto gelatin- and poly-L-lysine-coated slides were desiccated under vacuum overnight, fixed in 4% paraformaldehyde for 30 min and digested by

Table 1. Plasma ACTH and Corticosterone Levels in Rats Housed in Boxes Used for Alcohol Delivery, and in Absolute Controls (Bucket-Housed)

Group	1 hr	2 hr	3 hr	4 hr	6 hr
ACTH (pg/ml):					
Bucket-housed	6.48 ± 0.5	8.44 ± 0.83	8.79 ± 0.93	8.73 ± 1.68	19.83 ± 5.62
Chamber-housed	8.67 ± 2.20	9.94 ± 2.44	7.90 ± 1.57	10.59 ± 2.24	16.10 ± 3.51
Corticosterone (ng/ml):					
Bucket-housed	5.95 ± 1.14	15.67 ± 4.43	18.63 ± 4.4	19.94 ± 4.74	51.45 ± 4.45
Chamber-housed	7.75 ± 1.26	14.63 ± 6.82	18.13 ± 6.03	19.95 ± 5.87	59.50 ± 5.56

Mean ± SEM; $n = 8/\text{group}$. $p > 0.05$ between groups for each hormone and time point.

proteinase K (10 $\mu\text{g}/\text{ml}$ in 50 mM tris HCl, pH 7.5, and 5 mM EDTA, at 37°C for 25 min). Thereafter, brain sections were rinsed in sterile DEPC water followed by a solution of 0.1 M triethanolamine (TEA, pH 8.0), acetylated in 0.25% acetic anhydride in 0.1 M TEA, and dehydrated through graded concentrations of alcohol (50, 70, 95, and 100%). After vacuum drying for a minimum of 2 hr, 90 μl hybridization mixture (10⁷ cpm/ml) was spotted on each slide, sealed under a coverslip, and incubated overnight at 60°C in a slide warmer. Coverslips were then removed and the slides rinsed in 4 × SSC (1 × SSC: 0.15 M NaCl, 15 mM trisodium citrate buffer, pH 7.0) at room temperature. Sections were digested by RNase A (20 $\mu\text{g}/\text{ml}$, 37°C, 30 min), rinsed in descending concentrations of SSC (2 ×, 1 ×, 0.5 ×), washed in 0.1 × SSC for 15 min at 65°C and dehydrated through graded concentrations of alcohol. After being dried under vacuum, sections were exposed at 4°C to x-ray film (Kodak, Eastman Kodak, Rochester, NY) for 15–48 hr, defatted in xylene, and dipped in NTB2 nuclear emulsion (Kodak; diluted 1:1 with distilled water). Slides were exposed for 6–9 days, developed in D19 developer (Kodak) for 3.5 min at 15°C and fixed in rapid fixer (Kodak) for 6 min. Thereafter, tissues were rinsed in running distilled water, counterstained with thionin (0.25%), dehydrated through graded concentrations of alcohol, cleared in xylene, and cover slipped with DPX.

Quantitative Analysis of In Situ Hybridization Results of PVN Signals

Semiquantitative densitometric analysis of hybridization signals for RNAs of interest was carried out in nuclear emulsion-dipped slides. Brain paste standards containing serial dilutions of ³⁵S-UTP, used for quantification of mRNA signal, were prepared concurrently to ensure that optical density was found within the linear range of the standard curve (Chan and Sawchenko, 1995). In addition, analysis with emulsion-coated slides was carried out with 2–3 different exposure times in order to confirm that signals were not saturated. Densitometric analyses of autoradiographic signals was done over the confines of cells within the PVN using a Leitz optical system (Wetzlar, Germany) coupled to a Macintosh II computer and Image software (version 1.61, W Rasband, NIH). Dark-field measurements for the parvo- (p) and magnocellular (m) divisions of the PVN were obtained separately, as previously reported (Lee and Rivier, 1994b). Gray level measurements (optical density, O.D.) were taken under darkfield illumination of hybridized sections over the medial pPVN or mPVN, as defined by redirected sampling from the corresponding Nissl stained sections under brightfield images. Data were expressed in gray scale values of 1 to 256. All gray level measurements were corrected for background. Signals were measured in both sides of the brain, and mean values for all animals (4–6/group) were determined in 3–4 sections for each rat throughout the PVN. Both *c-fos* and NGFI-B transcripts were measured in the brains of rats exposed to shocks or LPS, or their corresponding controls. In order to illustrate the fact that both yielded comparable results, we chose to illustrate data obtained in the shock experiments with *c-fos* and those obtained in the endotoxemia paradigm with NGFI-B mRNA expression.

Statistical Analysis

Results of plasma ACTH levels were analyzed by ANOVA for single or repeated measures. Data of ZEME were analyzed by one- or two-way ANOVA followed by the Fisher Least Significant Difference test using the

NCSS2000 statistical software program (Kaysville, UT). ANOVA was preceded by testing for homogeneity of variance and for normality of residuals. Posthoc testing was only performed when the ANOVA yielded significant *F*-values. Data of in situ hybridization experiments are expressed as O.D. (arbitrary units), and were analyzed by ANOVA and posthoc comparisons using Scheffe test (Statview II, SAS, Cary, NC). All brain sections hybridized at the same time were included in the same statistical test.

RESULTS

Validation of the Alcohol Model

Environment. The first set of experiments was designed to show that exposing the rats to the individual boxes, at an air flow rate corresponding to the highest one used to deliver alcohol, was not stressful in the absence of the drug. Rats that had been implanted with i.v. cannulae 72 hr earlier were placed in the boxes and allowed to recover for 3 hr so that hormone levels that had been increased by the handling, returned to baseline. In order to provide a comparison with nonstressed conditions routinely used in our laboratory for the obtainment of blood samples [see for example (Lee and Rivier, 1994a, 1997b; Rivier, 1993, 1995)], a different group of rats of similar weights was placed in opaque buckets kept in a separate room (absolute controls). Plasma ACTH and corticosterone levels were measured between 1000 and 1600 in both groups of animals. As indicated in Table 1, there was no difference ($p > 0.05$) between the two groups for corresponding hormones at each of the studied time points. All levels remained at concentrations considered basal for these times of day and displayed the expected circadian rhythm-entrained increase of late afternoon (Dallman et al., 1987; Turnbull and Rivier, 1996).

Alcohol Exposure. For these experiments, we varied rat weights between 150 and 310 gm, flow rates between 50 and 135 ml/hr, and exposure times between 1 and 8 hr. Data were very similar across weights, and the results obtained in rats weighing 270–290 g are illustrated in Fig. 1 and 2. Although the 50 and 75 ml/hr flow rates did not cause condensation, 135 ml/hr did. This rate was therefore not used beyond pilot studies. To determine the influence of flow rate on BALs, alcohol was first delivered at 50 or 75 ml/hr for 60 min. As indicated in Fig. 1, virtually identical BALs ($p > 0.05$ between groups for each time) were obtained at the two flow rates in both weight groups. These results, which were subsequently extended to longer time frames, indicate that increasing the flow rate from 50 to 75

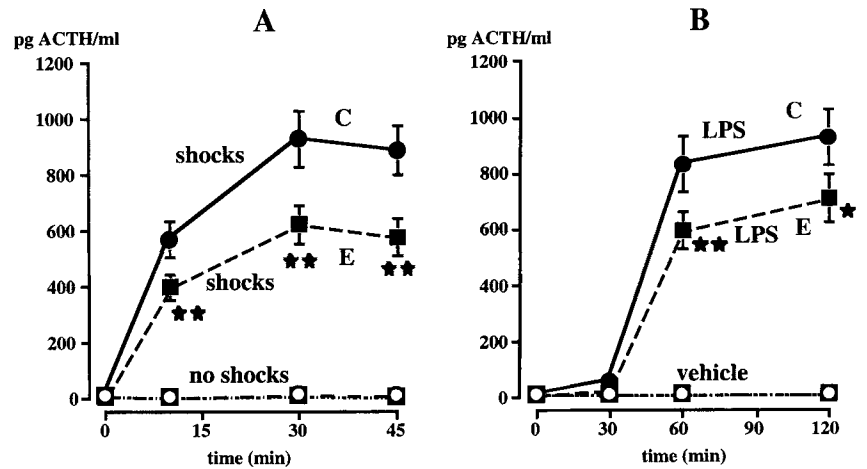


Fig. 5. Plasma ACTH levels in control rats or rats exposed to shocks (A) or LPS (B) at the end of a 8 day regimen of intermittent alcohol vapors. C, control, E, alcohol-exposed. Open symbols, no shocks (A) or vehicle (B); closed symbols, shocks (A) or LPS (B). Each point corresponds to the mean \pm SEM of 6–7 animals. *, $p < 0.05$; **, $p < 0.01$ vs. C.

ml/hr did not induce higher BALs at any given time and did not allow us to reach them more quickly. However, lower BALs can be achieved by decreasing the flow rate below 50 ml/hr (not shown). The flow rate of 50 ml/hr was then used to investigate increases in BALs over a 4 hr treatment period. Comparable results ($p > 0.05$ between groups for each time) were obtained in rats weighing 150–170 or 270–290 g body weight (Fig. 2), suggesting no obvious differences in alcohol metabolism as a function of weight. The next set of experiments was designed to determine whether, once a target BAL had been reached, we could maintain it for at least 2 hr. We illustrate here representative results chosen because they correspond to the conditions (270–290 gm rats, 50 ml/hr flow rate, 6 hr total daily exposure, BALs = 180–220 mg%) used for the experiments illustrated in Figs. 5–11. Alcohol was delivered at the set flow rate for 4 hr, and was then switched on and off every 30 min for an additional 2 hr. As expected from the data shown in Fig. 2, target BALs were obtained by 4 hr. They were then maintained without significant deviation ($p > 0.05$) for another 2 hr (Fig. 3), a time course that can be expanded to 4 hr with the same consistent BALs (not shown). Finally, we determined whether comparable BALs could be achieved and maintained when the rats were exposed to alcohol on a daily basis. BALs were measured hourly for the 1st day, then at 1 and 4 hr on subsequent days. As illustrated in Fig. 4, statistically comparable BALs ($p > 0.05$ between each day at $t = 1$ and 4 hr) were achieved and maintained over the time course of this experiment. Similar results were found when alcohol was delivered 6 hr daily for 8–9 days (not shown). Because we also wanted to examine the influence of alcohol on body weight gains, we included a control group not exposed to the drug. Alcohol hampered growth rates for the first 2 days only. On day 5, these rates were not different between groups (weight gains: controls, 3.4 ± 0.3 gm/day; alcohol-exposed, 3.1 ± 0.5 gm/day; $p > 0.05$. Absolute weights: controls, 258.3 ± 3.4 gm; alcohol-exposed: 249.3 ± 6.6 gm; $p > 0.05$).

ACTH Response to Shocks or LPS

Plasma ACTH levels were measured under basal conditions (i.e., in the absence of shocks or LPS injection, in animals whose last exposure to the chambers has ended at 1300 the day before), after shocks exposure or after LPS injection (1.0 μ g/kg, iv). There was no significant difference between resting ACTH levels of animals exposed to alcohol or those that had been placed in the chambers but not treated with the drug (Fig. 5). Both shocks (Fig. 5A) and LPS (Fig. 5B) significantly increased plasma ACTH levels but as expected (Lee and Rivier, 1993, 1994a), alcohol significantly blunted this response.

Pituitary CRF and VP Receptors

The pituitaries of rats exposed to alcohol showed a small, but not significant decrease in CRFR1 (CRFR1/GAPDH ratio: control, 0.258 ± 0.023 ; alcohol, 0.216 ± 0.06 ; $p > 0.05$) (Fig. 6) and VPR1 β mRNA levels (VPR1 β /GAPDH ratio: control, 2.240 ± 0.180 ; alcohol, 2.136 ± 0.165 ; $p > 0.05$).

CRF and VP Immunostaining in the ZEME

As shown in Fig. 7, alcohol treatment induced a significant ($p < 0.05$) increase in VP stores in the ZEME whereas the CRF stores significantly ($p < 0.01$) decreased. Although we did not analyze the fraction of terminals that co-expressed VP and CRF (a procedure that requires quantitative electron microscopy), previous studies (deGoeij et al., 1993; Schmidt et al., 1995; Whitnall et al., 1987) allow us to postulate that increases in the VP content in the ZEME were probably accompanied by increases in the fraction of CRF terminals that co-stored this peptide.

Basal and Stimulated PVN Neuronal Responses to Shocks

Based on previous findings regarding the time course of neuronal *c-fos* response to shocks or LPS, the rats were sacrificed at the end of a 30 min shock session or 1–3 hr

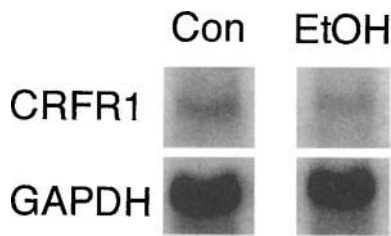


Fig. 6. Pituitary mRNA levels of CRFR1 in control (Con) and alcohol-exposed (EtOH) rats shown by Northern blot analysis. This representative autoradiogram was obtained with 20 μ g RNA. The size of the mature CRFR1 mRNA is 2.7 Kb.

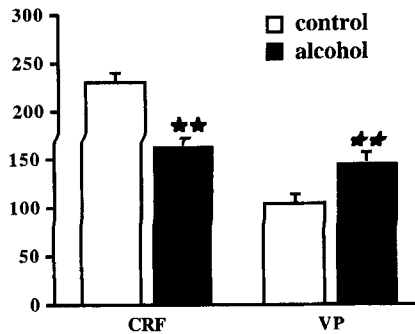


Fig. 7. CRF and VP levels in the external zone of the median eminence (ZEME) of control and alcohol-exposed rats. Data are expressed in arbitrary units of immunofluorescence and were determined by quantitative immunofluorescence microscopy. Each bar represents the mean \pm SEM ($n = 10$). **, $p < 0.01$.

after LPS, when peak levels of *c-fos* mRNA expression were found for the respective stimuli (Lee et al., 1995; Rivest and Rivier, 1994). After an 8-day alcohol exposure, or at a corresponding time in controls, basal *c-fos* mRNA levels were slightly, but not significantly, elevated in the pPVN of E, compared with C rats (Fig. 8 and 9). In contrast, they were markedly up-regulated in the mPVN of animals treated with alcohol. Exposure to shocks up-regulated *c-fos* mRNA levels, measured 30 min after the onset of the stress, in the pPVN of both groups of animals, but this response was significantly blunted ($p < 0.01$) in E animals. Shocks also increased *c-fos* mRNA levels in the mPVN of C animals, but did not alter these signals over baseline concentrations in E rats. Similar results were obtained with NGFI-B mRNA levels (not shown).

Basal and Stimulated PVN Neuronal Responses to LPS

As in the case of *c-fos*, NGFI-B mRNA levels measured in the absence of an acute stimulus were slightly, but not significantly, elevated in the pPVN of E, compared with C rats, but were markedly up-regulated in their mPVN (Fig. 10 and 11). The i.v. injection of LPS (100 μ g/kg, a dose we found to elicit significant and consistent PVN neuronal responses), measurably ($p < 0.01$) stimulated PVN neuronal activity over the usual time course of this response [30–180 min, see for example (Lee, et al., 1995)]. Peak responses were observed at 2 hr in both groups of animals and the response of E rats was significantly lower than in C animals.

DISCUSSION

We report here that chronic exposure of adult male rats to intermittent alcohol vapors produced significant changes in CRF and VP levels in the PVN and the ZEME that were associated with a blunted activity of the HPA axis. Because our results show, in particular, differences between responses in the pPVN and mPVN, and because peptide content in the ZEME is differentially influenced by the groups of cells, it may be useful to first briefly discuss the role of these two regions in the regulation of the HPA axis. The pPVN contains a variety of neuropeptides, including CRF and VP [see for example (Sawchenko and Swanson, 1990; Swanson, 1987; Watts, 1996; Whitnall, 1993)]. Neurons that actively participate in the regulation of ACTH release have terminals in the ZEME, from which the peptides are transported to the anterior lobe of the pituitary. Interestingly, pPVN CRF neurons can also express VP, and changes in the percentage of perikarya that contain both peptides occur during certain types of stresses. Although there are very few CRF cell bodies in the mPVN, this region contains a large number of neurons that synthesize VP (Antoni, 1993). The axons of most of these cells terminate in the posterior lobe of the pituitary, and while their terminals do not release VP into the portal system, some of the axons passing through the internal layer of the ME can release VP-containing secretory granules. This VP, which can gain access to the portal circulation, can therefore influence ACTH release. Consequently, three anatomically and functionally distinct hypothalamic pathways to the ME are involved in the regulation of ACTH secretion: CRF primarily derived from the pPVN, VP also derived from the pPVN, and some of the VP manufactured in the mPVN.

The most salient finding was that long-term alcohol impaired the neuronal response of the pPVN to shocks or LPS. To our knowledge, this is the first report that changes in HPA axis activity during chronic alcohol exposure are accompanied by significant changes in PVN neuronal activity. Interestingly this was not accompanied by changes in IEGs expression when the animals were sacrificed 20 hr after the last session of drug treatment, but in the absence of an acute stressor. There are many examples of stimuli which, although capable of altering HPA axis activity and PVN IEG expression in response to homeostatic challenges, do not significantly affect resting levels of a particular signal. For example, blockade of nitric oxide formation, which significantly blunts the response of PVN IEGs, CRF, and VP gene expression to neurogenic stresses, does not measurably modify baseline levels of these transcripts (Lee and Rivier, 1998). Also, the paradigm we presently use for alcohol exposure of pregnant dams (which is the same as the one described here) was found to increase the neuronal responses of the PVN to a variety of stresses without altering basal activity (Lee et al., unpublished data, 1999). It was therefore not surprising that resting concen-

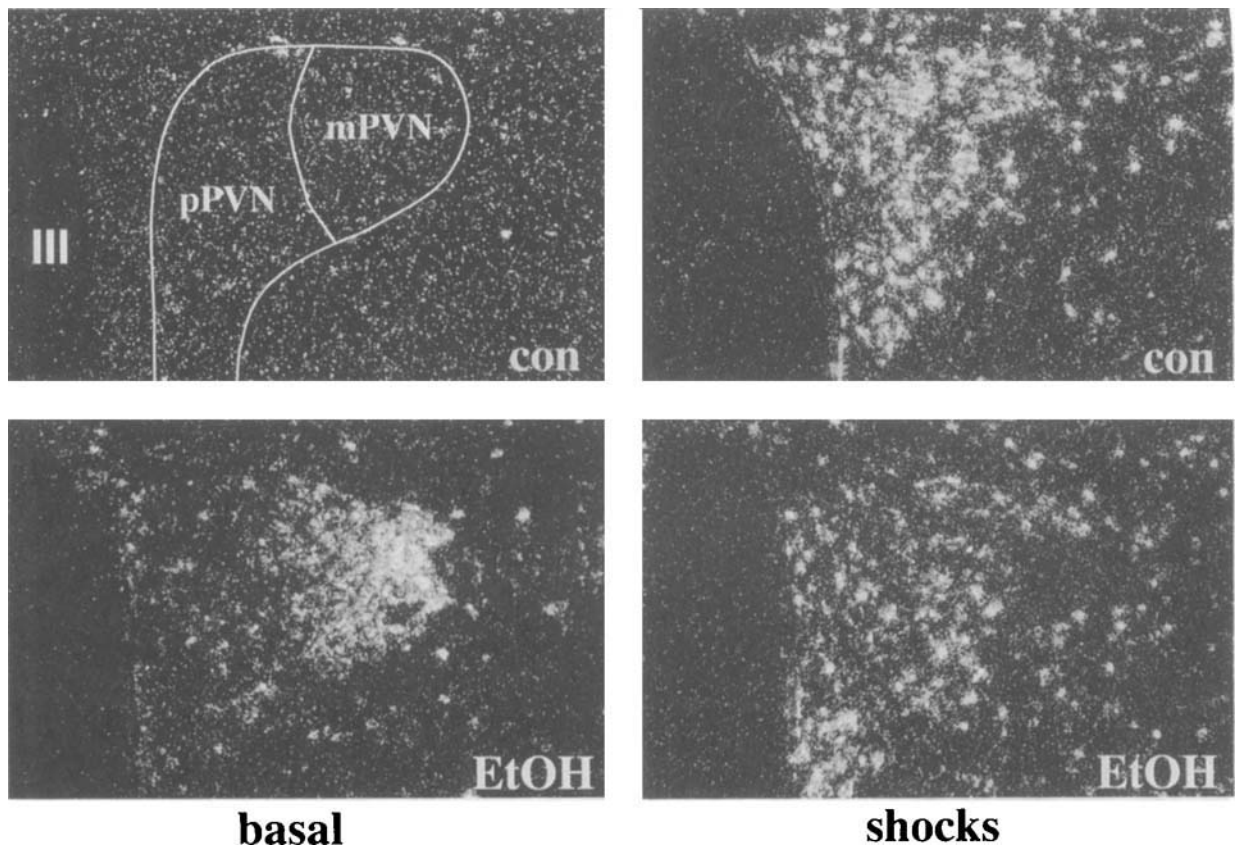


Fig. 8. Basal or stress-induced (0.5 mA electrofootshocks, 1 sec duration, 2 shocks/min for 30 min) mRNA levels of *c-fos* measured in the PVN of rats previously exposed to alcohol vapors (EtOH) (6 hr/day for 7–8 days), or control rats (Con). Brains, which are shown in coronal sections, were collected before (basal) and at the end of the shock session (shocks). III, third ventricle. Magnification, 340x. pPVN = parvocellular division of the paraventricular nucleus. mPVN = magnocellular division of the paraventricular nucleus.

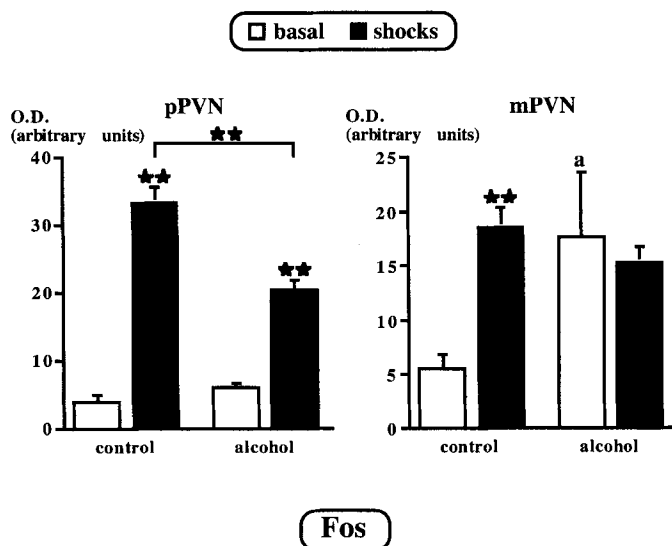


Fig. 9. Statistical analysis of the results of Fig. 8. **, $p < 0.01$ vs. basal unless otherwise mentioned; a, $p < 0.05$ vs. basal control. pPVN = parvocellular division of the paraventricular nucleus. mPVN = magnocellular division of the paraventricular nucleus.

trations of IEGs in the pPVN of rats exposed to alcohol for several days were essentially similar in C and E rats.

The pPVN of rats acutely exposed to shocks or LPS at

the end of a prolonged alcohol treatment showed a blunted increase in levels of IEG transcripts, compared with control animals. Although we did not identify the neurons that showed this decreased neuronal activity, we previously reported that alcohol exerts a significant stimulatory influence on CRF and VP perikarya (Lee and Rivier, 1997a,b; Rivier and Lee, 1996; Rivier et al., 1996). We have also shown that these two peptides represent essential modulators of the stimulatory effect of alcohol on the HPA axis [also see discussion in (Rivier, 1996)]. It therefore seems reasonable to propose that a significant percentage of the pPVN neurons that showed decreased response to shocks or endotoxemia manufacture CRF and/or VP. In the rat, changes in PVN levels of CRF peptide often remain undetectable unless the animals are exposed to blockers of axonal transport or to a stringent manipulation, such as adrenalectomy. As a result, many investigators use colchicine to block axonal transport and increase hypothalamic content of the peptide. However, this treatment is stressful and we prefer not to use it. Future studies will therefore address the contribution of changes in neurons that express CRF and VP by in situ hybridization and/or the identification of IEGs in neurons that contain these peptides. What mechanisms could account for this blunted PVN neuronal activity? Alcohol is unique as a drug because it seems to

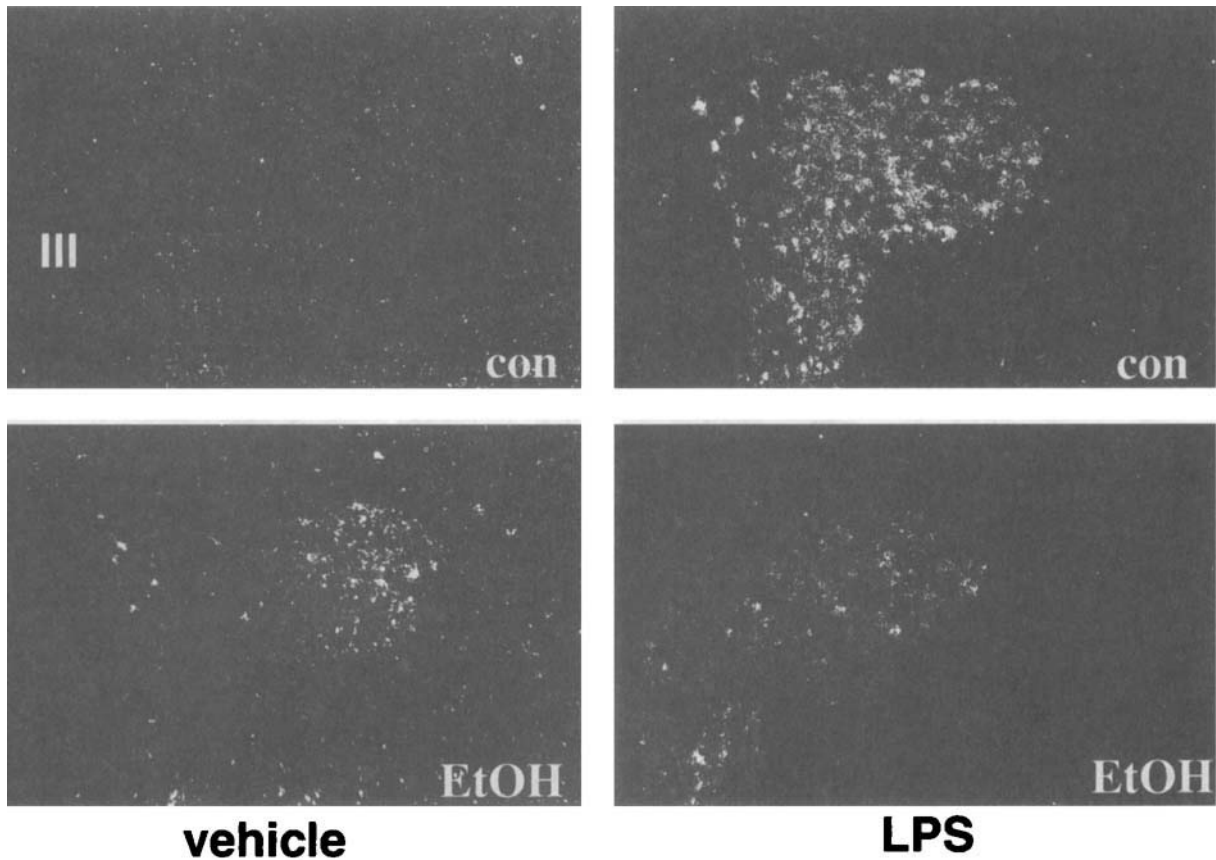


Fig. 10. Effect of the i.v. injection of the vehicle or LPS (100 $\mu\text{g}/\text{kg}$) on NGFI-B mRNA levels in the PVN of rats previously exposed to alcohol vapors (EtOH) (6 hr/day for 7–8 days), or vehicle control rats (Con). Brains, which are shown in coronal sections, were collected 2 hr after i.v. treatment. III, third ventricle. Magnification, 340x.

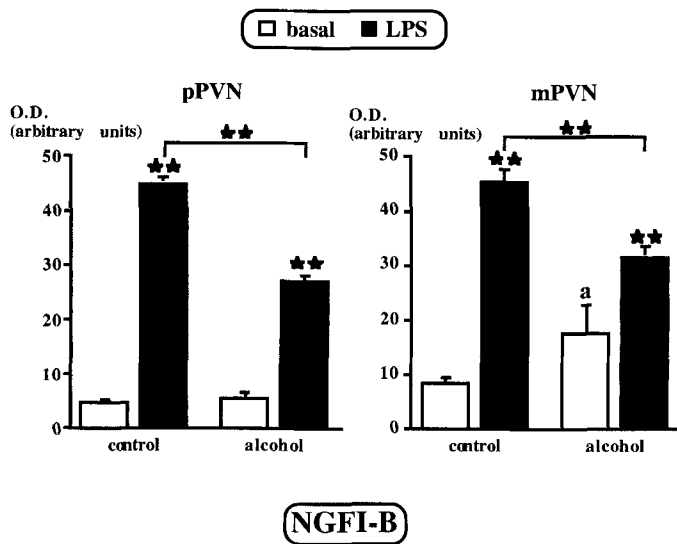


Fig. 11. Statistical analysis of the results of Fig. 10. **, $p < 0.01$ vs. basal unless otherwise mentioned; a, $p < 0.05$ vs. basal control.

have multiple primary targets that include ligand-gated ion channels (such as those associated with GABA, NMDA, 5-HT), transporters, catecholamines, serotonin, glutamate, opioids, dopamine, and acetylcholine (Eckardt et al., 1998; Faingold et al., 1998; Woods and Druse, 1996). As these neurotransmitters exert both stimulatory and inhibitory ef-

fects on the PVN, and as their responses to long-term alcohol exposure is not fully understood, it is very difficult to predict which ones are influential in our model. One hypothesis that we are presently pursuing is that nitric oxide (NO), a gas now recognized for its ubiquitous actions in the CNS, may be involved. The facts that NO stimulates CRF and VP synthesis in the PVN (Lee et al., 1999) and that prolonged alcohol ingestion reportedly decreases NO production in certain brain areas (Fitzgerald et al., 1995), suggests the possibility that at least part of the inhibitory action of long-term alcohol on the HPA axis may be due to decreased NO levels in the endocrine hypothalamus.

In contrast to its lack of effect on pPVN neuronal activity in the absence of additional stressors, alcohol markedly increased basal expression of IEG in the mPVN. These results are in agreement with previous reports suggesting that long-term alcohol treatment induced the accumulation of VP peptide levels in magnocellular neurons of the hypothalamus. In one of these reports (Carmona-Calero, et al., 1995), the authors attributed their finding to the inhibition of VP release induced by long-term alcohol exposure, but their hypothesis was not validated by measurement of plasma levels of this peptide, whereas a much shorter alcohol treatment (7 days) was reported not to alter this parameter (Gulya, et al., 1991). In contrast, several other investigators reported a down-regulation of hypotha-

lamic VP mRNA after chronic exposure to alcohol (Gulya et al., 1991; Ishizawa et al., 1990; Madeira and Paula-Barbosa, 1999; Rehbein et al., 1986), which may either indicate increased translation of the VP gene or relate to differences in experimental design. It should also be noted that as discussed above, changes in mPVN VP are also likely to influence ACTH release. We pointed out that there are several mechanisms through which VP manufactured in magnocellular axons can gain access to the pituitary portal circulation [see discussion in (Antoni, 1993; Kjaer, 1993; Sawchenko, 1991; Sawchenko et al., 1996)]. Nonterminal portions of magnocellular axons that pass through the internal layer of the ME may release secretory granules by exocytosis in proximity of the portal vasculature. Magnocellular VP might also reach the anterior pituitary via so-called short pituitary portal vessels. Finally, terminals from magnocellular VP cell bodies, by terminating in the neurohypophysis, contribute to the release of this peptide in the general circulation. Collectively, these findings are consonant with the concept that magnocellular vasopressinergic neurons influence ACTH secretion [see for example (Aguilera et al., 1994)], but they greatly complicate our understanding of the functional relationship between changes in hypothalamic activity, ZEME peptide stores, and pituitary receptors. Because the mPVN contains many neurons that produce peptides other than VP, we cannot, on the basis of our experiments, determine the nature of the peptide signal that is influenced by alcohol. Indeed, the purpose of our work was not to identify which secretagogues were altered in the hypothalamus of E rats, but to test whether their blunted ACTH responsiveness might at least in part result from decreased PVN activity. In view of the present findings, it is obvious that future experiments are needed to further explore this issue.

That alcohol can stimulate CRF and VP release from the ME is a fairly well accepted fact (Madeira and Paula-Barbosa, 1999). An unexpected finding was that of significant and opposite changes in levels of CRF and VP stores in the ZEME. It therefore seems that these changes can take place in the absence of significant alterations in basal neuronal activity in the pPVN or its afferent circuitry. In support of this hypothesis, recent studies indicate that intrinsic autonomic mechanisms within the CRF neurons are involved in the alterations of VP stores. Increased VP stores are inducible by single or repeated activation of these neurons by a variety of stimuli (Dijken et al., 1993a; Schmidt et al., 1995, 1996, 1997; Tilders et al., 1993), and even in the absence of extra-hypothalamic input (Bertini et al., 1993). Unexpectedly, because VP is known to potentiate CRF-induced ACTH secretion (Rivier and Vale, 1983, 1985), alcohol-induced increases in VP levels in the ZEME, and consequently increased delivery of VP to the corticotrophs (deGoeij et al., 1992b, 1993; Schmidt et al., 1995), were associated with blunted ACTH responses to alcohol, shocks, and LPS. However, the congruent alcohol-induced blunted ability of VP to elicit ACTH release, presumably

due to homologous receptor down-regulation [see for example (Carmona-Calero et al., 1995; Hauger et al., 1990; Madeira and Paula-Barbosa, 1999)], as well as decreased CRF drive/stores, may well be responsible for the decreased HPA axis responsiveness. In support of this concept, IL-1-induced increases in VP stores, which were associated with ACTH hyper-responsiveness, were not accompanied by decreased CRF stores (Schmidt et al., 1995), diminished VP binding, or blunted VP sensitivity of the pituitary (Tilders et al., unpublished data, 1999). As increases in VP levels, induced by single or repeated stressors, are often not accompanied by decreased CRF stores [see for example (Schmidt et al., 1996; Tilders and Schmidt, 1998; Tilders et al., 1993)], it seems that alcohol treatment may have rather unique effects on the transcription and/or translation of the CRF gene in the PVN.

We had previously reported that while corticotrophs' responsiveness to CRF appeared unaltered in rats exposed to alcohol for 7 days, VP-induced ACTH secretion was significantly blunted (Rivier, 1995). Here, we failed to observe significant alcohol-induced decreases in receptor transcripts for these two peptides. It should be noted, however, that attempts to correlate changes in the response of specific cells to a secretagogue with those of receptors for this secretagogue, are fraught with difficulties. This can be partly explained by the fact that in many situations, a partial decrease in ligand binding does not modify the maximal response to this ligand. This situation is linked to the concept of "spare receptors" [see for example (Camps et al., 1992; Dennis et al., 1992; Webb et al., 1992)], according to which only a small number of receptors need to be occupied for full biological activity. In addition, changes in receptor affinity, occupancy, and internalization also modulate receptor-mediated events, and further studies are needed to determine if these parameters were altered by alcohol. It is feasible, therefore, that decreased pituitary responsiveness can take place in the absence of measurable changes in receptor transcripts. If, on the other hand, changes in receptor transcripts do occur in parallel with changes in ligand-induced biological activities, the fact that pituitaries contain significantly larger amounts of CRFR1, compared with VPR1 β [(Aguilera et al., 1994; Morel et al., 1992; Ostrowski et al., 1992, 1994) and present work] may have relevance for our findings. Specifically, even though levels of both receptors showed comparable decreases in response to alcohol, the resulting absolute decrease may be much larger for VPR1 β . This might at least in part explain why these changes were accompanied blunted pituitary responsiveness to VP, but not to CRF (Lee and Rivier, 1995). Overall, however, we will need to establish the relationship between peptide receptor message and protein levels if we want to provide convincing evidence that changes in the density of these receptors play a role in the blunted ACTH response that we observed in rats chronically exposed to alcohol.

In conclusion, we have shown that exposure of intact rats

to intermittent alcohol vapors for several days increased the basal expression of IEG in cell bodies of the magnocellular, but not the parvicellular division of the PVN, indicating enhanced basal neuronal activity. This was accompanied by a reduced ability of the pPVN to respond to a neurogenic or systemic stressor, as indicated by blunted induction of IEG in this area, as well as by a diminished ACTH response to these stressors. In addition to an impaired responsiveness of the pPVN, evidence was found that blunted HPA activity may involve hampered VP and CRF signaling toward the pituitary, as indicated by decreased CRF stores in the ZEME and the decreased ability of VP to stimulate ACTH secretion. These results clearly deserve further investigation. It will be important, in particular, to determine whether alcohol acts directly on PVN neurons and/or on their afferent circuitry, to further uncover which neurotransmitters are involved, as well as to identify the molecular and cellular basis for these effects.

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