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Alcohol Selectively Attenuates Stress-Induced c-*fos* Expression in Rat Hippocampus

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The ability of ethyl alcohol to modify responses to stress has been well documented (cf. Pohorecky, 1990). However, the structural substrate mediating these effects of alcohol remains undefined. Using immediate early gene (IEG) expression in the brain as a marker of altered neuronal response, we investigated the effect of acute alcohol exposure on the activity of brain regions of rats exposed to 15 min of restraint stress. Immunocytochemical localization c-Fos protein demonstrated that restraint stress led to an induction of c-Fos expression in several brain structures including cingulate and piriform cortex, cortico-amygdaloid and hippocampo-amygdaloid transition zones, hippocampus, hypothalamus, supramammillary nucleus, and centromedial nucleus of thalamus. An intraperitoneal injection of 2 g/kg alcohol prior to stress decreased c-Fos expression in several but not all of these structures. In particular, alcohol strongly attenuated the stress-induced expression of c-Fos in hippocampus and cingulate cortex. Using slot-blot hybridization, significant induction of c-*fos* mRNA after restraint stress was demonstrated both in hippocampus and cortex, but prior alcohol exposure specifically attenuated c-*fos* induction only in the hippocampus. The response of c-*fos* mRNA expression to stress and alcohol differed from the effects on *jun*-B, c-*jun* and *jun*-D mRNA levels. Perhaps surprisingly, acute exposure to alcohol in otherwise unstressed rats did not induce significant changes in expression of IEGs in comparison to control (saline-injected) animals even with doses sufficient to elevate plasma corticosterone. In summary, these studies demonstrate a selective sensitivity of stress-induced activity of neurons of hippocampus and cingulate cortex to acute alcohol exposure.

[Key words: immediate early genes, c-fos, restraint stress, alcohol, hippocampus, corticosterone]

Alcohol has a dual effect on the response to stress: on the one hand, exposure is stressful as is shown by the elevation of peripheral corticosterone levels (Ellis et al., 1966), while on the other hand, alcohol has "tension reduction" effects (Conger, 1956) that antagonize stress-induced increases in blood level of corticosterone and catecholamines (Vogel and Deturck, 1983; Patel and Pohorecky, 1988) and produce anxiolytic behavioral responses (Masserman and Yum, 1946; Koob et al., 1984). Although several sites in the CNS have been proposed to participate in the interaction of alcohol with stress responses (Pohorecky, 1990), the primary structures mediating these effects of alcohol remain to be mapped. Recently, assays of immediate early gene (IEG) expression in the brain have been developed as useful tools to localize neural cells reactive to a variety of stimuli both within the normal range of physiological response as well as in neuropathological situations (Sagar et al., 1988; cf. Dragunow et al., 1989; Morgan and Curran, 1991).

The IEG c-*fos* encodes a nuclear protein regulating transcriptional activity of various genes via binding of the AP-1 promoter sequence (Curran and Franza, 1988; Sheng and Greenberg, 1990). Fos-protein alone can not bind the AP-1 site, but prior to binding must form a heterodimer with the product of other IEGs: *jun-B*, c-*jun*, or *jun*-D. In contrast to Fos, proteins of the Jun-family can form AP-1-binding homodimers of only Jun-proteins (Curran and Franza, 1988; Nakabeppu et al., 1988; Zerial, 1989). In normal physiological states, the basal level of IEG expression in the cell is low, but following stimulation, IEG transcription is rapidly and transiently induced through a cascade of second messengers (Krujer et al., 1985; Curran and Morgan, 1987). Such IEG induction has been observed in the CNS after various pharmacological, electrical, and other manipulative stimuli that are also known activate neurons electrophysiologically (Morgan et al., 1987; Saffen et al., 1988; cf. Dragunow et al., 1989). Of particular interest for the present investigation are studies reporting IEG expression in brain following several specific stress paradigms, including restraint stress (Cecatelli et al., 1989; Arnold et al., 1992; Bing et al., 1992; Honkaniemi, 1992, Melia et al., 1994), foot-shock (Campeau et al., 1991), tail-shock (Schreiber et al., 1991), swim stress (Duncan et al., 1993, Melia et al., 1994), or following stress-based learning induced in the shuttle-box (Maleeva et al. 1989), by step-down passive and active avoidance (Anokhin and Ryabinin, 1991, 1993), Y-maze active avoidance (Tischmeyer et al., 1990; Nikolaev et al., 1992), and conditioned fear (Pezzone, 1992; Smith et al., 1992). Pharmacological studies have suggested the importance of excitatory amino acids (Birder and de Groat, 1992), catecholamines (Bing et al., 1991, Stone et al., 1993), and cytokines (Rivest et al., 1992) for stress-mediated c-*fos* expression in brain.

Because of the dramatic ability of drug-induced seizures to generate an intense IEG response, the pharmacological regulation of seizure-mediated c-*fos* expression in brain has been more

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Table 1. Presence of c-Fos-positive cells in different regions of rat brain after restraint stress of saline- and alcohol-pretreated animals

	A	B	C	D
Cortex				
Cingulate	−	−	+++	+
Somatosensory	−	−	++	+++
Piriform	+	+	+++	++
Hippocampus				
CA1	−	−	−	−
Septal CA3	−	−	++	−
Temporal CA3	+	−	++	+
Septal DG	+	−	+	+
Temporal DG	−	−	++	−
Amygdala				
Central nucleus	++	++	++	++
CAA	+	−	+	+
AHA	+	+	++	+
Thalamus				
Bed nucleus	+	+	+	+
PV	++	++	+++	+++
CM	+	+	++	+
VLG	+++	+++	+++	+++
Hypothalamus				
AH	+	+	+	+
DM	+	−	+	+
VM	+	−	+	+
PH	−	−	+	+
SuM	+	+	++	+
Septum				
Lsi	−	−	+	+
SFi	−	−	+	+

Numbers of c-Fos–positive cells were counted per brain region on every sixth 40 μm coronal brain section and the highest number reached was indicated by a score: undetectable (−), less than 10 (+), 10–30 (++), more than 30 (+++). Animals ($N = 2$): A—saline-injected controls, B—alcohol-injected controls, C—saline-injected restraint-stressed, D—alcohol-injected restraint-stressed. Abbreviations: DG—dentate gyrus, CAA—corticoamygdaloid area, AHA—amygdalohippocampal area, PV—paraventricular nucleus of thalamus, CM—centromedial nucleus of thalamus, VLG—ventral lateral geniculate nucleus, AH—anterior hypothalamus, DM—dorsomedial hypothalamus, VM—ventromedial hypothalamus, PH—posterior hypothalamus, SuM—supramammillary nucleus, Lsi—lateral septal nucleus, SFi—septo-fimbrial nucleus.

extensively addressed than that of stress-induced c-*fos* expression. Thus, in early studies it was shown that c-*fos* expression after pentylene tetrazole seizures can be blocked by the NMDA antagonist MK-801, diazepam, or phenobarbital (Morgan et al., 1987, Sonnenberg et al., 1989). Moreover, Le et al. (1990) showed that the c-*fos* mRNA expression in brain induced by pentylene tetrazole seizures could be inhibited by prior exposure to alcohol. Pharmacological analysis of this attenuating effect of alcohol on IEG response indicated that it was mediated through both NMDA- and GABA-dependent processes, but not through kainate or adenosine receptors (Le et al., 1992). These findings suggested that similar mechanisms might mediate "the tension-reducing" effects of alcohol on stress responses. However, despite its clinical importance, the effect of alcohol on more physiologically relevant stress-induced patterns of neural activity using IEG expression has not yet been addressed.

In the present investigation, we studied the effect of restraint stress (as a primarily psychological stressor) on IEG expression in the brain of alcohol- or saline-pretreated rats. The neuronal populations exhibiting stress-induced c-Fos protein expression were localized by immunohistochemistry. Levels of c-*fos* mRNA in cortex and hippocampus were statistically compared using slot-blot analysis. To address whether changes in c-*fos* mRNA expression could affect possible Fos-Jun heterodimer formation, expression of c-*jun*, *jun*-B, and *jun*-D was studied in parallel with c-*fos* RNA levels. Our results show that alcohol attenuates restraint stress-induced IEG expression in particular populations of neurons including hippocampus. However, although alcohol can induce circulating levels of corticosterone in otherwise unstressed subjects, alcohol treatment alone does not affect basal IEG expression in hippocampus or cortex.

Materials and Methods

Animals. Adult male Sprague–Dawley rats (Charles River Laboratories), weighing 240–260 gm at the onset of each experiment, were used. Animals were housed two per cage in Plexiglas cages (45 × 22 × 20 cm) with sawdust bedding in a colony room kept at 22°C on a 12:12 hr light: dark cycle (lights on at 0600 hr) with ad libitum access to food and water throughout each experiment. Animals of different groups were kept in separate cages. For 1 week prior to each experiment, animals received daily intraperitoneal injections of 0.9% saline (0.3 ml) to facilitate adaptation to injection and handling procedure.

Experimental procedures. All animal manipulations were conducted between 0700 and 1100 hr. Animals received intraperitoneal injection of 16% (weight/volume) alcohol in 0.9% saline or an equal volume of 0.9% saline. In the experiments addressing alcohol effects on stress-induced c-*fos* expression, 10 min after injections restraint stress was imposed by placing the animals in hemicylindrical (20.5 × 9 × 6 cm), well-ventilated Plexiglas tubes for 15 min. During this stress procedure, animals were able to turn around easily within the tubes but were not able to walk. Animals were sacrificed for RNA analysis 1 hr after injection by decapitation and for protein analysis 2 hr after injection by chloral hydrate narcosis and intracardial perfusion. In the time between injections, restraint stress, and sacrifice, rats were returned to their home cages. *Blood alcohol levels* were determined in trunk blood collected immediately after decapitation. Samples were centrifuged, supernatant was removed and stored at −70°C. Samples (50 μl) of plasma were assayed for alcohol at 340 nm using a Sigma (St. Louis, MO) NAD-ADH kit.

Corticosterone assay. Ten microliters of plasma was used for determination of corticosterone levels by radioimmunoassay (ICN Biochemical, Costa Mesa, CA).

Immunohistochemical analysis. Five minutes after chloral hydrate narcosis (350 mg/kg, i.p.) rats were quickly perfused with 0.9% saline followed by 2% paraformaldehyde in isotonic sodium phosphate buffer (PBS), pH 7.4. Brains were dissected, fixed in the perfusion buffer for 2 hr, and stored for 3–7 d in 20% sucrose/PBS. Sections (40 μm) were cut on a Cryocut 1800 (Leica, Foster City, CA) and collected in PBS. Immunohistochemistry for c-Fos protein was performed with affinity purified rabbit polyclonal antibodies raised against a peptide corresponding to human c-Fos amino acid residues 3–16 (Santa Cruz Biotechnology, Santa Cruz, CA) that is not reactive to *fos*-B and *fra*-1 proteins. The immunoreaction was detected with Vectastain ABC kit (Vector Laboratory Inc., Burlingame, CA), according to the following procedure. Sections were incubated with goat anti-rabbit blocking serum in 0.3% Triton X-100/PBS solution for 2 hr at room temperature followed by anti-c-Fos serum (dilution 1:1000) in 0.3% Triton X-100/0.1% bovine serum albumin (BSA)/PBS for 20 hr at 4°C. Sections were then rinsed with PBS, incubated with secondary biotinylated antibody (50 μl in 10 ml of 0.3% Triton X-100/PBS) for 1 hr at 23°C, rinsed with PBS, incubated for 1 hr with the ABC reagent (100 μl of reagent A and 100 μl of reagent B in 10 ml of 0.3% Triton X-100/PBS), and rinsed again with PBS. Enzymatic development was done in 0.05% DAB in 0.5× sodium phosphate buffer containing 0.003% hydrogen peroxide for 7 min. After rinsing in PBS, sections were mounted on gelatin-coated glass slides. For a background control, sections were incubated without the primary antibody. To confirm that the immunocytochemical detection was not limiting, some sections were also incubated with two- and eightfold higher c-Fos antibody concentrations. Cell counting was performed manually at 100× magnification; questionable cells were checked for nuclear localization of staining using magnification of 400×.

RNA isolation and analysis. After decapitation, cortex and hippocampus were quickly dissected, frozen on dry ice, and stored at −70°C. Total RNA was isolated from brain structures of individual animals using the guanidine-isothiocyanate/phenol method (Chomczynski and Sacchi, 1987). RNA was dissolved in sterile distilled water, quantified by spectrophotometry at 260 nm and stored at −70°C. For quantitative measurements of mRNA levels, slot-blots of hippocampal and cortex RNA prepared from individual animals were performed on 1, 2, and 4 μg samples of total RNA to access the linearity of the hybridization signal. The RNA samples were denatured in 50% formamide, 10× SSC at 65°C and loaded onto Nytran nylon membranes using Schleicher and Schuell (Keene, NH) Manifold II slot-blot system, washed with 10× SSC and UV cross-linked using GS-Gene Linker (Bio-Rad, Hercules, CA). Replicate filters for hippocampus and cortex of each experiment were prepared and analyzed.

cDNA probes for rat c-*fos* (gift from T. Curran, Roche Institute), cyclophilin (gift from P. E. Danielson, The Scripps Research Institute), c-*jun*, *jun*-B, and *jun*-D (from ATCC) were used in this study. Probes were labeled by ^{32}P-dCTP and ^{32}P-dATP (Amersham, Arlington Heights, IL) using a Decaprime kit (Ambion Inc., Austin, TX). The specificity of the probes was initially determined by Northern Blot hybridization. Slot-blot or Northern blot filters were prehybridized overnight at 42°C in hybridization buffer (50% (v/v) formamide, 2× Denhardt's solution, 0.5% SDS, 1 mM sodium pyrophosphate, 200 μg/ml denatured salmon sperm DNA and 200 μg/ml denatured yeast tRNA), and then hybridized with approximately 2 ng/ml of the labeled probe in hybridization buffer for 24 hr at 42°C. Filters were washed in 0.5× SSC, 0.1% SDS, 1 mM sodium pyrophosphate at 37°C, 56°C, and 65°C and exposed to Amersham Hyperfilm-MP x-ray film. Time of exposure depended on the strength of the signal and was predetermined in a series of preliminary experiments to give bands in a linear range of intensity. Rehybridization of the filter with a new probe was done after eluting previously hybridized probe by boiling the filter in 0.1× SSC, 0.1% SDS. Quantitation of band intensities was done by scanning the films with a Microtek (Torrance, CA) Scanmaker 600ZS, digitizing with Microtek BLACK & WHITE software. Signal intensity was measured in pixels/band using NIH IMAGE 1.43 software. Data on IEG expression were normalized to corresponding cyclophilin values and the two normalized values from 4 and 2 μg RNA loading were averaged to obtain levels of IEG mRNA relative to cyclophilin. This value was used as a single data point for statistical analysis (data obtained on 1 μg concentration was too low to be quantified).

Statistical analysis. Statistical analysis of the obtained values was done using two-way and one-way factorial ANOVA and the Scheffe *F* test; for post hoc comparisons *p* values of less than 0.05 were considered statistically significant.

Results

Immunohistochemical localization of c-Fos expression in rat brain after restraint stress preceded by saline or alcohol injection

Preliminary experiments showed that exposure of unhandled rats to various stress paradigms including restraint, resident-intruder, as well as simple saline injections dramatically increased the number of c-*fos* immunoreactive neurons in hypothalamus, cortex, hippocampus, and other brain regions (data not shown), consistent with reports of other investigators (Giovanelli et al., 1990; Sharp et al., 1991; Covenas et al., 1993; Stone et al., 1993). However, if the animals were previously habituated to the stress of handling and saline injection, we observed that the amount of c-Fos immunoreactivity was substantially reduced, allowing for the detection of a more specific response, possibly reflecting activation of selected cell populations responding to the particular stressor.

To identify those cell populations exhibiting interactions between alcohol and their response to stress, c-Fos immunoreactivity was compared across the following groups of animals that were first habituated to injection procedures and handling: (1) *saline* control rats that received a saline injection and were returned to their home cage; (2) *alcohol* controls that received a 2 g/kg alcohol injection and were returned to their home cage; (3) *saline/stress* animals that received a saline injection, were returned to their home cage for 10 min, then placed into the restraint tube for 15 min, and finally returned to their home cage; and (4) *alcohol/stress* animals that received a 2g/kg alcohol injection, were returned to their home cage for 10 min, then placed into the restraint tube for 15 min, followed by being returned to their home cage again (two rats per group). All rats were sacrificed 2 hr after injection of either saline or alcohol resulting in a 95 min interval from the end of the restraint period in groups *saline/stress* and *alcohol/stress*. The alcohol dose of 2 g/kg was chosen as a commonly used dose exhibiting strong interaction with the effects of behavioral stress without a gross neurotoxic component (cf. Pohorecky, 1990).

Immunohistochemical analysis performed on coronal brain sections corresponding to bregma levels from 0 to −4.5 mm revealed a similar pattern of c-Fos expression in both saline- and alcohol-injected control rats (Table 1). Fos-positive cells were present in thalamus: in the paraventricular nucleus, the centromedial nucleus, and the ventral lateral geniculate nucleus, in the central nucleus of the amygdala and scattered through various hypothalamic areas. Localization of Fos-positive staining in these regions was specifically nuclear. However, in the paraventricular nucleus of hypothalamus, a substantial part of the staining was extranuclear that probably reflected cross-reactivity of the chosen antibody with cytoplasmic proteins. Consistent with this interpretation, extranuclear immunoreactivity was never observed using other antibodies recognizing both c-Fos and Fos-related antigens (own unpublished observations and Giovanelli et al., 1990). To avoid any misinterpretation of this staining, Fos-immunoreactivity in the paraventricular nucleus of hypothalamus was not further analyzed.

In animals subjected to restraint stress, a clear induction of c-Fos expression was observed, resulting in detection of additional sets of Fos-positive nuclei in cortical, hypothalamic, and hippocampal regions (Fig. 1). Comparison of restrained animals that received either alcohol or saline injections prior to the restraint stress showed major differences in c-*fos* expression in hippocampus and certain other, but not all, regions of the brain. Thus, in the animals of the *saline/stress* group, c-Fos–positive cells were abundant in the CA3 region of hippocampus and dentate gyrus (Fig. 1*C*,*E*,*F*). Interestingly, Fos-immunoreactive cells were unevenly distributed in hippocampus; that is, they were substantially more frequent in the temporal pole of hippocampus than in the septal pole. In the animals of the *alcohol/stress* group, c-Fos expression was dramatically attenuated such that no positive cells were found in the dentate gyrus of the temporal hippocampus (please compare Fig. 1*C* and *D*) and in the CA3 region (Table 1). The difference in c-Fos expression between alcohol- and saline-injected stressed animals could be also observed in particular regions of the neocortex; that is, c-Fos–expressing cells were more abundant in cingulate cortex and piriform of the animals from the *saline/stress* group than in the *alcohol/stress* animals. In contrast, consistently more c-*fos*–positive cells were found in the somatosensory regions of the cortex of *alcohol/stress* than in *saline/stress* animals (Table 1). Increasing the amount of primary antibody added to the immunohistochemical reaction did not increase the number of Fos-positive cells detected, but merely increased the background staining, suggesting that the relatively small number of stained cells reflected all cells expressing c-Fos protein and not a limited

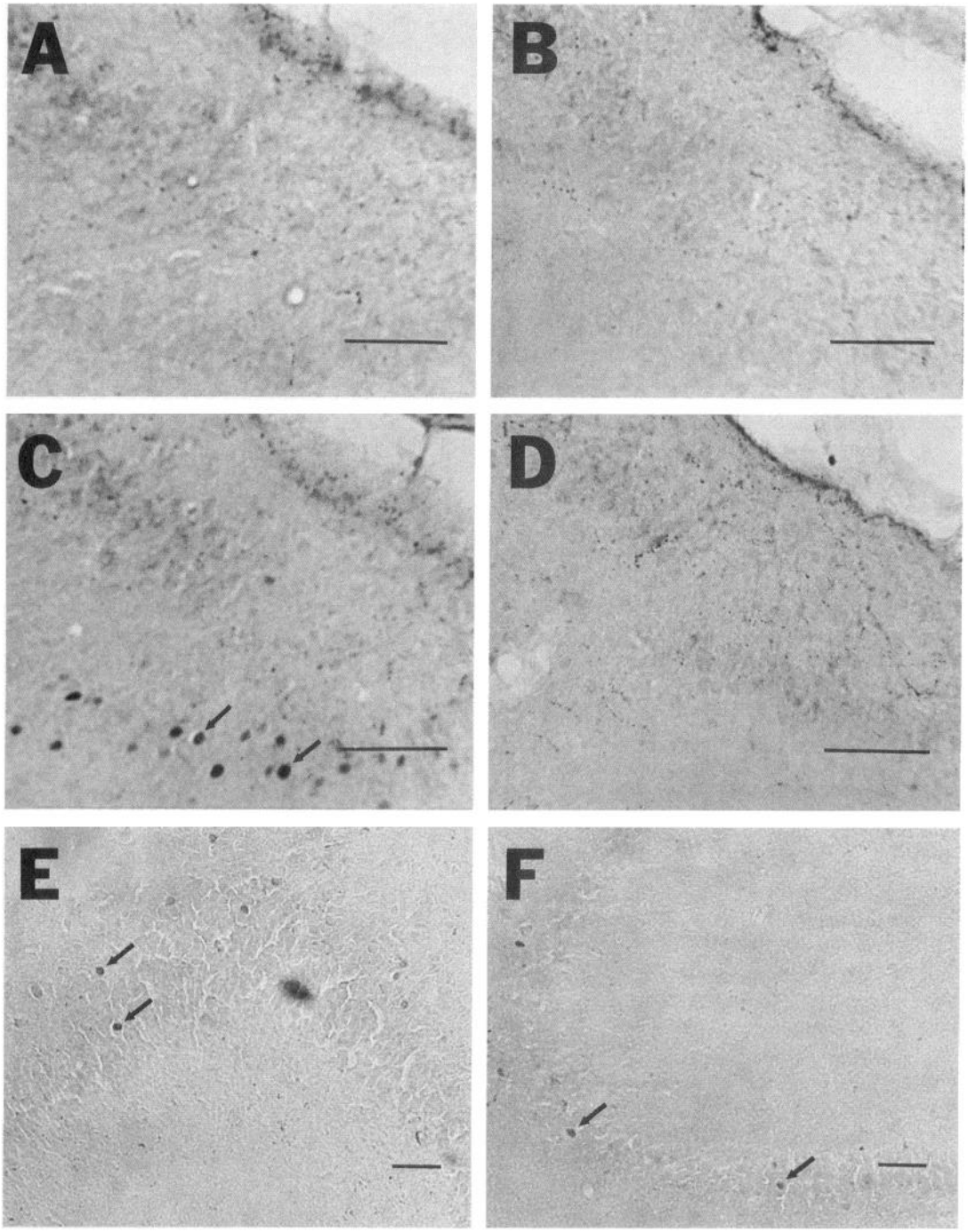

Figure 1. c-Fos protein is induced in hippocampal neurons after restraint stress in saline but not alcohol-pretreated animals. Representative coronal sections of the hippocampus stained immunohistochemically for c-Fos at approximate bregma position −4.3 mm are shown. *A–D* compare the dentate gyrus of the temporal pole of hippocampus of saline-injected control (*A*), alcohol-injected control (*B*), saline-injected restraint (*C*), and alcohol-injected restraint (*D*) animals. Lower magnification of the edge of the temporal (*E*) and septal (*F*) pole of the CA3 pyramidal layer of saline-injected restraint-stressed animals is demonstrated, where the corresponding to *E* and *F* areas of alcohol-injected restraint-stressed animals showed no c-Fos–positive staining. Bar indicates 100 μm. *Arrows* point to exemplary c-Fos–positive cells.

sensitivity or titer of the antibody. The difficulty of accurately aligning the precise brain regions with relatively small number of Fos-positive cells responding to the specific behavioral stimuli did not allow reliable statistical comparisons of the density of c-Fos–expressing cells between animals of experimental and control groups. To achieve a statistical evaluation of the interaction between alcohol and stress on c-*fos* expression mRNA levels were quantitatively analyzed in the following experiment.

IEG mRNA levels in rat hippocampus and cortex after restraint stress preceded by saline or alcohol injection

To investigate the significance of the alcohol effects on c-*fos* expression mRNA levels of IEG were analyzed by slot-blot hybridization of total RNA isolated from the entire hippocampus and cortex in an independent experiment comprised of the analogous groups of *saline* and *alcohol* controls and *saline/stress*

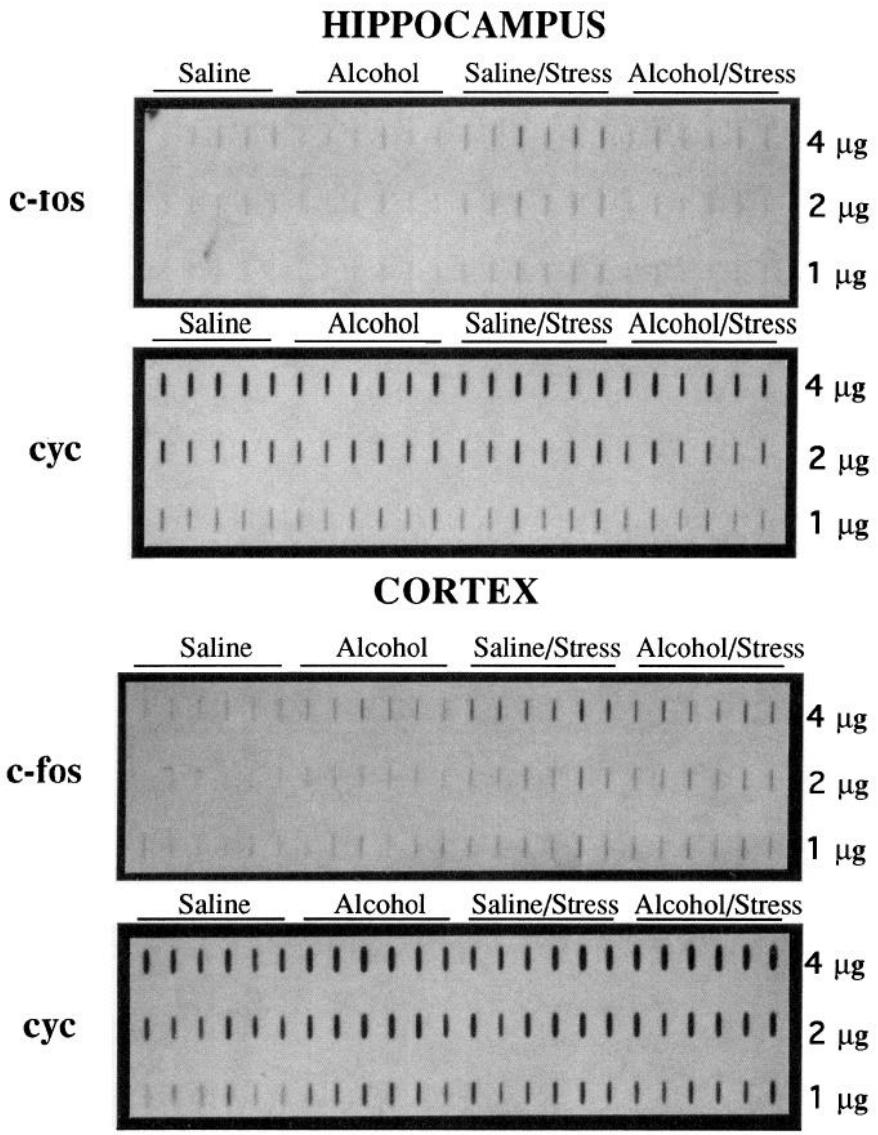

Figure 2. Slot-blots showing the level of c-*fos* mRNA in hippocampus and cortex of individual rats from experimental groups: saline-injected controls (*Saline*), alcohol-injected controls (*Alcohol*), saline-injected restraint animals (*Saline/Stress*), and alcohol-injected restraint animals (*Alcohol/Stress*); *4, 2, and 1 μg* indicated on the right show the three different amounts of total RNA from each animal loaded to ascertain the linearity of hybridization. Note that the higher level of c-*fos* hybridization in animals exposed to restraint alone (*Saline/Stress*) relative to all other groups is evident in the 1 μg lane on the blot with hippocampal but not in cortex RNA samples. *cyc*—result of rehybridization of the same filters with the cyclophilin cDNA to control for loading differences.

and *alcohol/stress* rats (six animals/group) to the described in the previous section. To assay the peak response in c-*fos* mRNA expression, animals were sacrificed 1 hr after injections (35 min after restraint stress).

Blood alcohol levels (BALs) measured in the trunk blood collected from *alcohol* animals reached an average of 119 ± 17.1, while BALs in the *alcohol/stress* group reached an average of 97 ± 13.1; control *saline* and the *saline/stress* groups had negligible BALs of 1 ± 0.4 and 1.5 ± 0.6, respectively. As expected, there was a significant effect of alcohol on blood alcohol concentration as confirmed by two-way factorial ANOVA [$F(1,19) = 89.2$, $p < 0.0001$]. More importantly, no effects of stress on blood alcohol levels [$F(1,19) = 0.92$, $p = 0.35$] or stress by alcohol interaction [$F(2,19) = 1.04$, $p = 0.32$] were observed, indicating that restraint stress and, therefore, metabolic differences between stressed and unstressed groups did not influence blood alcohol concentration in our experiment.

c-fos mRNA expression. As shown in Figure 2 and summarized in Figure 3*A* and *B*, slot-blot hybridization of the total RNA isolated from hippocampus and cortex of individual animals confirmed the previous immunocytochemical findings that there were no significant effects of alcohol on basal expression of c-*fos*. As expected, restraint stress significantly increased c-*fos* expression in both brain structures. However, c-*fos* induction in hippocampus was significantly lower in restraint-stressed animals pretreated with alcohol than in saline-injected animals subjected to the same stress. In contrast, the expression of c-*fos* mRNA in cortex of restraint-stressed animals was not significantly affected by alcohol pretreatment. A two-way factorial ANOVA of hippocampal levels of c-*fos* mRNA showed significant effects of stress [$F(1,19) = 17.1$, $p < 0.001$], alcohol [$F(1.19) = 15.8$, $p < 0.001$] and a significant stress by alcohol interaction [$F(2,19) = 13.8$, $p < 0.01$], reflecting a differential effect of alcohol on c-*fos* expression in hippocampus of restraint-stressed relative to nonstressed animals. In comparison, two-way factorial ANOVA showed a significant effect of stress in cortex [$F(1,19) = 22.4$, $p < 0.001$], but no significant effects of alcohol [$F(1,19) = 2.9$, $p = 0.1$], and no significant interaction of stress and alcohol [$F(2,19) = 1.7$, $p = 0.2$] on c-*fos* mRNA levels. This suggested that the dramatic attenuation of stress-induced c-*fos* expression in the cingulate cortex by alcohol was masked by other cortical cells not responsive to alcohol when the entire

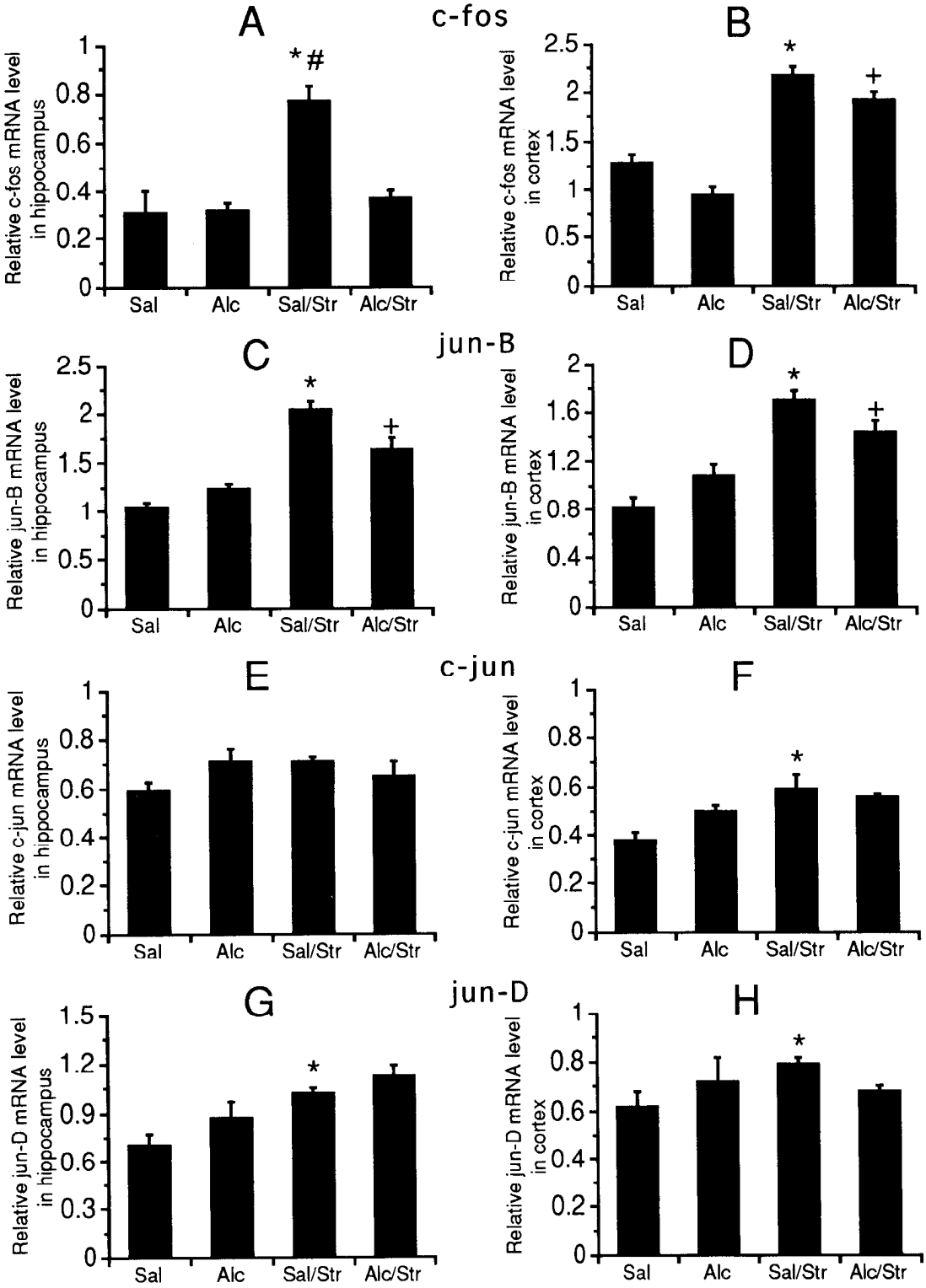

Figure 3. Differential effects of alcohol on restraint stress-induced IEG expression in hippocampus (*left*) and cortex (*right*). Densitometric data on IEG mRNA levels were normalized to the level of cyclophilin mRNA and expressed as mean ± SEM. Groups of animals: *Sal*—saline-injected controls; *Alc*—alcohol-injected controls; *Sal/Str*—saline-injected restraint animals; and *Alc/Str*—alcohol-injected restraint animals. $N = 6$ for all groups of animals, except $N = 5$ for hippocampus of Sal. *Significantly different from Sal; +Significantly different from Alc; #, significantly different from all other groups. $p < 0.05$. Note difference in Y-axis values for different probes and brain structures. Quantitative comparisons were analyzed only between groups within each histogram since the densitometry signal reflects not only the difference in the level of mRNAs but also the specific activity of the probes and hybridization efficiency.

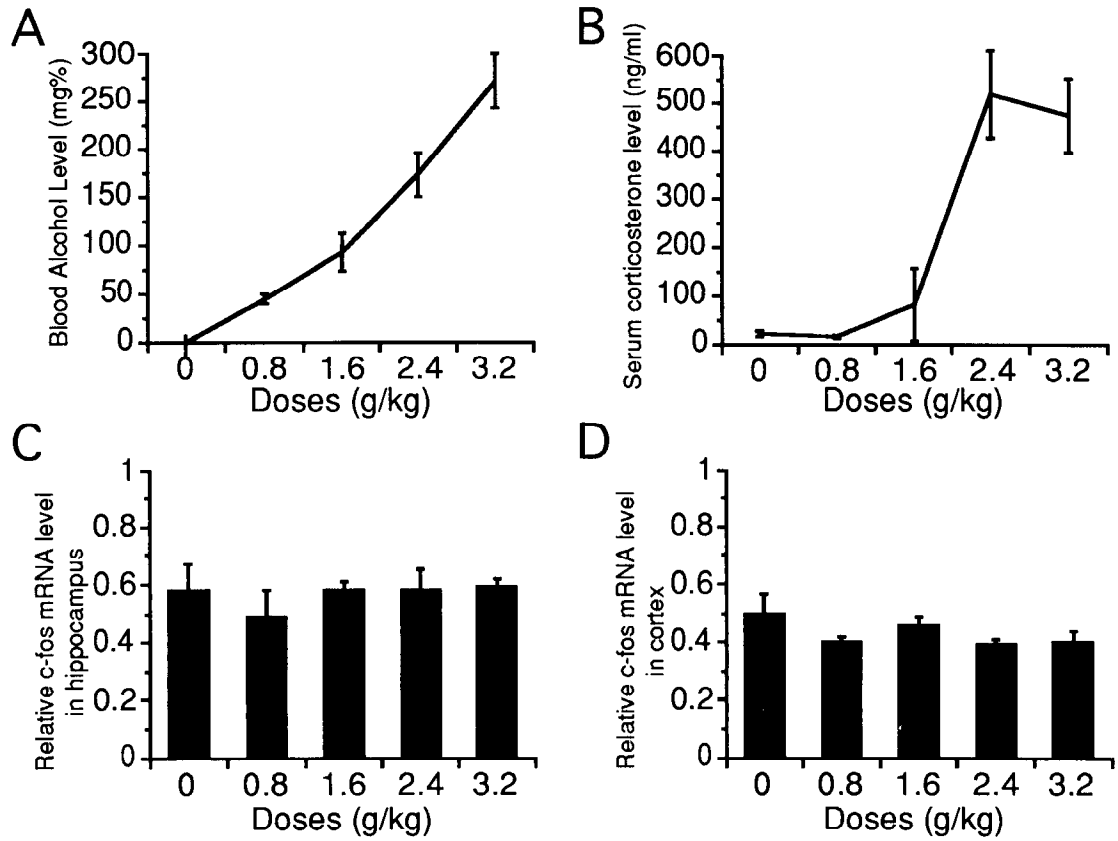

Figure 4. Increasing doses of alcohol lead to corresponding increases in blood alcohol levels, circulating corticosterone, but not c-*fos* mRNA expression in rat hippocampus and cortex. Rats were exposed to 0.8, 1.6, 2.4, and 3.2 g/kg alcohol by intraperitoneal injection of 1.5, 3, 4.5, and 6 ml of 16% alcohol ($N = 3$ for each dose) or isotonic saline (1.5, 3, and 6 ml—two for each volume). *A* shows that the BALs exhibit an expected dose response, *B* demonstrates that corticosterone response requires 1.6 g/ml or higher dose of alcohol. *C and D* show the normalized data obtained from slot-blot hybridizations of RNA isolated from hippocampus and cortex of individual rats. Densitometric data on c-*fos* mRNA levels were normalized to the level of cyclophilin mRNA. All data are expressed as mean ± SEM. As indicated in Figure 3, the quantitative differences were evaluated only between groups within each histogram.

cortex was assayed. Similar results were obtained in an independent replicate experiment. In a pilot study, reliable differences in c-*fos* expression in hypothalamus, amygdala, and cerebellum could not be discerned between saline- or alcohol-injected groups exposed to restraint stress.

jun-*B mRNA expression.* As for c-*fos*, both in hippocampus and cortex of saline-injected and alcohol-injected control animals, the basal levels of *jun*-B mRNA were not significantly different, and *jun*-B mRNA was increased in hippocampus and cortex of acutely stressed animals (Fig. 3*C,D*). However, in contrast to c-*fos*, alcohol produced only slight attenuation of *jun*-B expression in both these structures. In hippocampus, a two-way factorial ANOVA revealed a significant effect of stress [$F(1,19) = 77.3$, $p < 0.001$], no significant effects of alcohol [$F(1.19) = 1.1$, $p = 0.3$], but a significant stress by alcohol interaction [$F(2,19) = 11.5$, $p < 0.01$] on *jun*-B mRNA levels. As found for hippocampus, a two-way factorial ANOVA performed on mRNA levels of cortical RNA showed a significant effect of stress [$F(1,19) = 65.5$, $p < 0.001$], no significant effects of alcohol [$F(1,19) = 0.0004$, $p = 0.98$], and a significant stress by alcohol interaction [$F(2,19) = 11.1$, $p < 0.01$]. Thus, expression of *jun*-B mRNA in cortex and hippocampus was induced by restraint stress, whereas alcohol decreased stress-induced levels of *jun*-B expression. However, according to post hoc analysis, this attenuation was not statistically significant, in contrast to the more robust effect of alcohol on stress-induced c-*fos* expression in hippocampus.

c-jun *mRNA expression.* Normalized data obtained from slot-blot hybridization showed little difference in c-*jun* expression in hippocampus across all experimental groups (Fig. 3*E*). Whereas restraint-stressed animals had a higher level of c-*jun* expression in cortex compared with saline controls, no obvious effect of alcohol on this restraint induced gene expression was observed (Fig. 3*F*). A two-way factorial ANOVA showed no significant effect of stress [$F(1,19) = 0.47$, $p = 0.5$], alcohol [$F(1,19) = 0.42$, $p = 0.52$], or any stress by alcohol [$F(2,19) = 3.3$, $p = 0.09$] on c-*jun* mRNA levels in hippocampus. A two-way factorial ANOVA performed on cortical c-*jun* data did reveal a significant effect of stress [$F(1,19) = 13.7$, $p < 0.002$], no significant effects of alcohol [$F(1,19) = 1.3$, $p = 0.26$], but a significant stress by alcohol interaction [$F(2,19) = 15.4$, $p < 0.001$] on c-*jun* mRNA levels in cortex. The apparent significance of the stress by alcohol interaction probably reflected the fact that the level of c-*jun* mRNA expression in the *saline* controls was lower than in all other groups. This difference, moreover, was not repeated in a duplicate experiment and was not detected in further experiments evaluating alcohol dose effects (see below).

jun-*D mRNA expression.* The level of *jun*-D mRNA in hippocampus was significantly higher in restraint-stressed animals

than in control animals; however, alcohol did not significantly affect the expression of this gene (Fig. 3*G*). Restraint-stressed animals had also a higher level of *jun*-D expression in cortex than control animals and this level appeared to be lower in alcohol injected restraint-stressed animals (Fig. 3*H*). A two-way factorial ANOVA showed significant effect of stress [$F(1,19) = 16.8$, $p < 0.001$], but no significant effects of alcohol [$F(1,19) = 3.4$, $p = 0.08$] on *jun*-D mRNA levels in hippocampus and no significant interaction of stress and alcohol [$F(2,19) = 0.24$, $p = 0.63$]. A two-way factorial ANOVA performed on cortical data showed no significant effect of stress [$F(1,19) = 3.3$, $p = 0.09$] or alcohol [$F(1,19) = 0.003$, $p = 0.96$], but a significant stress by drug interaction [$F(2,19) = 8.7$, $p = 0.008$] reflecting a lower level of *jun*-D mRNA in alcohol pretreated than in saline-pretreated animals. However, post hoc analysis revealed that the difference in *jun*-D expression was only significant between *saline* control animals and *saline/stress* rats, again suggesting that *jun*-D expression in cortex after stress was not as sensitive to alcohol as clearly found for c-*fos* expression in hippocampus.

These data illustrate that the response of IEGs appears to be differentially affected by stress and alcohol stimuli and that c-*fos* mRNA expression is visibly reactive to restraint stress in both cortex and hippocampus, but sensitive to alcohol only in hippocampus.

Different doses of alcohol do not change basal IEG expression in hippocampus and cortex

Alcohol is known to exhibit differential effects on behavior and stress responses depending on the dose of the drug (Ellis et al., 1966; Engel and Liljequist, 1983). Therefore, although the dose of 2 g/kg used in the previous experiments did not change basal IEG expression, it is possible that a different dose of alcohol could reveal changes in neural activity not noticeable in the previous experiments. This question was addressed in a dose–response experiment where groups of animals received a ethanol doses of 0.8, 1.6, 2.4, or 3.2 g/kg of alcohol (three animals/group). *Saline* control rats received comparable volumes of 0.9% saline (1.5, 3, and 6 ml—two animals per dose). Animals were sacrificed 1 hr after either saline or alcohol injection, hippocampal and cortical brain regions were isolated for RNA analysis, and trunk blood was collected for assaying BALs and the level of corticosterone.

As expected, a one-way factorial ANOVA showed a significant effect of injected dose of alcohol on BALs [$F(4,17) = 54.61$, $p = 10^{-4}$] and corticosterone levels [$F(4,17) = 22.96$, $p = 10^{-4}$] (Fig. 4). A one-way factorial ANOVA showed no significant effect of alcohol on c-*fos* mRNA levels in hippocampus [$F(4,17) = 0.88$, $p = 0.5$] or in cortex [$F(4,16) = 0.22$, $p = 0.93$] (Fig. 4). Animals injected with different amounts of saline showed no difference in c-*fos* expression in hippocampus or cortex. *jun*-B, c-*jun*, and *jun*-D expression was also analyzed and, similarly, no significant changes were found (data not shown).

Discussion

In general, the results described here confirm previous observations of low basal levels of c-*fos* expression in the brain of naive animals and its inducibility by stressful behavioral stimuli (Cecatelli et al., 1989; Maleeva et al., 1989; Anokhin and Ryabinin, 1991). Based on studies showing that stress-induced c-*fos* expression in major brain structures can be downregulated by repeated exposure to the stressor but is readily activated by a novel stimulus (Melia et al., 1994), in the present experiments, animals were first habituated to the otherwise stressful injection and handling procedures. The immunohistochemical data presented demonstrates, in fact, that despite habituation the control groups still exhibited c-Fos expression in several brain regions, particularly in the paraventricular nucleus of thalamus, the ventral lateral geniculate nucleus, and the central nucleus of amygdala. It is not yet clear whether c-Fos expression in these structures reflects true basal activity, perhaps reflecting constant stimulation (i.e., visual) or is a reaction to the relatively large injection of saline or alcohol solutions in experimental animals compared to that during habituation (see Materials and Methods). Whereas the first explanation might account for expression in the paraventricular nucleus of thalamus and the ventral lateral geniculate nucleus, the second explanation seems more plausible for c-Fos expression in the central nucleus of amygdala since c-Fos has been shown to be induced in this brain region by stress (Honkaniemi, 1992). In contrast to these areas, control animals had a sparse density of Fos-immunoreactive cells in the other structures where c-Fos expression, including the hypothalamus, the amygdaloid complex, the thalamus, the hippocampus, and cortex. In response to restraint stress, certain subregions through these brain structures demonstrated a different extent of induction in c-Fos expression; in particular, a strong response to stress was found in hippocampus and cortex.

In the hippocampus of control animals, c-*fos* immunoreactive cells were almost undetectable (consistent with Hughes et al., 1992). Restraint stress led to a reliable increase in the number of Fos-positive cells. It is interesting that the localization of these responsive neurons was not distributed evenly across the CA3 area and the dentate gyrus. For example, Fos immunoreactive cells concentrated predominantly not in the septal, but in the temporal pole of hippocampus. It is possible that this distribution reflects a neuroanatomical and functional difference between these two parts of hippocampus, resulting in higher excitability of the temporal region (Grimes et al., 1989; Lee et al., 1990). Consistent with the increase in c-Fos protein expression observed by immunohistochemistry, the level of c-*fos* mRNA was found significantly increased when total RNA of the hippocampus was assayed.

In the neocortex the number of c-Fos–positive cells and the level of c-*fos* mRNA was low in control animals but significantly increased in animals exposed to restraint stress. As detected by immunohistochemistry, this induction was not equally distributed throughout cortex, but was higher in cingulate and piriform areas consistent with reports of Sharp et al. (1991), Giovanelli et al. (1992), and Stone et al. (1993).

In agreement with the studies of Le et al. (1990, 1992) we did not observe any significant effects of the alcohol treatment alone on levels of c-*fos* mRNA or c-Fos protein in hippocampus and in cortex. Even stressful doses of alcohol resulting in high blood alcohol levels and elevation of circulating corticosterone failed to induce c-*fos* expression in hippocampus and cortex. These data demonstrate a dissociation of the adrenal cortical response and c-*fos* expression in rat brain and supports the position that c-*fos* expression and its induction in response to certain physiological stimuli in hippocampus, cortex, and hypothalamus is not secondary to the activation of adrenal response and release of corticosteroids (Brown and Sawchenko, 1993; Melia et al., 1994).

Although there appeared to be no direct effects of alcohol on

basal IEG expression in cortex and hippocampus, alcohol strongly attenuated the induction of c-*fos* expression in hippocampus in response to restraint stress. This effect was clearly seen by immunohistochemical analysis in the dentate gyrus of the temporal pole of hippocampus and in the CA3 region, and was significant at the RNA level when the samples from the entire hippocampus were analyzed. In cortex, the picture that emerges appears more complex. Alcohol decreased the number of stress-induced c-Fos–expressing cells in cingulate cortex and, to a lesser extend, in piriform cortex, but not in the somatosensory cortex. When analyzed at the level of RNA, the attenuating effect of alcohol on stress-induced c-*fos* expression was not significant statistically, probably because a dissection of the entire cortex was used for RNA isolation.

Our findings strongly support the notion that alcohol in moderate doses does not act as a nonspecific depressant, but rather selectively affects only certain sensitive neurons. Recently, Le et al. (1992) demonstrated a receptor-specific effect of alcohol on seizure-induced c-*fos* expression in rat brain: only NMDA and GABA, but not kainic acid or caffeine, mediated induction was affected by alcohol. In the present experiments where c-*fos* was induced by a mainly psychological stressor, we were able to demonstrate that the effect of alcohol can also be population specific. The specificity of the neuronal populations responding to alcohol probably reflects the receptor specificity of these cells (Ticku et al., 1986; Lovinger et al., 1989; Hegarty and Vogel, 1993). The mechanism through which this receptor specificity is reached has yet to be characterized, especially regarding the possibility that these selective effects of alcohol can be mediated through neurons of other brain regions such as ventral tegmental area or locus coeruleus. For example, ethanol has recently been shown to inhibit facilitation of dentate gyrus responses to stimulation of dopaminergic inputs from the ventral tegmental area (Criado et al., 1994).

Our results also show that there is a difference in response to restraint stress and alcohol within the series of IEGs studied. This was not unexpected, since these IEGs differ in the mechanisms leading to their activation (Bartel et al., 1989; Auwerx et al., 1990) and in the general distribution of their activity throughout different brain regions (Wisden et al., 1990; Mellstrom et al., 1991). Consistent with observations showing parallel regulation of c-*fos* and *jun*-B by behavioral stimuli (Kornhauser et al., 1992; Rosen et al., 1992), we observed that these genes were, indeed, coactivated after stress in cortex and hippocampus. However, stress induction of *jun*-B in hippocampus appeared to be less sensitive to alcohol treatment than induction of c-*fos*. *jun*-D expression was also increased after restraint stress in both cortex and hippocampus, and this induction was not significantly attenuated by alcohol. In contrast, c-*jun* was induced by restraint stress in cortex but not in hippocampus and, like *jun*-D, was not sensitive to alcohol. Assuming that some of the activated IEGs were expressed in the same cells, it is likely that the primarily psychological stressor used in our experiments led to an increase in various Fos-Jun heteromers. The specific suppression of the c-Fos response by prior alcohol exposure suggests the interesting possibility that alcohol may lead to a relative deficiency of c-Fos, thus increasing the likelihood of Jun-Jun complexes in hippocampus. This may perturb the normal physiological cascade of responses to the stressor by reducing the efficiency of transcriptional activation or by redirecting AP-1 activation to other target genes. Clearly, studies closely evaluating the time course and cell specific expression of individual IEGs, in particular, members of the *jun* family, together with identification of genes targeted for these IEGs, will be necessary to fully evaluate this hypothesis.

In summary, using c-*fos* expression as a marker of neural activity we have demonstrated that alcohol can differentially alter stress response in selected neuronal populations including hippocampus and cingulate cortex. This interaction differentially affects expression of IEGs by principally suppressing the induction of expression of c-Fos in the CA3 region and dentate gyrus. It is intriguing to consider that this effect of alcohol on hippocampus, the primary structure participating in the formation of declarative memory and cognitive functions (cf. Squire, 1992), might reflect the anxiolytic effects of alcohol. However, it is not yet clear whether the attenuation of stress-induced IEG response in hippocampus reflects the cause of "tension reduction" by alcohol or is an epiphenomenon of the effects of alcohol on other CNS circuitry, in example, through catecholaminergic or dopaminergic transmission. Further investigations will be necessary to fully distinguish between these potential underlying mechanisms.

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