Modulation of Cellular Expression of Glucocorticoid Receptor and Glucocorticoid Response Element-DNA Binding in Rat Brain during Alcohol Drinking and Withdrawal

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ABSTRACT

To define the molecular mechanisms of abnormal hypothalamic pituitary adrenal (HPA) axis during ethanol dependence, we investigated the effect of chronic ethanol treatment (15 days) and its withdrawal (24 h) on the expression of glucocorticoid receptors (GRs) and glucocorticoid response element (GRE)-DNA binding in the rat brain. The effects of chronic mianserin [serotonin (5-HT2A/2C) antagonist] treatment on these parameters in various brain structures of control diet-fed and ethanol-fed rats were also investigated. It was found that ethanol treatment and withdrawal significantly decreased the GR protein levels in cortical (cingulate gyrus, frontal, parietal, and piriform cortex) and amygdaloid (central, medial, and basolateral) structures and paraventricular nucleus (PVN) of hypothalamus of rats. It was also observed that ethanol treatment produced significant reductions in GR protein levels in various hippocampal structures (CA1, CA2, CA3, and dentate gyrus), but these changes were normalized during ethanol withdrawal. Ethanol treatment also significantly decreased GRE-DNA binding in rat cortex and hippocampus, which remained decreased in the cortex but reverted to normal in hippocampus during ethanol withdrawal. Chronic mianserin (alone) treatment had no effect on cortical GRE-DNA binding and GR protein levels in cortical, amygdaloid, or PVN structures but significantly decreased the GR protein expression in various hippocampal structures and GRE-DNA binding in whole hippocampus. However, when administered concurrently with ethanol treatment, mianserin significantly antagonized the reductions in cortical GRE-DNA binding and in GR protein expression in cortical, PVN, and central, but not medial and basolateral, amygdaloid structures during ethanol withdrawal. On the other hand, mianserin treatment along with ethanol administration significantly decreased the hippocampal GRE–DNA binding and GR protein expression in various hippocampal structures during ethanol withdrawal. Furthermore, ethanol treatment and its withdrawal or mianserin treatment had no effect on the neuron-specific nuclear protein levels in the various brain structures. Taken together, these results indicate that interaction of 5-HT2A/2C receptors with GRs in cortical, central amygdaloid, and PVN structures may play a role in neural mechanisms of alcohol dependence. It is possible that decreased GR expression in PVN may be responsible for the abnormal HPA axis during ethanol exposure and withdrawal.

The hypothalamic pituitary adrenal (HPA) axis is one of the primary physiological systems activated during stress situations (Herman and Cullinan, 1997; Plotsky et al., 1998). The release of corticotropin-releasing factor (CRF) from the paraventricular nucleus (PVN) of the hypothalamus activates the release of adrenocorticotropic from pituitary, which stimulates the production of glucocorticoids from the adrenals. The glucocorticoids regulate the HPA axis through a negative feedback mechanism via binding to soluble glucocorticoid receptors at the pituitary, hypothalamic, and extra hypothalamic levels and, thus, inhibit the release of CRF and adrenocorticotropic (Plotsky, 1991; Jacobson and Sapolsky, 1991; Feldman and Weidenfeld, 1995). The glucocorticoid binding receptor has been divided into type 1-mineralocorticoid receptor (MR) and type 2-glucocorticoid receptor (GR) (Reul and De Kloet, 1986; Funder, 1992). The GR act as a ligand-inducible gene transcription factor, and after activation, the GR complex translocates into nucleus where it binds to glucocorticoid response element (GRE) of gene promoters and, thus, regulates the expression of selected genes (Simons et al., 1992; Gower, 1993).

It has been shown that acute and chronic ethanol consumption leads to a hyperactive HPA axis both in animals and humans. The primary physiological systems activated during stress situations (Herman and Cullinan, 1997; Plotsky et al., 1998). The release of corticotropin-releasing factor (CRF) from the paraventricular nucleus (PVN) of the hypothalamus activates the release of adrenocorticotropic from pituitary, which stimulates the production of glucocorticoids from the adrenals. The glucocorticoids regulate the HPA axis through a negative feedback mechanism via binding to soluble glucocorticoid receptors at the pituitary, hypothalamic, and extra hypothalamic levels and, thus, inhibit the release of CRF and adrenocorticotropic (Plotsky, 1991; Jacobson and Sapolsky, 1991; Feldman and Weidenfeld, 1995). The glucocorticoid binding receptor has been divided into type 1-mineralocorticoid receptor (MR) and type 2-glucocorticoid receptor (GR) (Reul and De Kloet, 1986; Funder, 1992). The GR act as a ligand-inducible gene transcription factor, and after activation, the GR complex translocates into nucleus where it binds to glucocorticoid response element (GRE) of gene promoters and, thus, regulates the expression of selected genes (Simons et al., 1992; Gower, 1993).

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humans, and this may be related to increased hypothalamic CRF secretion (Rivier et al., 1984; Wand, 1993; Rivier, 1996). The hyperactive HPA axis has also been shown during ethanol withdrawal after long-term exposure in animals and humans (Tabakoff et al., 1978; Wand, 1993). Furthermore, it has been shown that adrenalectomy prevents the development of ethanol preference in rats (Fahlke and Eriksson, 2000). These results indicate that the HPA axis seems to be one of the physiological systems implicated in the action of acute and chronic ethanol exposure. Ethanol exposure and withdrawal possibly disrupts the negative feedback mechanisms of HPA axis regulation, however, the molecular mechanism by which this process takes place is not well understood. It is also unknown how ethanol exposure and withdrawal modify the GRE-DNA binding in rat brain structures. Therefore, one goal of the present study was to examine the effect of chronic ethanol treatment and its withdrawal on the expression of GR in various brain structures including PVN and also on GRE-DNA binding in rat cortex and hippocampus.  

There are several lines of evidence, which indicate that the HPA axis is regulated by several neurotransmitters including serotonin (5-HT) (Feldman and Weidenfeld, 1995; Herman and Cullinan, 1997). It has been shown that drugs that have ability to enhance 5-HT function can stimulate the HPA axis (Van de Kar, 1991; Bagdy et al., 1989). Among multiple 5-HT receptors that exist in the brain, it was found that at least 5-HT1A, 5-HT2A, and 5-HT2C receptors in the PVN have stimulatory effects on HPA axis functions (Bagdy and Makara, 1994; Bagdy, 1996; Van de Kar et al., 2001). The neuroendocrine studies suggest that GRs in PVN are the major site for the feedback action of glucocorticoids (Plotsky, 1991). It is possible that 5-HT2A/2C receptors in the neural circuitry of PVN may interact with GR to maintain the normal HPA axis, and this interaction may be disturbed by ethanol exposure. We, therefore, also examined the effect of mianserin (5-HT2A/2C antagonist) on GR protein expression in various brain structures including PVN during chronic ethanol exposure and its withdrawal.

Materials and Methods

Animals and Treatment. Male Sprague-Dawley rats weighing 250 to 260 g (at the beginning of the experiment) were used in all experiments. All animal procedures were in accordance with the National Institute of Health “Guide for Care and Use of Laboratory Animals” and were approved by the Animal Care Committee of the University of Illinois at Chicago and VA Chicago Health Care System (West Side Division), Chicago. Ethanol administration to rats was performed by oral ethanol feeding as described previously (Pan- dy, 1996; Pandey et al., 1999, 2001). After a brief acclimation period, rats were housed in individual cages and offered 100 ml of Lieber-DeCarli control diet (Lieber-DeCarli Diet 82; Bioserve Inc., Frenchtown, NJ) as their sole source of food and fluid. Both control and ethanol liquid diets are nutritionally complete diets (Bioserve Inc.; Lieber and DeCarli, 1982). Fresh diet was provided between 5 and 6 PM every day. For chronic ethanol study, one group of rats was also introduced gradually to ethanol and maintained on the liquid control diet plus vehicle once daily at 8 to 9 AM for 15 days. It has been shown that the drug mianserin disappeared from the rat brain with a half-life of 1 to 3 h (Sanders-Bush et al., 1987). Mianserin-treated rats were used for neurochemical studies 24 h after the mianserin injection on the 15th day. For ethanol withdrawal studies, groups of ethanol-fed rats with and without mianserin treatment were withdrawn for 24 h after 15 days of ethanol treatment. Thus, there were five groups of rats in the chronic ethanol and chronic mianserin study: (1) control liquid diet-fed plus vehicle, (2) ethanol-fed (0-h withdrawal) plus vehicle, (3) ethanol-withdrawn (24 h after 15 days of ethanol treatment) plus vehicle, (4) control liquid diet-fed plus chronic mianserin-treated (15 days of i.p. 10 mg/kg/day), and (5) ethanol-withdrawn (24 h after 15 days of ethanol treatment) plus chronic mianserin-treated rats. In all experiments, we measured the daily ethanol-diet intake of the various ethanol-fed rats and control-diet intake in control rats. All these rats were anesthetized with pentobarbital (50 mg/kg, i.p.) and perfused with 4% paraformaldehyde, and the brains were collected for immunohistochemistry as described below. Another batch of rats was prepared as described above, the rats were decapitated, and their brains were removed. The cerebral cortices and hippocampi were dissected out and frozen at −80°C until used for measurement of GRE-DNA binding activities as described below. There were no differences in mean body weight among the different groups of rats.

Measurement of GRE-DNA Binding Activity by Gel Mobility Shift Assay: Preparation of Nuclear Extracts. Nuclear extracts from the cortices, and hippocampi areas were prepared according to the method of Pandey et al. (1999). Tissues were homogenized in buffer I [10 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES), pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 μg/ml pepstatin]. The homogenates were centrifuged at 100,000 g for 30 min. The resulting pellet was resuspended in buffer II (20 mM HEPES, pH 7.9, 0.84 M NaCl, 1.5 mM MgCl2, 0.4 mM EDTA, 0.5 mM DTT, 50% glycerol, and protease inhibitors as in buffer I). After 15 min of incubation on ice with frequent agitation, nuclear extracts were collected by centrifugation at 20,000 g for 15 min, and protein content of the nuclear extracts was determined.

Preparation of DNA Probes. Commercially available (Stratagene, La Jolla, CA) oligonucleotides carrying regulatory elements of GRE sequence (5′-GATCA GACAA CAGT GTTCTCTA-3′) were used. The probes were end-labeled with [γ-32P]ATP using T4 polynucleotide kinase according to the manufacturer’s method (US Biochemicals, Cleveland, OH).

Gel Mobility DNA Binding Assay. GRE-DNA binding reactions were carried out by incubating 15 μg of the nuclear protein with 1 μg of poly(dI-dC) and 6 μM of bovine serum albumin in a reaction mixture [20 mM HEPES, pH 7.9, 1 mM DTT, 0.3 mM EDTA, 0.2 mM ethylene glycol bis (β aminoethyl ether) N,N,N′,N′-tetraacetic acid, 80 mM NaCl, 10% glycerol, and 0.2 mM phenylmethylsulfonyl fluoride] for 15 min at room temperature. Approximately 40,000 dpm of 32P-labeled GRE oligonucleotide was added, and the incubation was continued for an additional 30 min. DNA-protein complexes were resolved on a 4% nondenaturing polyacrylamide gel in a buffer containing 25 mM Tris borate (pH 8.2) and 0.5 mM EDTA. The gel was dried and autoradiographed with intensifying Kodak film (Eastman Kodak, Rochester, NY) at −80°C. The optical densities of the bands of the DNA-protein complexes in the autoradiogram were determined.

Gold-Immunolabeling of Glucocorticoid Receptor and Neuron-Specific Nuclear (NeuN) Proteins. Rats were anesthetized...
and intracardially perfused with normal saline (100 ml) followed by 400 ml of 4% ice-cold paraformaldehyde fixative. Brains were removed and placed in the fixative for 20 h at 4°C. After postfixation, brains were soaked in 10% sucrose, followed by 20% sucrose, and then 30% sucrose (prepared in 0.01 M phosphate buffer, pH 7.4). Brains were then frozen, and 20-µm coronal sections were cut using a cryostat. The sections were placed in 0.1 M phosphate-buffered saline (PBS) at 4°C.

The gold immunolabeling procedure was performed according to the procedure described by Pandey et al. (2001). Brain sections were washed with PBS (twice for 10 min) and then blocked with RPMI 1640 with l-glutamine (Invitrogen, Carlsbad, CA) for 30 min followed by 10% normal goat serum (diluted in PBS containing 0.25% Triton X-100) for 30 min at room temperature. Sections were then incubated with 1% BSA (prepared in PBS containing 0.25% Triton X-100) for 30 min at room temperature. Sections were further incubated with anti-GR or anti-NeuN (Chemicon International, Temecula, CA) antibody (1:500 dilution for GR and 1:200 for NeuN in PBS containing 0.25% Triton X-100) for 18 h at room temperature. After two 10-min washes with PBS and two 10-min washes in 1% BSA in PBS, sections were incubated with gold particles (1 nm) conjugated with the secondary antibody (Ted Pella Inc., Redding, CA) for 1 h at room temperature. Sections were further rinsed several times with 1% BSA in PBS followed by water. Gold-immunolabeling was then silver enhanced for approximately 15 min and washed several times with water. Sections were then mounted on slides, dehydrated, and examined under a light microscope. For negative brain sections, an identical protocol was used except that 1% BSA in PBS was substituted for primary antibody. The quantification of gold-immunolabeled GR and NeuN proteins were performed using the Loats Image Analysis System (Loats Associates, Inc.) connected to the light microscope, which calculated the number of gold particles in a defined brain region at high magnification (100×). The threshold of each image was set up in such a way that areas without staining should give zero counts. Under this condition, gold particles in the defined areas of three adjacent brain sections of each rat were counted and then values were averaged for each rat. Results were expressed as number of gold particles/100 µm² area in a specific brain region. GR rabbit polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). We also characterized the GR antibody by Western blot using cytosolic fraction of cortex according to the procedure as described by Pandey et al. (2001).

Statistical Analyses. Differences among the groups were evaluated by using nonparametric Kruskal-Wallis test. Specific subgroup comparison (between two groups) were performed using the Mann-Whitney U test. A value of p < 0.05 was considered significant.

Results

Pattern of Alcohol Consumption and Blood Ethanol Levels. All rats gained weight during the 15 days of ethanol treatment or mianserin treatment, and there were no significant differences in mean ± S.E.M. (n = 12) body weight (at the end of 15 days of treatment) among the ethanol-fed plus vehicle (285 ± 5.1 g), ethanol-withdrawn plus vehicle (283 ± 5.4 g), ethanol-withdrawn plus mianserin (282 ± 4.7 g), control plus vehicle (284 ± 5.1 g), and control plus mianserin (292 ± 2.1 g)-treated rats. We also measured the daily intakes of control and ethanol diets during mianserin treatment. The patterns of ethanol consumption (milliliters per day or grams per kilogram per day) are shown in Fig. 1. As can be seen, mianserin treatment has no effect on daily ethanol intake in rats. Because animals were pair fed, the control groups also consumed similar amounts (ml/day) of liquid control diet. It was found that control groups consumed about 53 ± 0.6 ml/day (n = 11) control liquid diet. We also measured the morning (8–10 AM) blood ethanol levels (BELs) using an Analox alcohol analyzer (Lunenburg, MA) just before the initiation of withdrawal of ethanol-fed rats (15 days) concurrently treated with or without mianserin. There were no significant differences in mean BELs among the ethanol-fed rats plus vehicle (198 ± 32 mg %), and ethanol-fed rats plus mianserin (182 ± 19 mg %). The BELs in both groups after 24 h withdrawal was 0 mg % and in ethanol-fed rats plus vehicle (0 h withdrawal) was 210 ± 26 mg %. These blood ethanol values for chronic studies (15 days) are similar to the values reported by us in several previous publications (Pandey, 1996; Pandey et al., 1999). These results indicate that ethanol-fed rats treated with or without mianserin consumed similar amount of ethanol diet.

Time Course for Changes in GRE-DNA Binding in Rat Cortex and Hippocampus during Ethanol Withdrawal. We first characterized GRE-DNA binding in the nuclear extract of cortex using competitive experiment with excess of unlabeled GRE oligonucleotides (50 or 100 ng). It was found that unlabeled oligonucleotide dose dependently attenuated the GRE-DNA binding in the nuclear extract of rat cortex (data not shown). We then measured the GRE-DNA binding in the nuclear extracts of cortices and hippocampi of pair-fed control, ethanol-fed, and ethanol-withdrawn (12, 24, and 72 h) rats. The patterns of the GRE-DNA protein complexes in cortex and hippocampus are shown in Fig. 2A. It was found that chronic ethanol treatment significantly decreased GRE-DNA binding activity in the rat cortex and hippocampus, whereas ethanol withdrawal produced opposite effect in the cortex and hippocampus. GRE-DNA binding activity remained decreased at 12 and 24 h of ethanol withdrawal and returned to normal levels after 72 h of ethanol withdrawal in the rat cortex (Fig. 2B). In the hippocampus, GRE-DNA binding was not significantly altered at any time point (12, 24, and 72 h) of ethanol withdrawal after 15 days of ethanol treatment (Fig. 2B). These results indicate
that GRE-DNA binding is decreased in the nuclear extract of cortex during ethanol treatment and early phases of withdrawal. On the other hand, GRE-DNA binding is decreased in the nuclear extract of hippocampus during ethanol treatment and quickly reverted to normal levels during ethanol withdrawal.
Effects of Chronic Mianserin Treatment on GRE-DNA Binding in Rat Cortex and Hippocampus during Ethanol Withdrawal. We studied the effects of chronic mianserin treatment on GRE-DNA binding in the nuclear extracts of cortices and hippocampi of control liquid diet-fed and ethanol diet-fed rats. It was found that mianserin treatment alone has no effect on GRE-DNA binding in nuclear extract of the cortex but produced significant reduction in GRE-DNA binding in the nuclear extract of hippocampus (Fig. 3). Interestingly, when administered concurrently with ethanol treatment, mianserin significantly antagonized the down-regulation of cortical GRE-DNA binding during ethanol withdrawal (Fig. 3). As mentioned above, GRE-DNA binding in hippocampus returned to normal levels at 24 h of ethanol withdrawal, but mianserin treatment produced significant decrease in GRE-DNA binding in hippocampus of ethanol-withdrawn rats. These results indicate that mianserin treatment produced opposite effects in the cortex and hippocampus during ethanol withdrawal.

Effects of Ethanol Treatment and Withdrawal on the Expression of GR in Rat Cortex and Hippocampus. To determine whether decreased GRE-DNA binding in rat cortex and hippocampus is related to decreased protein levels of GR, we determined the protein expression of GR in rat cortex and hippocampus during ethanol treatment and withdrawal. We first characterized GR antibody using Western blot technique. It was found that this antibody recognized a single band of GR proteins (~95 kDa) in the cytosolic fraction of cortex (Fig. 4). We used this antibody to determine the subcellular distribution of GR in various structures of cortex and hippocampus during ethanol treatment and withdrawal. The gold-immunolabeling of GR protein is specific because negative brain sections do not show any labeling (data not shown). The GR-positive cell bodies can be seen in Fig. 5A for cortical and Fig. 6A for hippocampal structures. It was found that chronic ethanol treatment significantly decreased the protein expression of GR in layers II/III and IV/V of cingulate gyrus, frontal, and parietal and in layer II of piriform cortex and further decreased in these structures during ethanol withdrawal (24 h) (Fig. 5, A and B). On the other hand, the protein expression of GR was significantly decreased in CA1, CA2, CA3, and dentate gyrus (DG) of hippocampus during ethanol treatment but reverted to normal levels during ethanol withdrawal (Fig. 5, A and B). These results indicate that decreases in GRE-DNA binding in rat cortex during ethanol treatment and withdrawal may be related to decreases in GR protein expression.

Effects of Ethanol Treatment and Withdrawal on the Expression of GR in Hypothalamus and Amygdala. We also investigated the subcellular distribution of GR in the PVN and central, medial, and basolateral amygdaloid structures during ethanol treatment and withdrawal. The GR-positive cell bodies can be seen in central and medial amygdaloid (Fig. 7A) and PVN structures (Fig. 8A). It was found that chronic ethanol treatment significantly decreased the protein expression of GR in the PVN (Fig. 8B) and in the central,}

**Fig. 3.** Effect of ethanol withdrawal (0 and 24 h) after 15 days of ethanol treatment and the effect of chronic mianserin (5-HT₂ agonist) with or without ethanol administration on the GRE-DNA binding activity in the rat cortex and hippocampus. Values are the mean ± S.E.M. of 6 to 12 experiments and are represented as percents of the normal controls. * significantly different from the pair-fed control group (p < 0.05).

**Glucocorticoid Receptor**

**Fig. 4.** Representative Western blot of GR protein in the cytosolic fraction of rat cortex. Different concentrations of protein were resolved on SDS-polyacrylamide gel, transferred to nitrocellulose membrane, and incubated with GR primary antibody and then secondary antibody.
Fig. 5. A, low magnification views of GR gold-immunolabeling in various cortical structures of control diet-fed plus vehicle, ethanol-fed plus vehicle, ethanol-withdrawn plus vehicle, control diet-fed plus mianserin, and ethanol-withdrawn plus mianserin-treated rats. A through D, GR-positive cell bodies in layer IVV of cingulate gyrus (CG), the frontal cortex (Fr), the parietal cortex (Par), and layer II of piriform (Piri) cortex, respectively, of control diet-fed rats. E through H, GR-positive cell bodies in CG, Fr, Par, and Piri structures, respectively, of ethanol-fed rats. I through L, GR-positive cell bodies in CG, Fr, Par, and Piri structures, respectively, of ethanol-withdrawn rats. M through P, GR-positive cell bodies in CG, Fr, Par, and Piri structures, respectively, of ethanol-withdrawn plus mianserin-treated rats. Q through T, GR-positive cell bodies in CG, Fr, Par, and Piri structures, respectively, of mianserin (alone)-treated rats. Arrows indicate some of the GR-positive cell bodies. Scale bar, 40 μm in A through T. B, effect of ethanol withdrawal (0 and 24 h) after 15 days of ethanol treatment and the effect of chronic mianserin treatment with or without ethanol administration on GR protein levels in various layers of the cortical structures. Values are mean ± S.E.M. of five to six rats in each group. *, significantly different from the pair-fed control group (p < 0.05).
medial, and basolateral amygdala (Fig. 7B) and further decreased in these structures during ethanol withdrawal (Figs. 7B and 8B). These results indicate that GR protein expression is decreased in the PVN and in various amygdaloid structures during chronic ethanol treatment and its withdrawal.

**Effects of Chronic Mianserin Treatment on the Expression of GR in the Rat Brain during Ethanol Withdrawal.** We studied the effects of mianserin treatment on GR protein levels in the cortical, amygdaloid, hippocampal, and PVN structures of control liquid diet-fed and ethanol diet-fed rats. It was found that mianserin treatment alone had no effect on GR protein levels in cortical (Fig. 5, A and B), amygdaloid (Fig. 7, A and B), or PVN (Fig. 8, A and B) structures but produced significant reductions in the GR protein levels in hippocampal structures (CA1, CA2, CA3, and DG) (Fig. 6, A and B). It was also observed that mianserin treatment, when administered concurrently with ethanol treatment, significantly antagonized the decreased expression of GR in the PVN (Fig. 8, A and B), cortical (Fig. 5, A and B), and central but not in medial and basolateral amygdaloid (Fig. 7, A and B) structures during ethanol withdrawal. We observed that decreased expression of GR protein in various structures of hippocampus during ethanol treatment returned to normal levels during withdrawal.

Interestingly, mianserin treatment produced significant decreases in GR protein levels in hippocampal (CA1, CA2, CA3, and DG) structures of ethanol-withdrawn rats (Fig. 6, A and B). These results indicate that blockade of 5-HT2A/2C receptors during ethanol exposure antagonizes the ethanol-induced decrease in GR expression in PVN, cortical, and central amygdaloid structures but not in hippocampal, medial, and basolateral amygdaloid structures.

**Effects of Chronic Ethanol Treatment and Its Withdrawal on Number of Neurons in Rat Brain Structures.** To examine whether decreased expression of GR in various brain structures during ethanol exposure and its withdrawal is related to loss of neurons, we investigated the protein levels of NeuN in the nuclei. The NeuN-positive nuclei can be seen in Fig. 9A. It was found that chronic ethanol exposure and its withdrawal has no effects on the NeuN protein levels in the various structures of hippocampus, amygdala, cortex, and PVN (Fig. 9B). We also examined the effects of mianserin treatment on NeuN protein levels in brain structures of control and ethanol-withdrawn rats. It was found that mianserin treatment has also no effects on NeuN protein levels in various brain structures of control diet- and ethanol diet-fed rats. These results suggest that
neither ethanol treatment nor mianserin treatment is associated with loss of neurons in various brain structures.

Discussion

Neuroadaptation in the HPA Axis during Chronic Ethanol Exposure and Its Withdrawal. The first key finding of the present investigation is that expression of GR is decreased in PVN, cortical, hippocampal, and amygdaloid structures during chronic ethanol exposure. Furthermore, the reduction in GR protein levels is more in PVN, cortical, and amygdaloid structures but reverted to normal levels in hippocampus during ethanol withdrawal. It was also found that reduction in GR protein levels in cortical and hippocampal structures are associated with GRE-DNA binding in these structures during ethanol treatment.

The mechanisms by which ethanol treatment and withdrawal produced down-regulation of GR in various brain structures are not clear, but may be related to high levels of circulating glucocorticoids as reported by several investigators in rodent models and human alcoholics during ethanol exposure and its withdrawal (Tabakoff et al., 1978; Wand, 1993; Rivier, 1996). It has been shown that high levels of glucocorticoids leads to down-regulation of GR in several cell systems (Okret et al., 1986; Rosewicz et al., 1988). Hyperactive HPA axis has been shown in human alcoholics (Risher-Flowers et al., 1988; Wand, 1993). However, there are inconsistent reports about hyperactive HPA axis in animal models, and this may be due to variations in blood ethanol levels, methods, and duration of ethanol administration (Lee and Rivier, 1994; Rivier, 1996; Ogilvie et al., 1997; Rasmussen et al., 2000). In the present study, rats were treated with 9% ethanol for 15 days, which produced blood ethanol levels in the range of 210 to 198 mg %. Under this ethanol treatment paradigm, there are significant reductions in the protein levels of GR in the PVN and other nonhypothalamic regions (cortex, hippocampus, and amygdala).

The GRs in the PVN and hippocampus have been shown to be an important regulator of HPA axis via feedback mechanism (Jacobson and Sapolsky, 1991; Plotsky, 1991). In the present study, GR expression is decreased both in PVN and hippocampus during ethanol exposure. In PVN, GR expression further decreased whereas in hippocampus, GR expres-
sion reverted to normal levels during ethanol withdrawal. This suggests that GRs are regulated differentially in PVN and hippocampus during ethanol withdrawal. Furthermore, the hyperactive HPA axis during ethanol treatment and withdrawal may be related to decreased expression of GR in PVN, but may not be related to GR in hippocampus. The dichotomy between regulation of HPA axis by GR in PVN and hippocampus was shown earlier using local infusion of GR antagonist and agonist in stressed rats. It was found that GR antagonist and agonist infusion in hippocampus has no effect on corticosterone levels whereas GR antagonist and agonist infusion in PVN is able to increase or suppress the corticosterone levels, respectively (De Kloet et al., 1988; Kovacs and Makara, 1988). The results suggest that GR in PVN but not in hippocampus is crucial in feedback inhibition of HPA axis.

It has been established that effects of glucocorticoids in the brain are mediated by MR and GR and both of these receptors are important in mediating the negative feedback action of glucocorticoids (De Kloet, 1991). MR is rich in hippocampus and GR is located throughout the brain (Reul and De Kloet, 1986; Feldman and Weidenfeld, 1995). The chronic ethanol exposure has no effects on GR and MR levels as determined by binding techniques in the cytosolic fraction of hippocampus (Spencer and McEwen, 1990). One previous study investigated the effects of chronic ethanol exposure (10 days) on mRNA levels of GR in the hippocampal structures and found that mRNA levels of GR are significantly decreased in the CA1, CA3, and DG of hippocampus (Eskay et al., 1995). Here we found that chronic ethanol (15 days) exposure causes significant reductions in the GR protein levels in hippocampal structures and in other brain structures. Because GR protein levels are decreased in cortical, amygdaloid, hippocampal, and PVN structures during ethanol exposure, this suggests that GR in these brain structures may play a role in the neuromechanisms of alcohol tolerance. Furthermore, GR protein levels are normalized in the hippocampus whereas in PVN, cortical and amygdaloid structures remained decreased during ethanol withdrawal; this suggests that GR in nonhippocampal area such as PVN and central amygdala may play a role in the process of ethanol dependence. The one caveat of the present study is that we have not investigated the neuroadaptational changes in MR protein levels in various brain structures during ethanol exposure or its withdrawal. It is possible that the imbalance between MRs and GRs in hippocampus or PVN may be crucial in the abnormal HPA axis during ethanol dependence. Future studies will investigate this possibility.

**Hyperactive HPA Axis and Neuronal Loss in the Rat Brain during Chronic Ethanol Exposure.** It has been hypothesized that hippocampal structure is more vulnerable to endangering or weakening effects of elevated circulating levels of glucocorticoids (Walker et al., 1981; Eskay et al., 1995; Lukoyanov et al., 1999). Thus, other possible explanation for decreased GR protein expression in hippocampus or
other brain structures may be related to neurotoxicity in part to chronically elevated glucocorticoids during ethanol treatment. We tested this possibility by measuring neuronal marker (NeuN protein) in the neurons and found that neither chronic ethanol treatment nor withdrawal had any effects on the NeuN protein levels. These results indicate that decreased expression of GR in various brain structures is not due to loss of neurons during 15 days ethanol treatment or its withdrawal. It has been shown that long-term treatment with ethanol (more than 1 month) leads to loss of neurons in the hippocampus (Walker et al., 1981; Lukoyanov et al., 1999). Taken together, this suggests that long but not shorter ethanol exposure causes loss of neurons in the hippocampus.

**5-HT\(_{2A/2C}\) Receptor Interactions with HPA Axis during Chronic Ethanol Exposure.** The second key observation of the present study is that blockade of 5-HT\(_{2A/2C}\) receptors during alcohol drinking antagonized the ethanol-induced decreased expression of GR in PVN, cortical, and central but not medial and basolateral amygdaloid structures. On the other hand, mianserin treatment significantly decreased the GR expression in the neurocircuitry of hippocampus and other brain structures (cortex, PVN and central amygdala) behaves differentially during serotonergic manipulations. Because mianserin treatment has no effects on daily ethanol intake under forced treatment paradigm, this suggests that prevention of ethanol-induced decreases in GR protein levels in various brain structures by mianserin treatment may be related to direct action of drug on 5-HT\(_{2A/2C}\) receptors.

It has been shown that 5-HT\(_{2A/2C}\) receptor agonist stimulates HPA axis and this action, primarily due to stimulation of 5-HT\(_{2A}\) receptor, mediated CRF release in the PVN (Van de

![Fig. 9. A, low magnification views of NeuN gold-immuno-labeling in frontal cortex (layer IV/V), central amygdala, and PVN of control diet-fed plus vehicle, ethanol-fed plus vehicle, ethanol-withdrawn plus vehicle, control diet-fed plus mianserin, and ethanol-withdrawn plus mianserin-treated rats. A through E, NeuN-positive nuclei in frontal cortex of control diet-fed, ethanol-fed, ethanol-withdrawn, ethanol-withdrawn plus mianserin, mianserin (alone)-treated rats, respectively. F through J, NeuN-positive nuclei in central amygdala of control diet-fed, ethanol-fed, ethanol-withdrawn, ethanol-withdrawn plus mianserin, mianserin (alone)-treated rats, respectively. K through O, NeuN-positive nuclei in PVN of control diet-fed, ethanol-fed, ethanol-withdrawn, ethanol-withdrawn plus mianserin, mianserin (alone)-treated rats, respectively. Scale bar, 40 μm in A through O. B, effect of ethanol withdrawal (0 and 24 h) after 15 days of ethanol treatment and the effect of chronic mianserin treatment with or without ethanol administration on NeuN levels in various brain structures. Values are mean ± S.E.M. of five rats in each group.](https://jpet.aspetjournals.org/content/49/6/782.full.pdf)
Kar, 1991; Feldman and Weindenfeld, 1995; Van de Kar et al., 2001). The notion that 5-HT2A/C receptors in the PVN may regulate the HPA axis is supported by the fact that lesioning of PVN prevents this receptor-mediated release of corticosterone in rats (Bagdy and Makara, 1994). Regardless of mechanisms, these results indicate that interaction between 5-HT2A/C receptors and GRs in the PVN, cortical, or central amygdaloid structures may play an important role in the pathophysiology of HPA axis during ethanol dependence. The decreased GRs in the limbic structures such as central amygdala and frontal cortex may be involved in anxiety developing during ethanol withdrawal. We have shown earlier that ethanol withdrawal (24 h) after 15 days treatment produced anxiety-like behavior in rats (Pandey et al., 1999). It has been also shown that local infusion of corticosterone in to central amygdala increase CRF mRNA levels and also anxiety-like behaviors in rats (Shepard et al., 2000). Increased CRF levels in the central amygdala have been shown to be involved in anxiety-like behaviors in rats during ethanol withdrawal (Koob et al., 1998). Furthermore mianserin treatment is also able to prevent anxiety during ethanol withdrawal (Lal et al., 1993). It is possible that 5-HT2A/C receptor interaction with GRs and CRF in the central amygdala may be one of the mechanisms responsible for the ethanol withdrawal-related anxiety. Future experiments are needed to explore such relationship during ethanol withdrawal.

Conclusions

The data presented here provide the first evidence that decreased GR protein levels in PVN, cortical, hippocampal, and amygdaloid structures may be associated with neuroadaptational mechanisms to chronic ethanol exposure. The GR expression normalized in hippocampal but further decreased in cortical, amygdaloid, and PVN structures during ethanol withdrawal, this suggests that GR in these nonhippocampal structures may be associated with the process of alcohol dependence. Because GRs in the neural circuitry of PVN are the important regulator of HPA axis, it is possible that decreased GRs in this brain structures may be responsible for the compromised HPA axis during ethanol exposure and withdrawal. Moreover the decreased expression of GR in various brain structures during ethanol exposure and withdrawal is not related to neuronal loss.

References

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