Potential Role of the Gene Transcription Factor Cyclic AMP-Responsive Element Binding Protein in Ethanol Withdrawal-Related Anxiety

SUBHASH C. PANDEY, DAOLONG ZHANG, NAVDHA MITTAL and DEEPAK NAYYAR

The Psychiatric Institute, Department of Psychiatry, College of Medicine, University of Illinois, and Psychiatry Research Service, Veterans Administration Chicago Health Care System (West Side Division), Chicago, Illinois

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ABSTRACT

This investigation examined the effects of acute and chronic ethanol exposure and its withdrawal on the cAMP-responsive element binding protein (CREB) and the activator protein-1 (AP-1) gene transcription factors in the rat brain. The anxiogenic effects of ethanol withdrawal after acute or protracted ethanol treatment of rats were measured by the elevated plus-maze (EPM) test. It was observed that ethanol withdrawal after acute ethanol treatment has no effect on open-arm activity (percent of open-arm entries and the mean percent of time spent on the open arms) of rats on the EPM test. On the other hand, the time course studies of the development of anxiety during ethanol withdrawal (0, 12, 24, and 72 h) after 15 days of ethanol treatment indicate that peak anxiety (significant decrease in open-arm activity) occurred at 24 h of ethanol withdrawal in rats. It was observed that acute ethanol treatment and its withdrawal (24 h) had no effect on CRE- or AP-1 DNA-binding activities in the rat cortex as determined by the electrophoretic gel-mobility shift assay. It was also found that chronic ethanol treatment and its withdrawal (24 h) had no effect on AP-1 DNA-binding activity in the rat cortex. Investigation of the time course studies of changes in CRE-DNA-binding activity during ethanol withdrawal (0, 12, 24, and 72 h) after 15 days of ethanol treatment indicated that the peak reduction of CRE-DNA-binding activity occurred at 24 h of ethanol withdrawal. The changes in the immunolabeling of the CREB-related target, that is, brain-derived neurotrophic factor (BDNF), in the rat cortex during chronic ethanol treatment and its withdrawal (24 h) were examined using western blotting. It was found that 24 h but not 0 h of ethanol withdrawal after 15 days of ethanol treatment caused a significant decrease in the immunolabeling of BDNF in the rat cortex. Fluoxetine (alone) treatment of rats for 1 or 15 days had no effect on open-arm activity and cortical CRE-DNA-binding activity. However, when fluoxetine was administered concurrently with ethanol treatment for 15 days, it caused a reversal of the anxiogenic effects of ethanol withdrawal and antagonized the down-regulation of CRE-DNA-binding activity and of the decrease in immunolabeling of BDNF in the cortices of ethanol-withdrawn rats. On the other hand, acute fluoxetine treatment produced normalization of the reduction of cortical CRE-DNA binding in ethanol-withdrawn rats (24 h) but did not reach the level of significance compared with normal control rats. Acute fluoxetine treatment had no effect on anxiety in ethanol-withdrawn rats. Taken together, these results suggest the possibility that decreased CRE-DNA-binding activity in the rat cortex may be associated with the molecular mechanisms of ethanol dependence (i.e., ethanol withdrawal-related anxiety).

Cessation of chronic ethanol consumption is often accompanied by signs and symptoms characteristic of ethanol withdrawal syndromes (Koob and Bloom, 1988; Harris and Buc, 1990). Anxiety is a common early symptom of ethanol withdrawal and is considered an important factor in the continued use of alcohol by alcoholics because this relieves the symptoms of ethanol withdrawal (Roelofs, 1985; Wilson, 1988; Kushner et al., 1990). Study of the cellular mechanisms associated with the development of anxiety during ethanol withdrawal after protracted ethanol exposure is important to provide a basis for developing better drugs to treat ethanol withdrawal-related anxiety.

The cellular actions of various neurotransmitters [serotonin (5-HT), norepinephrine, dopamine, acetylcholine] in the brain are mediated through the activation of adenylate cyclase, which causes the formation of the intracellular second messenger cAMP and subsequently leads to the activation of cAMP-dependent protein kinase A (PKA) (Gilman, 1989). The activation of PKA releases the catalytic subunits of PKA,

ABBREVIATIONS: AP-1, activator protein-1; BDNF, brain-derived neurotrophic factor; CREB, cyclic AMP-responsive element-binding protein; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; CRE, cyclic AMP-response element; EPM, elevated plus-maze test; G_i, inhibitory guanine nucleotide-binding protein; G_s, stimulatory guanine nucleotide-binding protein; PBS, phosphate-buffered saline; 5-HT, serotonin; PKA, protein kinase A.
which migrate to the nucleus and phosphorylate the cAMP-responsive element binding protein (CREB) gene transcription factor, which then regulates the expression of cAMP-inducible genes (Gonzales et al., 1989; Gonzales and Montminy, 1989; Hunter and Michael, 1992; Meyer and Habener, 1993). Alterations in the various steps of the cAMP signal transduction pathway in the brain and in other cell systems during ethanol tolerance and dependence have been demonstrated by several investigators (Mochly-Rosen et al., 1988; Valverius et al., 1989; Hoffman and Tabakoff, 1990; Wand and Levine, 1991; Coe et al., 1996).

Several earlier studies have demonstrated that chronic ethanol treatment decreases the activity of adenylate cyclase and decreases stimulatory guanine nucleotide-binding protein (Gs) and increases inhibitory guanine nucleotide-binding protein (Gi) protein expression and function in the rodent brain (Valverius et al., 1989; Hoffman and Tabakoff, 1990; Wand and Levine, 1991). It has also been shown that PKA-mediated phosphorylation is decreased in the brain of chronic ethanol-treated rats compared with controls (Ruis et al., 1988). Because various components (neurotransmitter receptors, G proteins, adenylate cyclase, PKA) of the cAMP signal transduction cascade are decreased in the rodent brain during ethanol dependence (Shen et al., 1983; Ruis et al., 1988; Valverius et al., 1989; Hoffman and Tabakoff, 1990; Wand and Levine, 1991), it is possible that the function of the CREB gene transcription factor may also be altered during ethanol dependence. Furthermore, the expression of the brain-derived neurotrophic factor (BDNF) has been shown to be regulated by the CREB gene transcription factor (Condorelli et al., 1994; Duman et al., 1995, 1997). This suggests that alterations in CRE-DNA-binding activity might modulate the expression of BDNF in the rat brain during ethanol dependence.

Activator protein-1 (AP-1) is another gene transcription factor that has been shown to recognize a specific DNA sequence (TGACTCA) in the promoter region of several genes (Hai and Curran, 1991; Hughes and Dragunow, 1995). The immediate-early genes, c-fos and c-jun, encode proteins that are known to form homodimeric or heterodimeric protein complexes (Sonnenberg et al., 1989; Kobierski et al., 1991; Hughes and Dragunow, 1995). These protein complexes recognize the AP-1 DNA-binding elements that regulate the transcription of genes containing this DNA element (Sonnenberg et al., 1989; Hughes and Dragunow, 1995). Although changes in CRE- and AP-1 DNA-binding activities have been demonstrated in various brain structures of rats during morphine or cocaine treatment (Hope et al., 1992; Nestler, 1992; Maldonado et al., 1996; Pich et al., 1997), their roles in ethanol dependence are not fully understood. Recently, it was shown that AP-1 DNA-binding activity is increased in brain of ethanol-withdrawn rats after chronic ethanol inhalation (Beckmann et al., 1997). Whether acute or chronic ethanol treatment produces similar or dissimilar effects on AP-1 DNA-binding activity in the rat brain, however, is unknown.

5-HT neurotransmission has been shown to play an important role in anxiety (Eison and Eison, 1994; Graeff et al., 1996), and 5-HT uptake blockers (e.g., fluoxetine) are useful in the treatment of anxiety and depression in alcoholics undergoing detoxification (LeJoyeau, 1996; Pettinati, 1996). It has been shown that long-term fluoxetine treatment increases the function of CREB and the expression of BDNF in the rat hippocampus (Nibuya et al., 1996). These observations raise the possibility that although the initial action of fluoxetine could be related to increases in 5-HT levels and to alterations in 5-HT receptor subtypes (Wong et al., 1995), the CREB signaling cascade could also be involved in the anxiolytic or the antidepressive actions of fluoxetine.

The objectives of the present study were to elucidate (1) the effects of acute and chronic ethanol treatment and its withdrawal on the CRE- and AP-1 DNA-binding activities in the rat cortex, (2) whether changes in CRE-DNA-binding activity are associated with changes in CREB targets (e.g., BDNF, in the rat cortex during ethanol treatment and its withdrawal), (3) whether ethanol withdrawal after acute or protracted ethanol treatment is associated with the development of anxiogenic behaviors in rats, (4) whether the time course of the development of anxiogenic behaviors during ethanol withdrawal after protracted ethanol intake is associated with the time course of changes in CRE-DNA-binding activity in the rat cortex, and (5) the effects of acute and chronic fluoxetine treatment on anxiogenic behaviors and on the cortical CRE-DNA-binding activity in ethanol-withdrawn rats after 15 days of ethanol intake.

Materials and Methods

Animals and Ethanol Administration

Virus-free male Sprague-Dawley rats weighing 225 to 250 g were used in all experiments. Ethanol administration to rats was performed by oral ethanol feeding as described previously (Pandey, 1996; Pandey et al., 1996). After a brief acclimation period, rats were individually housed and offered 100 ml of the Lieber-DeCarli control diet (Lieber-DeCarli Diet 82; Bioserve, Inc., Frenchtown, NJ) as their sole source of food or fluid. On the next day, rats were randomly selected for the acute or the chronic study. All animals were pair-fed and were weighed twice a week. Fresh diet was provided between 5:00 and 6:00 PM every night. All animal procedures were in accordance with the National Institutes of Health “Guide for the 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) on Chicago.

For the acute ethanol study, one group of rats received the control liquid diet and a second group of rats was gradually introduced to the ethanol diet by receiving a liquid diet containing 4.5% (v/v) ethanol on the first day, 7.5% (v/v) ethanol on the second day, and 9% (v/v) ethanol on the third day. Ethanol-treated rats were withdrawn from ethanol for 0, 14, and 24 h. All rats were tested for anxiogenic behaviors using the elevated plus-maze test (EPM) (see later EPM discussion). All of these rats were decapitated, and their brains were collected. The cerebral cortices were dissected onto an ice-chilled plate and frozen at −80°C until used for measurement of the CRE and the AP-1 DNA-binding activities.

For the chronic ethanol study, one group of rats were also introduced gradually to ethanol (see previous acute study discussion) but were maintained on the ethanol-containing (9% v/v) Lieber-DeCarli liquid isocaloric diet ad libitum for 15 days (ethanol-fed group). Another group of rats received the control liquid diet for 15 days (pair-fed control group). For the time course studies, ethanol-treated rats were withdrawn for 0, 12, 24, and 72 h. For the chronic fluoxetine studies, the control liquid diet-fed and the ethanol-fed groups also received fluoxetine (5 mg/kg/day i.p.) or vehicle treatment for 15 days. Thus, there were five groups of rats in the chronic ethanol and chronic fluoxetine study: (1) control liquid diet-fed plus vehicle, (2) ethanol-fed (0-h withdrawal), (3) ethanol-withdrawn (24 h after 15 days of ethanol intake) plus vehicle, (4) liquid diet-fed plus chronic fluoxetine-treated (15 days of i.p. 5 mg/kg/day), and (5)
ethanol-withdrawn (24 h after 15 days of ethanol intake) plus chronic fluoxetine-treated. All five rat groups were tested for anxiogenic behaviors using the EPM. In the fluoxetine-treated rats, the behavioral measurements were performed 24 h after the fluoxetine injection on the 15th day. For the acute fluoxetine studies, control liquid diet-fed and ethanol-withdrawn rats (24 h) received a single injection of fluoxetine (5 mg/kg i.p.) just 1 h before the EPM. After behavioral measurements, the rats were decapitated and their brains were collected. The cerebral cortices were dissected and frozen at −80°C until used for measurement of CRE- and AP-1 DNA-binding activities and for the immunolabeling of BDNF.

Measurement of Anxiogenic Behaviors by EPM

The EPM has been used frequently to demonstrate the anxiogenic behaviors occurring in rats during ethanol withdrawal after chronic ethanol treatment (Baldwin et al., 1991; Rassnick et al., 1993). The elevated plus-maze is constructed of white Plexiglas and black metal and consists of two open arms and two closed arms arranged directly opposite each other and interconnected to a central platform (Lafayette Instrument Company, IN). The test procedure was the same as that described by other investigators (Baldwin et al., 1991; Rassnick et al., 1993; Wallis et al., 1999). Each rat was taken to the test room and allowed a 5-min pretest habituation period before the EPM was performed. After the habituation period, the test rat was placed gently on the central platform facing toward an open arm. The rat was observed for a 5-min test period, and the number of entries made onto each type of arm (open versus closed) and the time spent on each type of arm were recorded. The EPM test results are expressed as the mean ± S.E.M. of the percent of open-arm entries and the mean percent of time spent on the open arms. Anxiogenic behaviors are defined as the decrease in the percent of time spent exploring the open arms as well as in the percent of open-arm entries into the EPM.

Determination of CRE- and AP-1 DNA-Binding Activities by Electrophoretic Gel-Mobility Shift Assay

Preparation of Nuclear Extracts. Nuclear extracts from the cortical area were prepared according to the methods of Ishige et al. (1996). Tissues were homogenized in 5.0 ml of buffer I (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.9, 1.5 mM MgCl$_2$, 10 mM KCl, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, 10 μg/ml of the AP-1-binding domain) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and diluted 1:1000 in PBS containing 1.0% Tween 20 for 30 min at room temperature. The nitrocellulose membrane was then incubated overnight at 4°C with the BDNF antibody (Biotechnologie, Santa Cruz, CA) and diluted 1:1000 in PBS containing 1.0% Tween 20. After repeated washing (three times for 15 min) in PBS containing 1.0% Tween 20, the nitrocellulose membrane was incubated with a horse-radish peroxidase-linked secondary anti-rabbit antibody (1:2000) for 4 h. The nitrocellulose membranes were washed as described above, and the bound antibody was detected by the enhanced chemiluminescence method according to the manufacturer's instructions (Amersham, Arlington Heights, IL). The blots were stripped at 50°C for 20 min using a stripping buffer (100 mM Tris–HCl, 2 mM 2-mercaptoethanol, 20% SDS, and 62.5 mM Tris–HCl, pH 6.7) and then incubated with the β-actin antibody and the secondary anti-mouse antibody according to the procedure described above. The bands on the autoradiograms were quantified by using the Las-3000 Image Analysis System. Values were normalized to the β-actin immunoreactivity in each sample and expressed as a percent of the control value.

Statistical Analysis

Differences among groups were evaluated by using analyses of variance or the Kruskal-Wallis test where appropriate. Specific subgroup comparisons were performed by using Student's t test. A value of P < .05 is considered significant.

Results

Body Weight and Chronic Ethanol Intake. In the acute ethanol study, there were no differences in the mean ± S.E.M. body weight (g) among ethanol-fed (272 ± 3.0), ethanol-withdrawn (24 h) (273 ± 3.9), and pair-fed control (279 ± 2.0) rats. Blood ethanol levels were found to be 101 ± 23 mg/100 ml in the acutely ethanol-fed rats. In the chronic...
studies, all rats gained weight during the 15 days of ethanol treatment, and there were no significant differences in mean ± S.E.M. body weight (g) among ethanol-fed (297 ± 3.7), ethanol-withdrawn (292 ± 3.9), pair-fed control (280 ± 4.7), and fluoxetine-treated groups (ethanol-withdrawn plus fluoxetine, 290 ± 6.5; fluoxetine (alone)-treated, 290 ± 10.0). Blood ethanol levels were found to be 183 ± 19 mg/100 ml in the chronically ethanol-fed rats. The blood ethanol level after 24 h of withdrawal in the ethanol-fed group was 0 mg/100 ml. We also measured the daily ethanol intake of the various chronically ethanol-fed rat groups and found that the mean ethanol intake (g/kg/day) was similar in all rat groups (ethanol-fed, 14.65 ± 0.32; ethanol-withdrawn, 14.94 ± 0.36; ethanol-withdrawn plus fluoxetine, 14.72 ± 0.25). These findings indicate that chronic fluoxetine treatment concurrent with ethanol administration had no effect on the daily intake of ethanol.

Anxiogenic Behaviors in Rats during Ethanol Withdrawal after Acute Ethanol Intake. Rats were treated with ethanol (4.5% v/v, first day; 7.5% v/v, second day; and 9% v/v, third day) or control liquid diet. Ethanol-treated rats were withdrawn from ethanol for 0, 14, and 24 h. Anxiogenic behaviors in ethanol-withdrawn (0, 14, and 24 h) rats and in pair-fed control rats were measured by EPM. As shown in Fig. 1, ethanol withdrawal after acute ethanol intake had no significant effect on either the open- or closed-arm activities (number of entries and time spent on open or closed arms) of rats into the EPM test. We also observed that acute ethanol treatment and its withdrawal had no significant effect on the total number of arm entries (open plus closed arms) on the EPM (Fig. 1). These results suggest that ethanol withdrawal after acute ethanol treatment does not cause anxiogenic behaviors in rats.

Effects of Acute Ethanol Treatment and Its Withdrawal on CRE- and AP-1 DNA-Binding Activities in the Rat Cortex. We characterized CRE-DNA binding by using two methods: (1) incubation of the nuclear extract (10 μg) with CREB antibody (0.76 μg) resulted in the formation of a supershift band in the gel-mobility shift assay, which indicates that the CRE-DNA-binding complex contains CREB protein (Fig. 2a); and (2) a competitive experiment with unlabeled normal CRE oligonucleotide completely blocked the CRE-DNA-binding activity, whereas incubation with mutated CRE oligonucleotides had no effect on CRE-DNA-binding activity (Fig. 2b). In a similar manner, we also characterized AP-1 DNA-binding activity in the rat cortex. The competitive experiments performed with unlabeled normal or mutated AP-1 oligonucleotides indicate that AP-1 DNA binding is attenuated by the unlabeled normal AP-1 oligonucleotide (higher concentrations) but not by the mutated AP-1 oligonucleotide (Fig. 2b).

We studied the effects of acute ethanol treatment and its withdrawal (24 h) on CRE- and AP-1 DNA-binding activities in the rat cortex and found that neither acute ethanol feeding (9% v/v for 1 day) nor 24 h of ethanol withdrawal after acute treatment had any significant effect on CRE- or AP-1 DNA-binding activities in the rat cortex (Fig. 3, a and b). Thus, our results indicate that ethanol withdrawal (0 or 24 h) after acute ethanol intake does not modulate CRE- or AP-1 DNA-binding activities in the rat cortex.

Time Course of Development of Anxiogenic Behaviors during Ethanol Withdrawal after 15 Days of Ethanol Intake. We studied the effects of ethanol withdrawal (0, 12, 24, and 72 h) on the anxiogenic behavior (open-arm activity) of rats treated with ethanol for 15 days (Fig. 4). The percent of open-arm entries and the time spent on the open arms and the time spent on the closed arms on the EPM by ethanol-fed rats (0-h withdrawal) are similar to those of the pair-fed control rats. At 12 h of ethanol withdrawal, there was a slight decrease in the percent of open-arm entries and in the time spent on the open arms, and a slight increase in the time spent on the closed arms by the ethanol-withdrawn rats (Fig. 4). It was observed that the percent of open-arm entries was significantly decreased in ethanol-withdrawn rats at 24 and 72 h of withdrawal. The percent of time spent on the open arms was also significantly decreased, and the time spent on the closed arms was also significantly increased at 24 h of withdrawal. It was also observed that...
although ethanol-withdrawn rats (72 h) made fewer entries onto the open arms compared with pair-fed control rats, the time spent on the open arms by ethanol-withdrawn rats (72 h) was similar to that of pair-fed control and ethanol-fed (0-h withdrawal) rats. The total number of entries on the EPM (closed-arm plus open-arm entries) was significantly lower in 12-, 24-, and 72-h ethanol-withdrawn rats compared with pair-fed control rats (Fig. 4). These results suggest that peak anxiety in rats occurs at 24 h of ethanol withdrawal after 15 days of ethanol treatment.

**Time Course of Changes in CRE-DNA-Binding Activity during Ethanol Withdrawal after 15 Days of Ethanol Intake.** We examined CRE-DNA-binding activity in the cortices of chronic ethanol-fed, ethanol-withdrawn (12, 24, and 72 h), and pair-fed control rats using the gel-mobility shift assay. The patterns of the DNA-protein complexes were similar to those reported by other investigators (Lukasiuk and Kaczmarek, 1994; Ishige et al., 1996). As can be seen in Fig. 5, a and b, there were no significant changes in CRE-DNA-binding activity in the rat cortex during ethanol consumption, but CRE-DNA-binding activity was slightly decreased at 12 h yet significantly decreased at 24 h of ethanol withdrawal compared with pair-fed control rats. Although the changes observed in cortical CRE-DNA-binding activity at 24 h of withdrawal approached normal levels after 72 h of ethanol withdrawal, the levels remained significantly lower compared with pair-fed control rats (Fig. 5b). These results suggest that peak reduction in CRE-DNA-binding activity occurred at 24 h of ethanol withdrawal after 15 days of ethanol exposure.

**Effects of Protracted Ethanol Treatment and Its Withdrawal on AP-1 DNA-Binding Activity in the Rat Cortex.** To examine whether the changes in CRE-DNA binding are unique, we determined AP-1 DNA-binding activity in the cortices of chronic ethanol-fed (0-h withdrawal), ethanol-withdrawn (24-h withdrawal), and pair-fed control rats using the gel-mobility shift assay. The patterns of the DNA-protein complex of AP-1 were similar to those reported by other investigators (Lukasiuk and Kaczmarek, 1994; Ishige et al., 1996). There were no significant changes in AP-1 DNA-binding activity in the cortices of ethanol-fed or withdrawn (24 h) rats compared with pair-fed control rats (Fig. 6, a and b). These results suggest that AP-1 DNA-binding activity is not changed and that only CRE-DNA-binding activity is decreased in the rat cortex during ethanol withdrawal (24 h) after protracted (15 days) ethanol intake.

**Effects of Acute and Chronic Fluoxetine Treatment on Anxiogenic Behaviors and on CRE-DNA Binding Activity during Ethanol Withdrawal.** We studied the effects of acute (5 mg/kg once i.p.) and chronic (5 mg/kg/day i.p. for 15 days) fluoxetine treatment on the anxiogenic behaviors (Fig. 7) of rats as well as on CRE-DNA-binding activity in the rat cortex. It was observed that although acute fluoxetine treatment antagonized the reduction in cortical CRE-DNA-binding activity in ethanol-withdrawn rats, CRE-DNA-binding activity in the cortices of ethanol-withdrawn plus acute fluoxetine-treated rats is lower compared with normal control rats (Fig. 8, a and b). On the other hand, acute fluoxetine treatment had no effect on the reduction of open-arm activity (anxiogenic behaviors) in rats during ethanol withdrawal after 15 days of ethanol intake (Fig. 7).

Interestingly, chronic fluoxetine treatment, when administered concurrently with ethanol treatment, significantly antagonized the reduction of open-arm activity (percent of
open-arm entries and the mean percent of time spent on the open arms) measured by EPM (Fig. 7) and significantly blocked the down-regulation of cortical CRE-DNA-binding activity (Fig. 9, a and b) during ethanol withdrawal. We also observed that chronic fluoxetine treatment of the ethanol-withdrawn rats tended to increase but not completely normalize the total number of entries onto the arms (closed plus open) of the EPM (Fig. 7). It was observed that ethanol-withdrawn rats chronically treated with fluoxetine made fewer entries onto the open arms, but time spent in exploring the open arms was similar to that of pair-fed control or ethanol-fed (0-h withdrawn) rats. Furthermore, acute as well as chronic fluoxetine treatment alone had no effect on the open- and closed-arm activities of rats (Fig. 7) or on CRE-DNA-binding activity in the rat cortex (Figs. 8, a and b, and 9, a and b). Taken together, these results suggest that blockade of the anxiogenic effects of ethanol withdrawal by chronic fluoxetine treatment may be due to constant stimulation of CRE-DNA-binding activity in the cortices of ethanol-fed rats.

**Immunolabeling of BDNF: Effects of Chronic Ethanol and Chronic Fluoxetine Treatment.** We examined whether (1) decreased CRE-DNA-binding activity in the rat cortex during ethanol withdrawal (24 h) after 15 days of ethanol treatment is associated with a decrease in the protein expression of BDNF and (2) the blockade of anxiogenic behaviors by chronic fluoxetine treatment is associated with normalization of changes in protein levels of BDNF during ethanol withdrawal after chronic ethanol intake. We used 60 μg of protein from the cortices of each group (pair-fed control, ethanol-fed, ethanol-withdrawn (24 h), ethanol-withdrawn plus fluoxetine-treated, and fluoxetine (alone)-treated rats) for the immunoblotting of BDNF. We used antibodies for BDNF and β-actin to demonstrate the alterations in protein levels of BDNF in the rat cortex during ethanol treatment and its withdrawal. We used β-actin as a normalizing factor for the BDNF immunolabeling studies because we have shown that the immunolabeling of β-actin in the rat cortex is not altered by chronic ethanol treatment and its withdrawal (Pandey, 1996). Autoradiograms (Fig. 10a) were analyzed by densitometric analysis, and BDNF values were normalized using the values for β-actin protein. We observed a significant decrease (40%) in the immunolabeling of BDNF in the rat cortex at 24 h of ethanol withdrawal but no significant decrease (20%) at 0 h of ethanol withdrawal after 15 days of ethanol treatment (Fig. 10b). It was also observed that chronic fluoxetine (15 days) treatment concurrent with ethanol treatment significantly blocked the down-regulation of the immunolabeling of BDNF in the rat cortex during ethanol withdrawal (Fig. 10b). These results suggest that decreased CRE-DNA-binding activity is associated with decreased immunolabeling of the CREB-target (i.e., BDNF, in the rat cortex during ethanol withdrawal after chronic ethanol intake).

**Discussion**

The novel findings of the present investigation are that both CRE-DNA-binding activity and immunolabeling of BDNF are decreased in the rat cortex during ethanol withdrawal (24 h) after protracted ethanol consumption (15 days)
and that these changes may be related to the anxiogenic behaviors occurring in rats during ethanol withdrawal after chronic ethanol intake.

CRE- and AP-1 DNA-Binding Activities in the Rat Cortex: Effects of Ethanol Exposure. Our results indicate that acute or chronic ethanol treatment has no significant effect on CRE- or AP-1 DNA-binding activities in the rat cortex, whereas ethanol withdrawal after chronic ethanol treatment significantly decreased CRE-DNA-binding activity without modulating AP-1 DNA-binding activity in the rat cortex. It was found that ethanol withdrawal after acute ethanol treatment had no effect on CRE- or AP-1 DNA-binding activities in the rat cortex. In contrast, Yang et al. (1996) reported that acute ethanol treatment significantly increased CRE-DNA-binding activity as well as the phosphorylation of CREB in the rat cerebellum. The differences between the present study and the studies of Yang et al. (1996) in the

Fig. 4. Time course of the development of anxiety during ethanol withdrawal after 15 days of ethanol treatment. Rats were treated with ethanol (9% v/v) or control liquid diet. Ethanol-treated rats were withdrawn from ethanol for 0, 12, 24 and 72 h, and open- and closed-arm activities of these rats along with pair-fed controls were measured by the EPM test. Values are the mean ± S.E.M. from 5 to 8 rats. *Significantly different from the pair-fed control groups (p < .01–.001).

Fig. 5. A, representative autoradiogram of the gel-mobility shift assay showing the time course of changes in nuclear CRE-DNA-binding activity in the rat cortex during ethanol withdrawal after 15 days of ethanol treatment. Rats were treated with ethanol (9% v/v) or control liquid diet. Ethanol-treated rats were withdrawn from ethanol for 0, 12, 24, and 72 h, and cortices from these rats were used for the measurement of CRE-DNA-binding activity. Ten micrograms of nuclear extract protein were incubated with 32P-labeled CRE oligonucleotides, and CRE-DNA protein complexes were separated out by gel electrophoresis as described in Materials and Methods. B, the effect of various time points of ethanol withdrawal (0, 12, 24, and 72 h) after 15 days of ethanol exposure on CRE-DNA-binding activity in the rat cortex. Values are the mean ± S.E.M. of five to six experiments and are represented as percents of the normal controls. *Significantly different from the pair-fed control group (p = .003 for control group versus 24-h ethanol-withdrawn group; p = .038 for control group versus 72-h ethanol-withdrawn group).
findings regarding CRE-DNA-binding activity during acute ethanol exposure may be related to the difference in brain regions (cortex versus cerebellum) investigated or to the blood ethanol levels attained. Acute i.p. injection of ethanol (Yang et al., 1996) produced high blood ethanol levels (252 ± 26 mg/100 ml), whereas the acute oral ethanol feeding paradigm (Pandey, 1996; blood ethanol level 90 ± 12 mg/100 ml) used in the present study produced low blood ethanol levels (101 ± 23 mg/100 ml). Similar to our results, Yang et al. (1996) observed that chronic ethanol treatment had no effect on CRE-DNA-binding activity in the rat cerebellum. The effect of ethanol withdrawal after chronic ethanol intake had not been investigated previously. Our study clearly suggests that CRE-DNA-binding activity is decreased in the rat cortex during ethanol withdrawal after chronic ethanol intake.

The mechanism by which CRE-DNA-binding activity is decreased during ethanol withdrawal after chronic ethanol intake is unknown at the present time but may be due to decreased mRNA and protein levels of CREB. It has been
shown that CREB-binding protein (CBP) may regulate CRE-DNA binding by interacting with phosphorylated CREB (Chrivia et al., 1993). It is possible that decreased CBP levels...
during ethanol withdrawal after chronic ethanol intake may be one of the mechanisms involved in the decrease of CRE-DNA-binding activity in the rat cortex. The decrease in CRE-DNA-binding activity in the rat cortex during ethanol withdrawal appears to be specific because AP-1 DNA-binding activity is not altered during ethanol withdrawal after chronic ethanol intake. Our finding of no change in AP-1 DNA-binding activity in the rat cortex during ethanol withdrawal (24 h) after 15 days of ethanol treatment is dissimilar to the findings of Beckmann et al. (1997), who reported an increase in AP-1 DNA-binding activity in the rat cortex during ethanol withdrawal after chronic ethanol treatment. The reasons for this discrepancy are not clear but may be related to the difference in ethanol treatment paradigms (oral feeding with liquid diet versus ethanol inhalation) and/or to the differences in blood ethanol levels between these two studies, which may account for the difference in the findings regarding AP-1 DNA-binding activity in the rat cortex during ethanol withdrawal. For example, blood ethanol levels in the present study averaged 183 ± 19 mg/100 ml, whereas the blood ethanol levels in the study by Beckmann et al. are greater than 400 mg/100 ml. Taken together, these studies suggest the possibility that the regulation of AP-1 DNA-binding activity in the rat cortex may be dependent on the blood ethanol levels achieved during ethanol exposure.

**CRE-DNA-Binding Activity and Ethanol Withdrawal-Related Anxiety.** To establish whether changes in CRE-DNA-binding activity are associated with the development of anxiety during ethanol withdrawal after acute or protracted ethanol intake, we studied the cortical CRE-DNA-binding activity and the anxiogenic behaviors of rats undergoing ethanol withdrawal after acute and chronic ethanol intake. Our findings indicate that ethanol withdrawal after acute ethanol intake did not cause changes in CRE-DNA-binding activity in the rat cortex and did not produce any anxiogenic behaviors. An important finding of the present investigation is that the time course of the decrease in cortical CRE-DNA-binding activity is correlated with the time course of the development of anxiogenic behaviors in rats during ethanol withdrawal after 15 days of ethanol exposure. The peak anxiety and the peak reduction in cortical CRE-DNA activity occurred at 24 h of ethanol withdrawal after 15 days of ethanol treatment of rats. These results suggest that there is a temporal correlation between the reduction in cortical CRE-DNA-binding activity and the development of anxiety during ethanol withdrawal after chronic ethanol intake.

Acute and long-term fluoxetine (5-HT uptake blocker) treatment normalized partially and fully, respectively, the decrease in CRE-DNA-binding activity in the rat cortex during ethanol withdrawal after 15 days of ethanol treatment. However, at the behavioral level, long-term but not acute fluoxetine treatment blocked the anxiety during ethanol withdrawal. Decreased CRE-DNA-binding activity during ethanol withdrawal would be expected to cause decreased expression of cAMP-inducible genes. It is possible that the partial normalization of the decrease in CRE-DNA-binding activity by acute fluoxetine treatment may not fully normalize the changes in downstream cAMP-inducible gene expression in ethanol-withdrawn rats and therefore not be able to prevent the development of anxiety during ethanol withdrawal. On the other hand, the reduction in cortical CRE-DNA-binding activity is fully compensated by chronic...
fluoxetine treatment, thereby normalizing changes in CREB-related targets and relieving anxiety in ethanol-withdrawn rats. Our results, which show a significant reduction in open-arm activity (percent of open-arm entries and the mean percent of time spent on open arms) on the EPM during ethanol withdrawal (24 h) after 15 days of ethanol treatment in rats, are similar to the results reported by other investigators (Baldwin et al., 1991; Lal et al., 1993; Rassnick et al., 1993). Moreover, chronic fluoxetine treatment significantly antagonized the reduction in the percent of time spent on open arms and in the percent of open-arm entries but did not completely reverse the reduction in total arm entries (open plus closed) into the EPM during ethanol withdrawal after 15 days of ethanol treatment. This suggests that chronic fluoxetine treatment antagonizes the anxiogenic effects of ethanol withdrawal after chronic ethanol intake but not the reduction in overall activity during ethanol withdrawal. Other serotonergic drugs (e.g., the 5-HT2A/2C receptor antagonist mianserin, the 5-HT1A receptor partial agonist buspirone) and the corticotropin-releasing factor antagonist also have been shown to block the development of the anxiogenic effects of ethanol withdrawal, but these agents had no effect on the reduction in total arm entries into the EPM during ethanol withdrawal after protracted ethanol intake by rats (Baldwin et al., 1991; Lal et al., 1993; Wallis et al., 1993).

The mechanism by which fluoxetine treatment blocks the down-regulation of cortical CRE-DNA-binding activity in ethanol-withdrawn rats is unknown. Previous studies have demonstrated that chronic but not acute fluoxetine treatment increases CRE-DNA-binding activity, CREB mRNA levels, and immunoreactivity in the normal rat hippocampus (Nibuya et al., 1996). Here, we observed that acute and chronic fluoxetine treatments have no effect on CRE-DNA-binding activity in the cortices of normal rats, but when CRE-DNA-binding activity is decreased in the cortices of ethanol-withdrawn rats, both acute and chronic treatments are able to increase cortical CRE-DNA-binding activity in ethanol-withdrawn rats. It is possible that blockade of the down-regulation of CRE-DNA-binding activity by fluoxetine during ethanol withdrawal may be due to an increase in mRNA and in protein levels of CREB in the rat cortex. It is also possible that fluoxetine treatment may increase the levels of phosphorylated CREB via the activation of PKA. Further studies are needed to explore these possibilities.

**CREB-Targeted BDNF Genes: Effects of Ethanol Exposure and Fluoxetine Treatment.** BDNF and somatostatin are cAMP-inducible genes and are regulated by the CREB gene transcription factor (Comb and Hyman, 1987; Con-dorelli et al., 1994; Duman et al., 1995, 1997). It has been demonstrated that the infusion of an antisense oligonucleotide to CREB into the rat hippocampus decreases the basal level of BDNF and blocks the induction of BDNF expression caused by electroconvulsive shock (Duman et al., 1995). It has also been shown by some (Hughes and Dragunow, 1995; Gaiddon et al., 1996) but not all (Sano et al., 1996) investigators that AP-1 DNA-binding activity and BDNF expression may be interrelated. It is noteworthy that mRNA levels of BDNF and the immunolabeling of somatostatin in the rat hippocampus are decreased during ethanol withdrawal after protracted ethanol exposure (Andrade et al., 1992; MacLennan et al., 1995). In our study, we observed that decreased CRE- but not AP-1 DNA-binding activity is associated with decreased immunolabeling of BDNF in the rat cortex during ethanol withdrawal after chronic ethanol intake. Interestingly, when CRE-DNA-binding activity is normalized by chronic fluoxetine treatment, the level of BDNF is also normalized in the rat cortex during ethanol withdrawal. Furthermore, chronic fluoxetine treatment also antagonizes the anxiogenic effects during ethanol withdrawal after chronic ethanol intake. Currently, it is unknown whether BDNF is involved in anxiety, but the antidepressive properties of BDNF have been demonstrated in animal models of depression (Siucci et al., 1996; Duman et al., 1997). Available evidence indicates that BDNF has neuromodulatory and neurotrophic influences on 5-HT neurons in the brain (Mamounas et al., 1995; Eaton and Whittemore, 1996). Also, chronic but not acute treatment with fluoxetine increases the expression of BDNF in the rat hippocampus (Nibuya et al., 1996). Taken together, these studies indicate that BDNF and serotonin may modulate each other’s functions, so that the decreased function of both in the rat brain may be associated with the anxiogenic effects of ethanol withdrawal. Thus, changes in CRE-DNA-binding activity in the rat cortex undergoing ethanol withdrawal after chronic ethanol intake can modulate the expression of CREB targets (i.e., BDNF). Although these data support a relationship between CREB and BDNF in the rat cortex during ethanol withdrawal, they do not establish that the changes in the expression of BDNF are explicitly due to changes in CREB.

Another issue is whether the decreases in CRE-DNA-binding activity and in protein levels of BDNF in the cortex during ethanol withdrawal after 15 days of ethanol treatment are related to the development of other withdrawal symptoms (e.g., seizures, tremor) and, furthermore, whether chronic fluoxetine treatment blocks the other ethanol-withdrawal symptoms. The data collected in the present study do not address this issue. It is possible, but unlikely, that decreased CRE-DNA-binding activity and decreased BDNF protein levels are associated with the development of seizures during ethanol withdrawal because increases in AP-1 and CRE-DNA-binding activities in the cortex and the hippocampus have been found during electrical- and drug-induced seizure activity (Hope et al., 1994; Lukasiuk and Kaczmarek, 1994; Ishitge et al., 1996). Expression of BDNF has also been shown to increase in various brain structures of rats during seizures induced by electrical or pharmacological manipulations (Ernfors et al., 1991; Timmusk et al., 1993). It is likely that long-term fluoxetine treatment may not be able to block other withdrawal symptoms because the decrease in the total number of arm entries (open plus closed) into the EPM by ethanol-withdrawn rats is not completely normalized by this drug. Future studies are needed to establish whether there is a cause-and-effect relationship of CREB and CREB-related genes in specific brain regions to anxiety or other withdrawal symptoms during ethanol withdrawal after chronic ethanol consumption.

**Conclusions.** The data presented here provide the first evidence that a reduction in CRE-DNA-binding activity may be associated with behavioral manifestations of ethanol withdrawal after chronic ethanol intake. CRE-DNA-binding activity and the expression of CREB targets (i.e., the protein levels of BDNF) are significantly decreased in the rat cortex during ethanol withdrawal after chronic ethanol intake. More importantly, chronic treatment of rats with a
5-HT-uptake blocker (fluoxetine) concurrent with ethanol treatment significantly blocks changes in CRE-DNA-binding activity and in BDNF levels in the cortex, as well as antagonizing the development of anxiogenic behaviors during ethanol withdrawal after chronic ethanol intake. Recently, using pharmacological and genetic manipulations, it was shown that the cAMP signaling cascade is involved in the behavioral response to ethanol exposure in Drosophila (Moore et al., 1998). Our results suggest that the CREB in the brain may be one of the possible molecular loci associated with the anxiogenic effects of ethanol withdrawal after protracted ethanol intake. CREB has been shown to be involved in the neuroadaptational mechanisms to chronic exposure to morphine or cocaine in rodents (Nestler, 1992; Maldonado et al., 1998). Our results suggest that the CREB in the brain may be one of the possible molecular loci associated with the mediation of long-term effects of drugs of abuse in general.

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Send reprint requests to: Dr. Subhash C. Pandey, Psychiatry Research Service (M/C 151), Veterans Administration Chicago Health Care System (West Side Division), 820 S. Damen Ave., Chicago IL 60612.