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Neuroadaptational changes in DNA binding of stimulatory protein-1 and nuclear factor-kB gene transcription factors during ethanol dependence

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Abstract

To define the molecular basis of ethanol dependence, the changes in gene transcription factor stimulatory protein-1 (SP1) and nuclear factor-kB (NF-kB) DNA binding activities were investigated in the rat cortex and hippocampus during ethanol treatment (15 days) and its withdrawal. It was found that both protracted ethanol treatment and its withdrawal (12, 24, or 72 h) had no effect on NF-kB DNA binding activity in the rat cortex and hippocampus. Time-course studies of the changes in SP1 DNA binding activity during ethanol withdrawal (0, 12, 24, and 72 h) after protracted ethanol exposure indicated that SP1 DNA binding in the rat cortex was significantly decreased at 0 h, and that it remained decreased at 12, 24, and 72 h of withdrawal. On the other hand, SP1 DNA binding activity did not change in the rat hippocampus during ethanol treatment but was significantly decreased at 12, 24, and 72 h of withdrawal. These results suggest the possibility that decreased SP1-dependent gene transcription in the rat cortex and hippocampus may be associated with the molecular mechanisms of ethanol dependence.

Keywords: SP1 (stimulatory protein-1) gene transcription factor; NF-kB (nuclear factor-kB) gene transcription factor; Brain; Ethanol tolerance; Ethanol dependence

1. Introduction

Long-term exposure to ethanol produces multifaceted neuronal responses, which include changes in the sensitivity of neurotransmitter receptors, in effector enzymes of the signaling cascades, and ultimately, changes in gene expression (Pandey, 1996, 1998; Tabakoff and Hoffman, 1996; Koob et al., 1998; Yang et al., 1998). In spite of numerous neurochemical studies, the exact molecular and cellular mechanisms of alcohol dependence are still unclear. Recent studies have focused on elucidating further the mechanisms of gene transcription during ethanol dependence and on identifying the genes related to alcoholism (Goldman, 1995; Tabakoff and Hoffman, 1996; Crabbe et al., 1999; Schuckit et al., 1999). To this end, the regulation of gene transcription factors appears to be an important issue because changes in transcription factor activity may lead to alterations in the gene transcription program during alcohol dependence.

The mapping of promoters of various genes has revealed the presence of GC boxes (GGGCGG). The stimulatory protein-1 (SP1) gene transcription factor binds to GC boxes, and then regulates DNA transcription (Briggs et al., 1986; Zhu et al., 1994; Nielsen et al., 1996). The nuclear factor (NF)-kB is another gene transcription factor, a member of the family of NF-kB/Rel proteins, and the heterodimers of p50 and p65 kDa proteins bind to the NF-kB element (GGGACTTTCC) in the promoter region of genes, thereby regulating the DNA transcription program (Baeuerle and Baltimore, 1996; O'Neill and Kaltschmidt, 1997). It has been shown that brain insults caused by pharmacological manipulations are associated with the induction of NF-kB DNA binding in rat cortex and hippocampus (Meberg et al., 1996; O'Neill and Kaltschmidt, 1997). We recently have shown that cyclic 3′–5′ adenosine monophosphate (CAMP)-responsive element binding protein (CREB) gene transcription factor

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DNA binding activity is decreased in the rat cortex during ethanol withdrawal after 15 days of ethanol treatment. These results suggest that CREB-dependent events are involved in the neuroadaptational mechanisms of alcohol dependence (Pandey et al., 1999). The regulation of other transcription factors, such as SP1 and NF-kB, in the brain during ethanol dependence is currently unknown. Therefore, in the present investigation, we examined the time course of changes in SP1 and NF-kB DNA binding in the rat cortex and hippocampus during ethanol withdrawal after 15 days of ethanol treatment. A better understanding of the regulation of these gene transcription factors may help to elucidate the molecular mechanisms of the neuroadaptational processes in long-term ethanol exposure.

2. Materials and methods

2.1. Long-term treatment with ethanol

Male Sprague–Dawley rats weighing 300–350 g were used in all experiments. Rats were given control or ethanol Lieber–DeCarli liquid diet as described previously (Pandey, 1996; Pandey et al., 1999). Rats were individually housed and received 80.0 ml of Lieber–DeCarli control diet (Bioserv, Frenchtown, NJ, USA) as their sole source of food and liquid. One group of rats continued to receive control liquid diet, and another group was gradually introduced to ethanol and was then maintained on the ethanol (9% v/v)-containing Lieber–DeCarli liquid diet for 15 days. The rats were pair-fed and were weighed twice a week. The ethanol-fed rats were withdrawn for 0, 12, 24, or 72 h after 15 days of ethanol treatment. There were no significant differences in body weight (g) among the various groups of rats. The ethanol-dependence phenomenon in these rats was established by measuring anxiety during ethanol withdrawal using the elevated plus-maze test (recently published data; Pandey et al., 1999). Blood ethanol levels measured after 15 days of ethanol treatment were 172 ± 30 mg/100 ml. Following 12 or 24 h of ethanol withdrawal after 15 days of ethanol treatment, the blood ethanol levels were 0 mg/100 ml. Pair-fed control and ethanol-withdrawn (0, 12, 24, or 72 h) rats were decapitated and brains were removed. Cortices (all cortical areas) and hippocampi (all hippocampal areas) were dissected out and immediately frozen at −80°C until used for an electrophoretic gel-mobility shift assay. All animal use procedures were in accord with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care Committee of the University of Illinois at

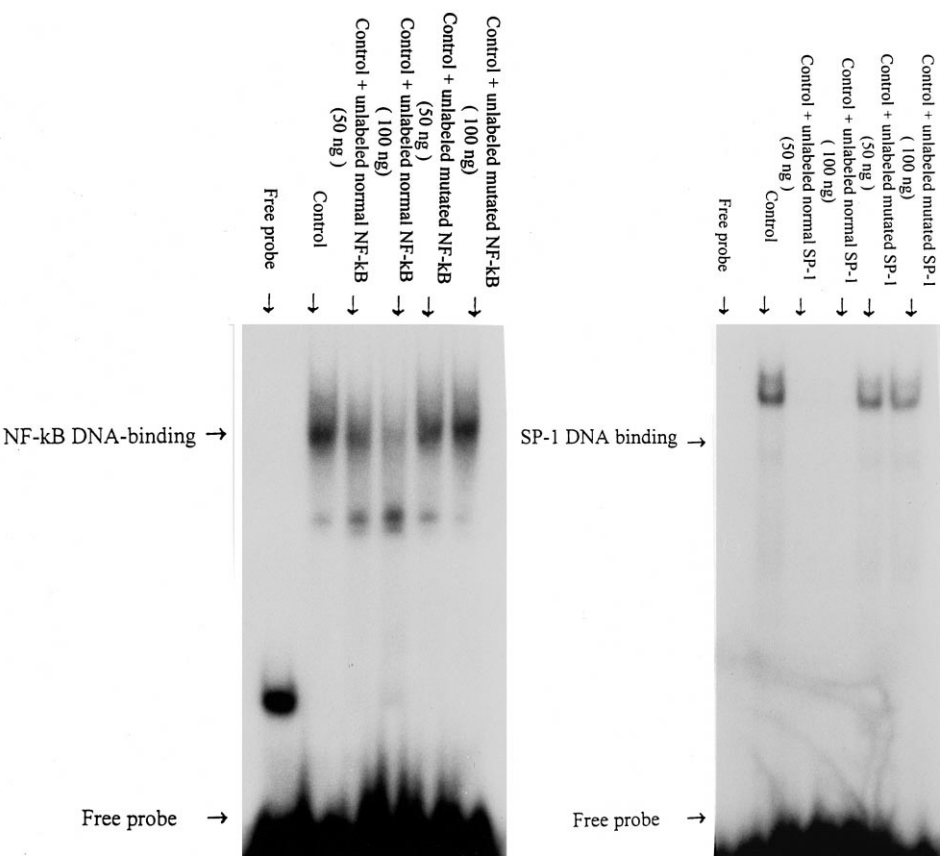

Fig. 1. Results of the competitive experiment performed with the nuclear extract obtained from normal rat cortex. The binding reactions were performed in the presence of normal unlabeled SP1 (50 and 100 ng) or NF-kB (50 and 100 ng) oligonucleotides, and of mutated unlabeled SP1 (50 and 100 ng) or NF-kB (50 and 100 ng) oligonucleotides.

Chicago and by the VA Chicago Health Care System (West Side Division).

2.2. Determination of SP1 and NF-kB DNA binding activities by electrophoretic gel-mobility shift assay

Nuclear extracts from cortices and hippocampi were prepared by a procedure we described previously (Pandey et al., 1999). Tissue was homogenized in 2.0 ml of buffer I (10 mM HEPES, pH = 7.9; 1.5 mM $MgCl_2$; 10 mM KCl, 1 mM dithiothreitol; 0.5 mM phenylmethylsulfonyl fluoride; 10 μg/ml aprotinin; 10 μg/ml leupeptin; and 1 μg/ml pepstatin). The homogenates were centrifuged at $100{,}000 \times g$ for 30 min. The pellet thus obtained was suspended in buffer II (20 mM HEPES, pH = 7.9; 0.84 M NaCl; 1.5 mM $MgCl_2$; 0.4 mM EDTA; 0.5 mM dithiothreitol; 50% glycerol; protease inhibitors as in buffer I). Tissue suspensions were incubated on ice for 15 min with continuous shaking. Nuclear extracts were removed by centrifuging the homogenate at $20{,}000 \times g$ for 15 min. Protein content of the nuclear extracts was estimated by the method of Lowry et al. (1951).

Binding reactions were carried out by incubating 5 μg of the nuclear extract with 1 μg of poly (dI-dC) and 6 μg of bovine serum albumin in 20 μl of a reaction mixture containing binding buffer (20 mM HEPES, pH = 7.9; 1 mM dithiothreitol; 0.3 mM EDTA; 0.2 mM EGTA; 80 mM NaCl; 10% glycerol; 0.2 mM PMSF) for 15 min at room temperature. Approximately 80,000 disintegrations per minute (dpm) of ^{32}P-labeled SP1 (5′GATCGATCGGGCGGGGCGATC3′) or NF-kB (5′GATCGAGGGGACTTTCCCTAGC3′) oligonucleotides were added and incubation was continued for an additional 30 min. These DNA oligonucleotides were purchased from Stratagene (La Jolla, CA) and end-labeled with γ^{32}P-ATP using T4 polynucleotide kinase according to the manufacturer's methods (U.S. Biochemical, Cleveland, OH). DNA–protein complexes were resolved on a 4.0% nondenaturing polyacrylamide gel 25 mM Tris-borate (pH = 8.2) buffer containing 0.5 mM EDTA. The gel was dried and autoradiographed with intensifying Kodak film. The optical densities of the bands of the DNA–protein complexes on the autoradiogram were measured by the Loats Image Analysis system using the Inquiry program. The results are expressed as a percent of the control values.

The competitive experiments were performed by incubating the nuclear extract protein (5 μg) using a procedure similar to that described above except that the binding reactions were performed in the presence and in the absence of unlabeled normal SP1 (50 and 100 ng) and mutated SP1 (50 and 100 ng) oligonucleotides, and of unlabeled normal NF-kB (50 and 100 ng) and mutated

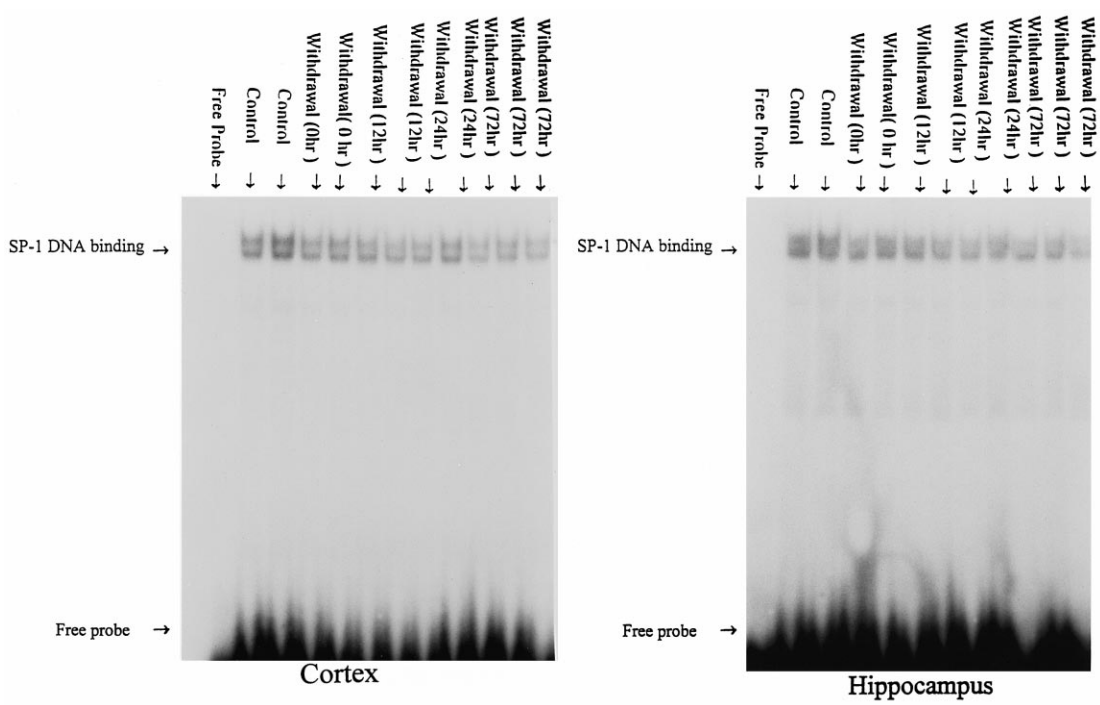

Fig. 2. A representative autoradiogram of the gel-mobility shift assay showing the time-course of changes in nuclear SP1 DNA binding activity in the rat cortex and hippocampus during ethanol withdrawal after 15 days of ethanol treatment. Each lane represents the SP1 DNA binding in the brain of an individual rat. Rats were treated with ethanol (9% v/v) or control liquid diet. Ethanol-treated rats were withdrawn from ethanol after 0, 12, 24, or 72 h, and cortices and hippocampi from these rats were used for the determination of SP1 DNA binding activity according to the procedure described in the Materials and Methods section.

NF-kB (50 and 100 ng) oligonucleotides. Mutated SP1 oligonucleotides (GG → TT substitution in the SP1 binding domain) and mutated NF-kB oligonucleotides (G → C substitution in the NF-kB binding domain) were purchased from Santa Cruz Biotechnology, (Santa Cruz, CA).

2.3. Data analysis

Differences among groups were evaluated by the Kruskal–Wallis test. Specific group comparisons were performed by Student's *t*-test.

3. Results

3.1. Determination of the specificity of SP1 and NF-kB DNA binding in the rat cortex

We characterized SP1 and NF-kB DNA binding using competitive experiments with unlabeled mutated and normal oligonucleotides (50 or 100 ng). A competitive experiment with unlabeled normal SP1 oligonucleotides (50 or 100 ng) revealed completely blocked SP1 DNA binding activity, whereas incubation with mutated SP1 oligonucleotides (50 or 100 ng) had no effect on SP1 DNA binding activity in the rat cortex (Fig. 1). Competitive experiments performed with unlabeled normal and mutated NF-kB oligonucleotides indicate that NF-kB DNA binding is attenuated by unlabeled normal NF-kB oligonucleotides (50 or 100 ng) but not by mutated NF-kB oligonucleotides (50 or 100 ng) (Fig. 1). These results indicate that SP1 and NF-kB DNA binding activities are specific to the SP1 and the NF-kB gene transcription factor, respectively.

3.2. Effects of chronic ethanol treatment and its withdrawal on SP1 DNA binding in the rat brain

SP1 DNA binding activity was measured in nuclear extracts of cortices and hippocampi of pair-fed control, ethanol-fed, and ethanol withdrawn (12, 24, 72 h) rats. The patterns of the SP1 DNA and protein complexes are shown in Fig. 2, and are similar to the patterns reported by other investigators (Briggs et al., 1986). As seen in Fig. 2, SP1 DNA and protein complexes are present in two bands. This may be due to the presence of two forms of SP1 proteins, with different molecular weights (Ammendola et al., 1992). It was found that long-term ethanol treatment (15 days) significantly decreased SP1 DNA binding in the rat cortex, which was further decreased at 12 h and then remained at the same level at 24 and 72 h of ethanol withdrawal (Figs. 2 and 3). On the other hand, SP1 DNA binding activity in the rat hippocampus was not changed at 0 h of ethanol withdrawal but was significantly decreased at 12, 24, and 72 h of ethanol withdrawal after 15 days of ethanol treatment (Figs. 2 and 3). These results indicate that ethanol withdrawal after protracted ethanol treatment produced reductions in SP1 DNA binding activity in the rat brain.

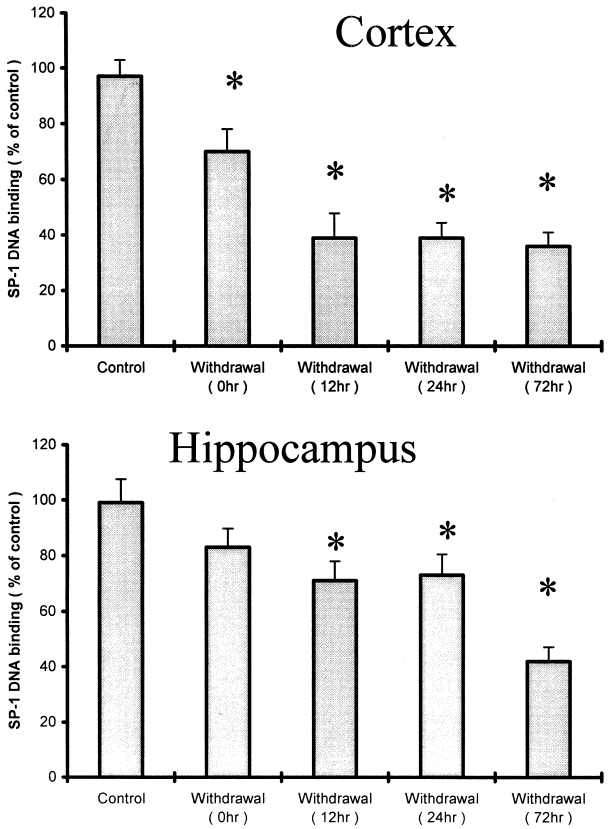

Fig. 3. The effects of various time points of ethanol withdrawal (0, 12, 24, and 72 h) after 15 days of ethanol exposure on SP1 DNA binding activity in the rat cortex and hippocampus. Data are mean ± S.E.M. (bars) values derived from 8 to 14 experiments (rats) in the cortex and 7 to 8 experiments in the hippocampus, and are represented as percentages of the control. * Significantly different from the control group ($P < 0.01$–0.001).

3.3. Effects of protracted ethanol treatment and its withdrawal on NF-kB DNA binding in the rat brain

NF-kB DNA binding activity was determined in nuclear extracts of cortices and hippocampi of pair-fed control, ethanol-fed, and ethanol-withdrawn (12, 24, or72 h) rats. The patterns of NF-kB DNA and protein complexes in cortex and hippocampus are shown in Fig. 4. It was found that chronic ethanol treatment or its withdrawal had no effect on NF-kB DNA binding in the cortex or in the hippocampus (Fig. 5). The results suggest that neuronal excitability during ethanol withdrawal after protracted ethanol treatment does not produce any alterations in NF-kB DNA binding in the rat cortex or hippocampus.

4. Discussion

The novel findings of this investigation are that ethanol withdrawal produced a significant reduction of SP1 DNA

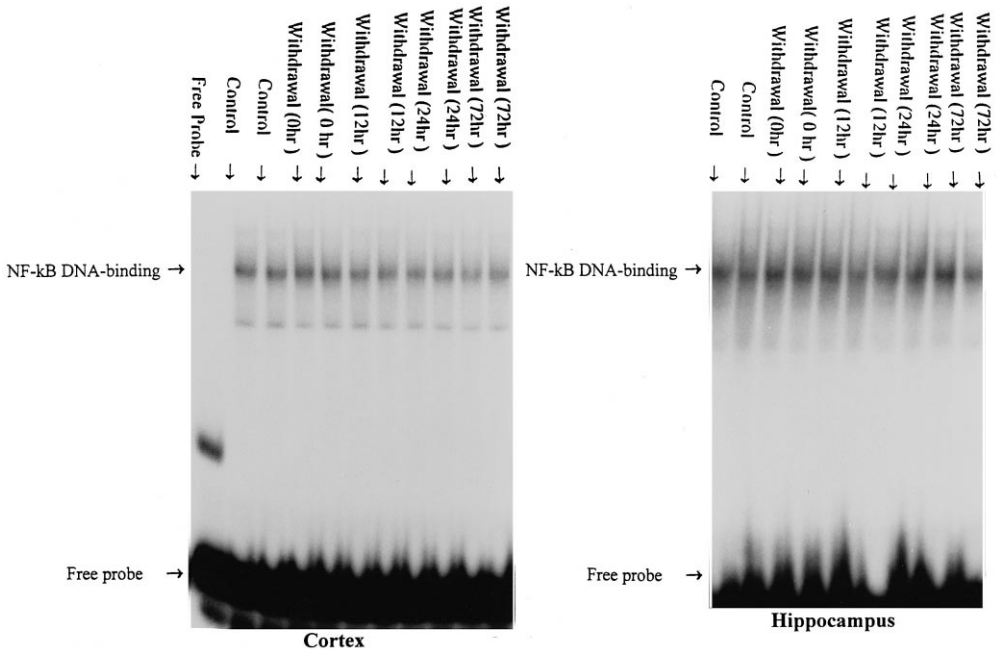

Fig. 4. A representative autoradiogram of the gel-mobility shift assay showing the time-course of changes in nuclear NF-kB DNA binding activity in the rat cortex and hippocampus during ethanol withdrawal after protracted ethanol exposure. Each lane represents the NF-kB DNA binding in the brain of an individual rat. Rats were treated with ethanol (9% v/v) or control liquid diet. Ethanol-treated rats were withdrawn from ethanol for 0, 12, 24, or 72 h, and cortices and hippocampi from these rats were utilized for the determination of NF-kB DNA binding activity according to the procedure described in the Materials and Methods section.

binding activity in the rat cortex and hippocampus. On the other hand, NF-kB DNA binding activity was not changed in cortex or hippocampus of both ethanol-fed and -withdrawn rats. The mechanism by which ethanol withdrawal after protracted ethanol administration produces decreased efficacy of SP1 binding to the GC box of cognate DNA in the rat cortex and hippocampus is not known at present, but may be related to decreased protein levels of SP1, or may be caused by changes in posttranslational regulatory mechanisms, such as phosphorylation by SP1 kinase (Jackson et al., 1990). Similarly, it has been shown that SP1 DNA binding efficacy is significantly decreased in nuclear extracts of aged rat brain, and it has been suggested that this decrease is not due to different protein levels but may be related to posttranslational alterations in SP1 proteins (Ammendola et al., 1992, 1994).

SP1 gene transcription factors are widely expressed and are involved in controlling the expression of gene-containing GC boxes in their promoters (Briggs et al., 1986). Many genes, such as monoamine oxidases A and B, and cholecystokinin, have been shown to be regulated by this gene transcription factor (Zhu et al., 1992, 1994; Nielsen et al., 1996). Monoamine oxidase A preferentially oxidizes serotonin (5-HT) to 5-hydroxyindole acetic acid (5-HIAA), and alterations in the serotonergic system have been implicated in alcohol dependence (LeMarquand et al., 1994; Pandey and Pandey, 1996). It is possible that changes in monoamine oxidase activity in platelets and brain during ethanol dependence may be mediated via changes in SP1 transcription factor activity. In the past, considerable interest developed related to studies of monoamine oxidase, most specifically in platelets of alcoholics (VonKnorring et al., 1985, 1991). It was suggested that low platelet monoamine oxidase activity may be a biological marker for vulnerability to alcoholism (VonKnorring et al., 1991). Monoamine oxidase activity in the brain of alcoholics has been shown to be decreased compared with normal control subjects (Oreland et al., 1983). Monoamine oxidase activity in the brain of ethanol-treated rats was found to be normal in most studies (Wiberg et al., 1977; Sheriff et al., 1993), but one study reported decreased activity (Murthy et al., 1989). It has been shown that the catalytic activity of monoamine oxidase A and the mRNA levels of monoamine oxidase A are dependent on the concentration of SP1 proteins in cultured cells (Zhu et al., 1994). Since we observed that SP1 DNA binding activity is decreased in rat cortex and hippocampus during ethanol withdrawal and that SP1 DNA binding activity is decreased in rat cortex but not in the hippocampus during ethanol treatment, it is possible that different changes in monoamine oxidase A gene expression may be occurring during ethanol treatment and withdrawal in the various brain regions. Further studies are needed to establish the relationship between changes in SP1 gene transcription factor activity and SP1-related gene expression in different brain regions during ethanol treatment and its withdrawal.

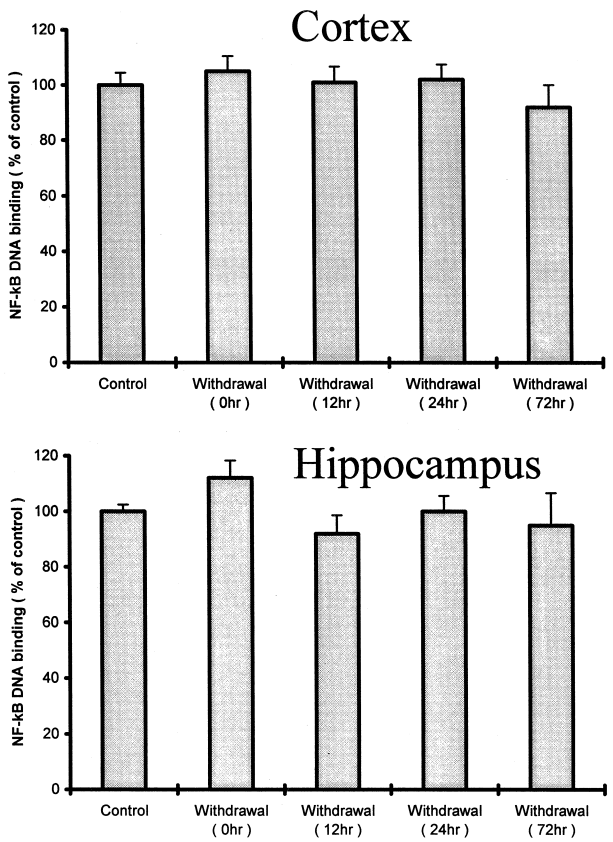

Fig. 5. The effects of various time points of ethanol withdrawal (0, 12, 24, and 72 h) after 15 days of ethanol exposure on NF-kB DNA binding activity in the rat cortex and hippocampus. Data are mean ± S.E.M. (bars) values ($n = 5–10$) and are represented as percentages of the control.

The cholecystokinin (CCK) gene is another important gene that is regulated by the SP1 gene transcription factor (Nielsen et al., 1996), and the cholecystokininergic system has been implicated in ethanol dependence (Crespi, 1998). In fact, CCK receptor antagonists are able to prevent ethanol-withdrawal-related anxiety (Wilson et al., 1998). In addition, CCK binding sites have been shown to be increased in the frontal cortex of rats treated with ethanol on a long-term basis (Harro et al., 1994). Recently, the higher frequency of genetic mutations (C → T) in the SP1 binding Cis element of the cholecystokinin gene have been found in some but not all of the Japanese alcoholic population (Harada et al., 1998; Ishiguro et al., 1999). Furthermore, it has been shown that the frequency of allelic mutations (-45T) in the promoter regions of the CCK gene are found to be higher in those alcoholic subjects who appear to be more prone to delirium tremens due to ethanol abuse (Okubo et al., 1999). These results suggest that the SP1 Cis-element in the CCK gene may play a crucial role in ethanol dependence. More recently, we reported that ethanol-fed rats (0 h withdrawal) do not exhibit anxiety and that anxiety developed at 24 h of ethanol withdrawal and disappeared after 72 h of withdrawal (Pandey et al., 1999). In the present study, we found that in the brain of these same rats, the SP1 DNA binding activity in the cortex was decreased at 0 h, further decreased at 12 h, and remained decreased up to 72 h of ethanol withdrawal, whereas in the hippocampus, SP1 DNA binding was not changed at 0 h but was decreased at 12, 24, and 72 h of ethanol withdrawal. It is possible that the decrease in SP1 gene transcription factor activity in the rat cortex and hippocampus may lead to alterations in SP1-related gene expression during ethanol dependence.

NF-kB gene transcription factor activity is altered in human monocytes during ethanol exposure (Zakhari and Szabo, 1996; Mandreker et al., 1997). Recently, it was suggested that NF-kB is not only involved in the function of immune cells but also plays an important role in neuronal and glial cell functions (O'Neill and Kaltschmidt, 1997). It has also been shown that brain injury and neuronal excitation are associated with an increased expression of NF-kB proteins and increased NF-kB DNA binding in rat cortex and hippocampus (Meberg et al., 1996; O'Neill and Kaltschmidt, 1997). It has been pointed out that glutamate is one of the brain-specific activators of NF-kB, and thus the NF-kB gene transcription factor is also implicated in synaptic plasticity (O'Neill and Kaltschmidt, 1997). Ethanol withdrawal after chronic ethanol intake is associated with neuronal excitability in the brain (Grant and Lovinger, 1995), and supersensitive *N*-methyl-D-aspartate (NMDA) receptors represent important neurochemical correlates in this process and also in the behavioral manifestations of ethanol withdrawal, e.g., seizures (Grant et al., 1990; Fadda and Rossetti, 1998). We had thought this property might cause NF-kB DNA binding activity induction in the rat brain; however, it was found that NF-kB DNA binding is not altered in the rat cortex or hippocampus by chronic ethanol treatment, or by acute (12 or 24 h) or protracted (72 h) ethanol withdrawal after 15 days of ethanol treatment. Thus, NF-kB DNA binding in the rat brain appears to be insensitive to ethanol treatment and its withdrawal.

In conclusion, protracted ethanol treatment produced a reduction in SP1 DNA binding activity in the rat cortex but not in the hippocampus, whereas ethanol withdrawal after 15 days of ethanol treatment produced a significant decrease in SP1 DNA binding activity in both the cortex and the hippocampus. These results suggest that the SP1-related gene transcription program in the cortex and the hippocampus may play a role in the molecular mechanisms associated with the process of ethanol dependence. Future studies on SP1 gene transcription factor-related genes may provide further insight into the molecular mechanisms of ethanol dependence.

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