Changes of phosphorylation of cAMP response element binding protein in rat nucleus accumbens after chronic ethanol intake: naloxone reversal[1]

LI Jing[2], LI Yue-Hua, YUAN Xiao-Ru[3]

Department of Pathology and Pathophysiology, [3]Department of Physiology, Nanjing Medical University, Nanjing 210029, China

KEY WORDS ethanol; nucleus accumbens; cAMP response element binding protein; naloxone

ABSTRACT

AIM: To study the changes in the expression and phosphorylation of cAMP response element binding protein (CREB) in the rat nucleus accumbens after chronic ethanol intake and its withdrawal. **METHODS:** Ethanol was given in drinking water at the concentration of 6 % (v/v), for one month. Changes in the levels of CREB and phospho-CREB (p-CREB) protein in the nucleus accumbens were measured by immunohistochemistry methods. **RESULTS:** Ethanol given to rats in drinking water decreased the level of p-CREB protein in the nucleus accumbens (-75 %) at the time of exposure to ethanol. The decrement of p-CREB protein in the nucleus accumbens remained at 24 h (-35 %) and 72 h (-28 %) of ethanol withdrawal, which recovered toward control level after 7 d of ethanol withdrawal. However, chronic ethanol, as well as ethanol withdrawal failed to produce any significant alteration in the level of CREB protein in the nucleus accumbens. Naloxone (alone) treatment of rats had no effect on the levels of CREB and p-CREB protein in the nucleus accumbens. However, when naloxone was administered concurrently with ethanol treatment, it antagonized the down-regulation of p-CREB protein in the nucleus accumbens (142 %) of rats exposed to ethanol. **CONCLUSION:** A long-term intake of ethanol solution down-regulates the phosphorylation of CREB in the nucleus accumbens, and those changes can be reversed by naloxone, which may be one kind of the molecular mechanisms associated with ethanol dependence.

INTRODUCTION

The molecular mechanisms of the addictive properties of ethanol are poorly understood. Clinical, biochemical, pharmacological, and behavioral studies have shown that opioid systems are involved in some of ethanol effects[1]. Ethanol could modulate the activity of this system *via* alteration of the synthesis, release or degradation of the opioids, or of opioid receptor binding or signaling[2]. Thus, it has been hypothesised that ethanol reward effect is mediated, at least in part, by the increase in the opioid activity. In line with this notion, non-selective opioid receptor antagonists (eg, naloxone) could attenuate ethanol consumption in animal tests and nalmefene could prevent relapse from heavy drinking in alcohol-dependent patients[3,4].

The nucleus accumbens, a major component of the ventral striatum, is currently under experimental scrutiny because of its likely involvement in self-

[1] Project supported by the Natural Science Foundation from Committee for Education of Jiangsu Province of China, No 01KJB 310005.
[2] Correspondence to Dr LI Jing. Phn 86-25-666-2886. Fax 86-25-650-8960. E-mail wlbianjs@163.com
Received 2002-07-02 Accepted 2003-03-19

administration of heroin, alcohol, and other drugs of abuse[5], and because of its role in regulating extrapyramidal motor function and the motor activity associated with motivation[6]. The nucleus accumbens contains abundant opioid peptides, as well as three different opiate receptor subtypes. It may be an important substrate for the acute reinforcing properties of opiates and possible also for the long-term motivational changes associated with opiate addicton[7]. Recently, several evidences suggest that changes in the expression and function of gene transcription factors in the nucleus accumbens, such as the cAMP response element binding protein (CREB), plays an important role in the mechanism of drug addiction[8-10]. CREB is a nuclear protein that modulates the transcription of genes with cAMP response element (CRE) sites in their promotors[11,12]. CREB can be phosphorylated by several kinases, such as protein kinase A (PKA), Ca^{2+}- and calmodulin-dependent protein kinases (CaM kinases), and ribosomal S_6 kinase *via* mitogen-activated protein kinase[13-15]. Phosphorylated CREB then forms homodimers or heterodimers with cAMP response element modulator protein or activator transcription factor that bind to the promotor regions of genes containing CRE sites, and thus, regulate gene expression[12]. This suggests that phosphorylation of CREB at the Ser 133 site is an essential step in the regulation of the transcription of many cAMP-inducible genes. However, little is known about the effects of ethanol on the phosphorylation of CREB in the nucleus accumbens. Whether the action of ethanol on CREB in the nucleus accumbens being associated with opioid system is also unknown.

The aim of the present study was to elucidate (1) the effects of chronic ethanol intake and its withdrawal on the expression of CREB and p-CREB in the nucleus accumbens of rats, (2) the reversal effects of naloxone on changes in the CREB and p-CREB induced by chronic ethanol.

MATERIALS AND METHODS

Drugs and reagents Ethanol was produced by Nanjing Chemical Plant, China. Total CREB antibody and phospho-CREB (at 133 serine) antibody were from New England Biolabs, USA. Naloxone hydrochloride was from Jingmei Biotech Co Ltd, China. Vectastain ABC kit and 3',3-diaminobenzidine (DAB) were from Vector Laboratories, Burlingame, CA, USA.

Animals and drug administration Fifty-four male Sprague-Dawley rats (Grade II, Certification No 97001, Nanjing Medical University) weighing 180 to 210 g were used at the beginning of experiments and weighing 360 to 410 g at the end of experiments. Animals were caged in a quiet department that was nature light and temperature was controlled (18-24 ºC). For chronic ethanol experiments, ethanol was given in drinking water at the concentration of 6 % (v/v) for one month[16]. Control animals received tap water for an identical period of time. The amount of liquid intake was measured daily: rats consumed ethanol 6.5-8.0 $g \cdot kg^{-1} \cdot d^{-1}$. All animals were weighed once a week. There were no significant differences in body weight between the control and ethanol-withdrawal animals. For the chronic naloxone studies, the control and ethanol-administration groups received naloxone (1.0 $mg \cdot kg^{-1} \cdot d^{-1}$, ip) or saline treatment for 10 d from d 20 of ethanol administration. Thus, there were eight groups of rats in the chronic ethanol administration and chronic naloxone treatment: (1) intake of tap water for 30 d (control), (2) chronic intake of ethanol for 30 d (chronic ethanol), (3) withdrawal 24 h after 30 d of ethanol intake, (4) withdrawal 72 h after 30 d of ethanol intake, (5) withdrawal 7 d after 30 d of ethanol intake, (6) control plus chronic naloxone-treated (naloxone only), (7) chronic ethanol intake plus chronic saline-treated, and (8) chronic ethanol intake plus chronic naloxone-treated (chronic ethanol+naloxone).

Immunohistochemistry of CREB and p-CREB Rats from these eight groups were anesthetized with sodium pentobarbital (40 mg/kg, ip) and then perfused intracardially with saline (0.9 % NaCl, 150 mL), followed by 400 mL of 4 % ice-cold paraformaldehyde prepared in 0.1 mol/L phosphate buffer solution (pH=7.4). Brains were dissected out and placed in the same fixative solution at 4 ºC for 3-4 h. After postfixation, brains were soaked in 20 % sucrose (prepared in 0.1 mol/L phosphate buffer, pH=7.4), at 4 ºC overnight. Brains were then frozen and consecutive 25 μm thick coronal sections containing the nucleus accumbens between the Bregma 1.60 mm and the Bregma 0.70 mm were prepared on a cryostat, according to the Stereotaxic Atlas of Paxinos and Watson[17]. Those sections were placed in 0.01 mol/L phosphate-buffered saline (PBS, pH=7.4) at 4 ºC.

Sections were washed twice with PBS at room temperature for 10 min and then in PBS containing 0.2 % Triton X-100 for 30 min. Non-specific binding sites

were blocked in 2 % goat serum in PBS/0.2 % Triton X-100 for 30 min. Sections were further incubated with CREB or p-CREB antibodies (diluted in PBS/0.2 % Triton X-100: 1:200 for CREB and p-CREB) at 4 ºC for 72 h. After incubation, the sections were washed three times at room temperature for 10 min and were incubated with biotinlyated secondary antibodies at room temperature for 1 h. Then, the sections were incubated with avidin-biotin-peroxidase complex (1:100, room temperature, 45 min) from a Vector ABC Kit. The sections were subsequently incubated with DAB (0.5 g/L) at room temperature for 10-15 min. The brown color reaction with DAB was stopped after checking the staining with the microscope. For the negative control sections an identical protocol was used, except that 1 % bovine serum albumin in PBS was substituted for the primary antibody.

Quantitative analysis The quantification of immunoreactive positive particles was performed using the Leica Image Analysis System connected to a light microscope that calculated the number of positive particles/100 μm^2 area of defined nucleus accumbens. The threshold of each image was set up in such a way that areas without staining should give zero counts. Under this condition, we counted the positive particles in the nucleus accumbens areas surrounding to the anterior commissure (Fig 1). Three adjacent brain sections of each rat were counted and then values were averaged for each rat.

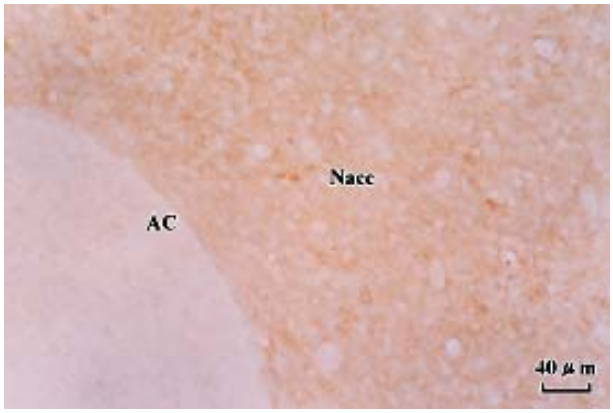

Fig 1. One typical negative section containing the nucleus accumbens. The section was incubated with 1 % BSA in PBS containing 0.2 % Triton X-100 without primary antibodies for p-CREB and CREB. Nacc, nucleus accumbens; AC, anterior commisure. ×200.

Statistical analysis Data from 6 rats of each group were presented as the mean±SD. Statistical analysis of data was performed with *t* test.

RESULTS

Changes in CREB and p-CREB protein levels in the nucleus accumbens during chronic ethanol intake and its withdrawal The immunostaining of CREB and p-CREB protein was specific because we did not observe any positive staining in the negative brain sections (Fig 1). Ethanol given to rats in drinking water led to a significant reduction in p-CREB protein level in the nucleus accumbens (-75 %; t=17.89) (Fig 2B, Fig 3A) compared to control. The decrement of p-CREB protein in the nucleus accumbens remained at 24 h (-35 %; t=6.76) and 72 h (-28 %; t=6.37) (Fig 2C, D, Fig 3A) after ethanol withdawal compared to control rats, and p-CREB protein level in the nucleus accumbens approached control levels after 7 d of ethanol withdrawal (t=1.66) (Fig 2E). However, ethanol given to rats in drinking water had no effect on the expression of CREB protein in the nucleus accumbens compared to control (t=1.33) (Fig 2G, Fig 3B). The level of CREB protein in the nucleus accumbens did not change during ethanol withdrawal yet (t=0.87) (Fig 2H, Fig 3B). These results suggest that the phosphorylation status of CREB is attenuated in the specific neurocircuitry of nucleus accumbens during chronic ethanol intake and its withdrawal, but the expression of CREB protein did not change.

Effects of naloxone on the expression of CREB and p-CREB during chronic ethanol intake Chronic naloxone treatment, when administered concurrently with ethanol treatment, significantly blocked the down-regulation of p-CREB protein level in the nucleus accumbens at the time exposure to ethanol (Fig 4B, C). The level of p-CREB in the nucleus accumbens of ethanol-intake plus naloxone treatment rats was 142 % higher than that in ethanol-intake plus saline treatment rats (t=8.74) (Fig 5A). However, chronic naloxone treatment alone had no apparent direct effect on the phosphorylation of CREB in the rat nucleus accumbens, as compared to control rats (t=0.50) (Fig 4D, Fig 5A). Chronic naloxone treatment failed to produce significant alteration in CREB protein level in the nucleus accumbens of ethanol-intake rats (t=1.02) (Fig 4G, Fig 5B), and chronic naloxone treatment alone did not modify the expression of CREB in the nucleus accumbens compared with control rats (t=1.17) (Fig 4H, Fig 5B).

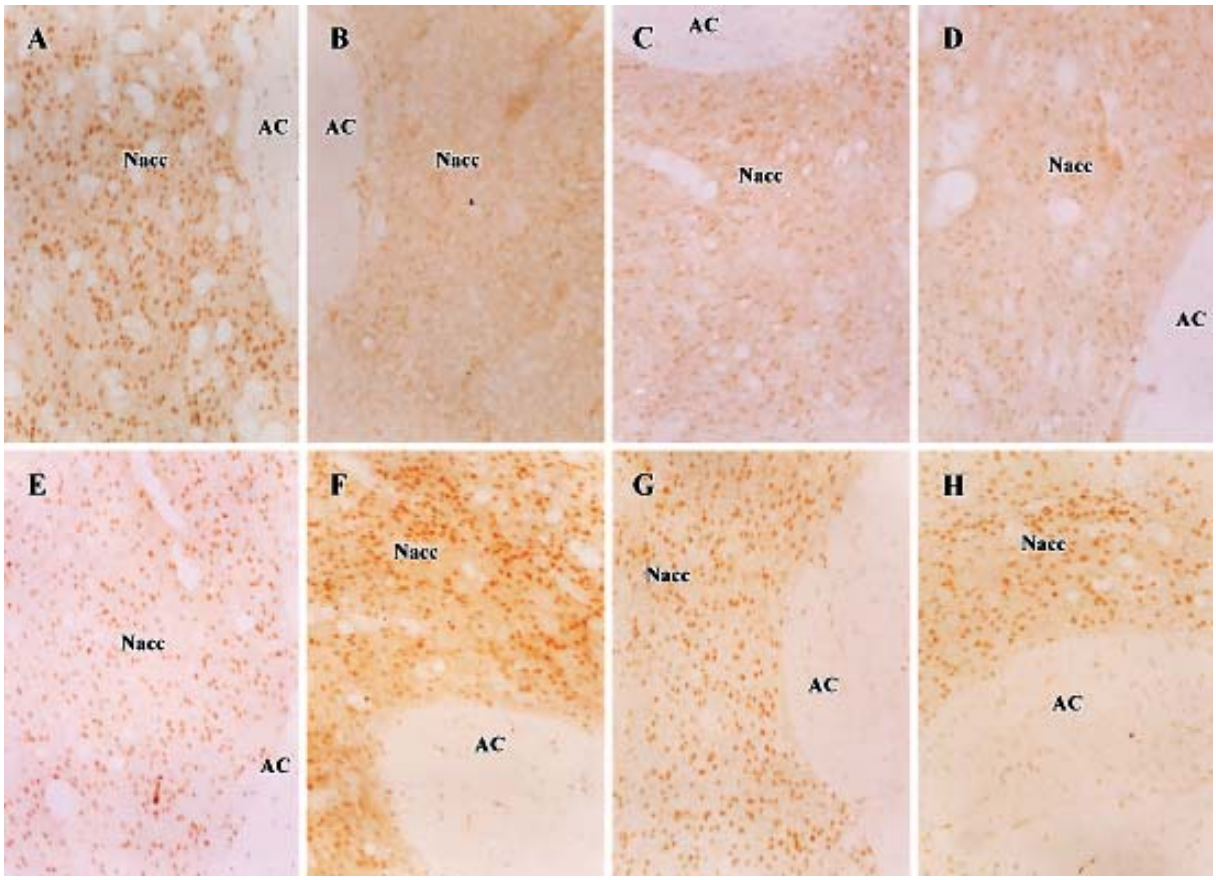

Fig 2. Immunostaining of p-CREB (A-E) and CREB (F-H) protein in the nucleus accumbens of control, ethanol-intake, and ethanol-withdrawal rats. A, F: control; B, G: ethanol-intake for 30 d; C, H: ethanol-withdrawl for 24 h; D, E: ethanol-withdrawl for 72 h and 7 d. ×200.

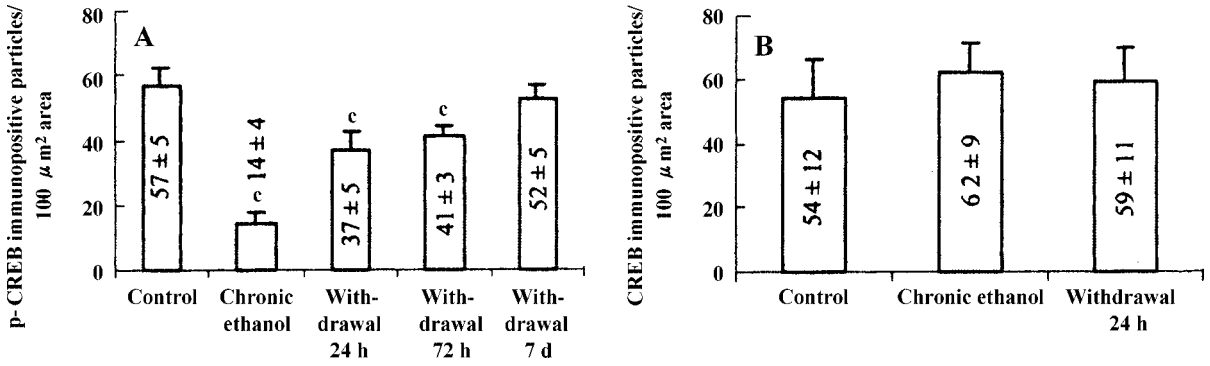

Fig 3. Effects of chronic intake of ethanol and its withdrawal (24 h, 72 h, 7 d) on the levels of p-CREB (A) and CREB (B) protein (number of immunopositive particles per 100 μm² area) in the rat nucleus accumbens. *n*=6 rats. Mean±SD. $^{c}P<0.01$ *vs* control.

DISCUSSION

The cAMP signaling pathway is a major target for ethanol in intact cells[18]. It has been showed that various components (neurotransmitter receptors, G protein, adenylate cyclase, PKA) of the cAMP signal transduction cascade were decreased in the rodent brain during ethanol dependence. It is possible that the function of the CREB gene transcription factor may also be altered during ethanol dependence. Since the nucleus accumbens is an important brain region for reinforcement and motivational aspects of ethanol dependence, we explored the effects of chronic ethanol on CREB in the nucleus accumbens of rats in this study. The results

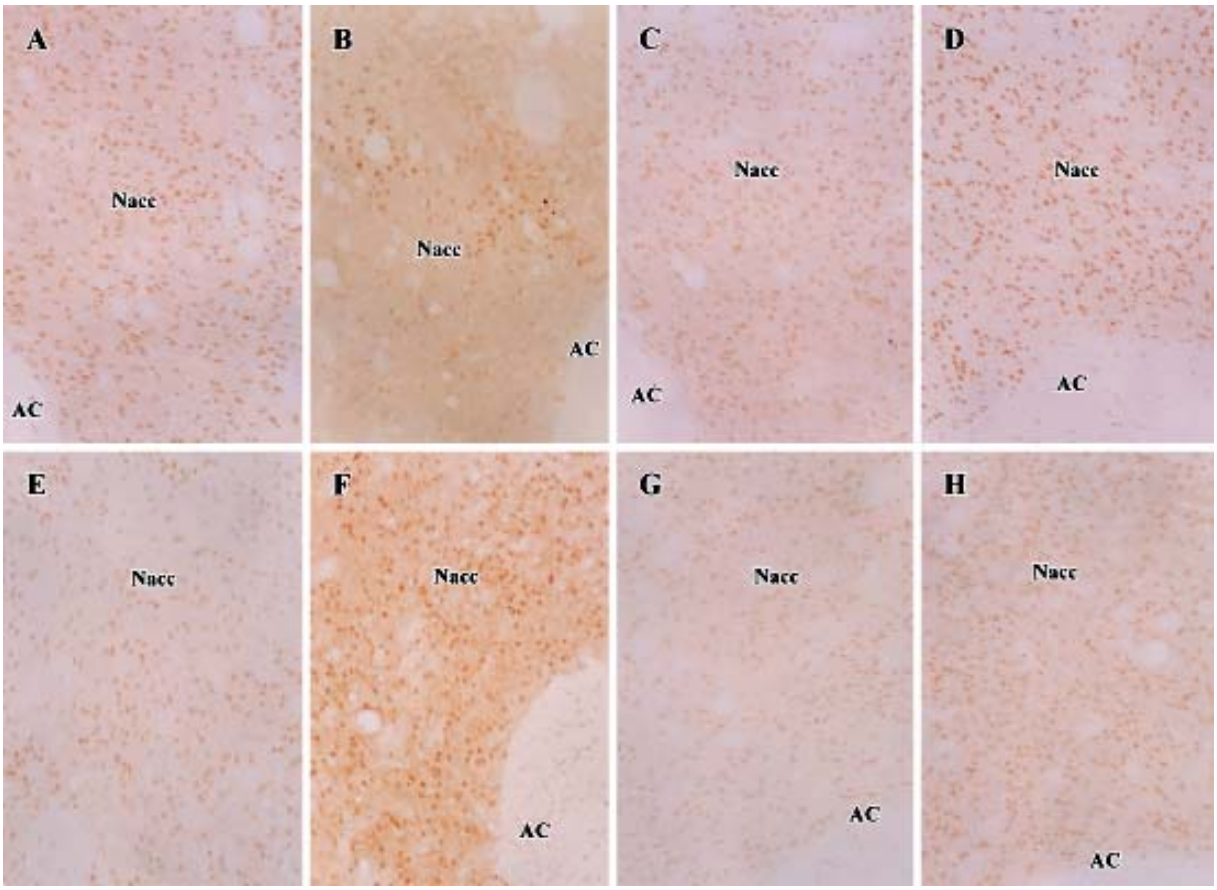

Fig 4. Immunostaining of p-CREB (A-D) and CREB (E-H) protein in the nucleus accumbens of control, ethanol-intake, and naloxone-treated rats. A, E: control; B, F: ethanol-intake for 30 d; C, G: chronic ethanol-intake plus naloxone-treated; D, H: naloxone-treated alone. ×200.

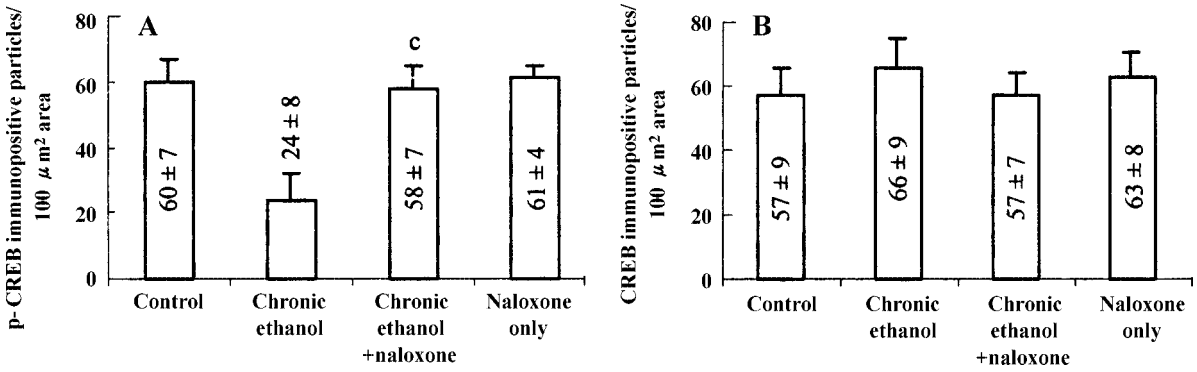

Fig 5. Effects of chronic intake of ethanol and naloxone with or without concurrent ethanol on the levels of p-CREB (A) and CREB (B) protein in the rat nucleus accumbens. *n*=6 rats. Mean±SD. $^{c}P<0.01$ *vs* chronic ethanol.

indicated that though a long-term intake of ethanol did not effect the expression of CREB in the nucleus accumbens, it produced a significant decrease in p-CREB immunoreactivity in the rat nucleus accumbens, and the decrement of p-CREB protein remained at 24 h and 72 h of ethanol withdrawal, which implied that the decreased p-CREB in the nucleus accumbens at the time of exposure to ethanol may underlie some of long-term adaptation assiociated with ethanol dependence. Our findings that chronic ethanol intake led a decrease of p-CREB protein in the nucleus accumbens are similar to the studies of Ikemoto *et al*[19], who found that chronic

morphine treatment decreased CREB-DNA binding activity in the amygdala, hypothalamus, and cortex, and the CREB-DNA binding activity still reduced in those brain regions after 14 d of morphine withdrawal, which may underlie some of the long-term adaptation of opiate. In contrast, Pandey *et al*[20] reported that chronic ethanol treatment had no effect on p-CREB immunoreactivity in rat cortex such as frontal cortex, piriform cortex and parietal cortex, and the level of p-CREB significantly decreased only at 24 h of ethanol withdrawal. The differences between the present study and the studies of Pandey *et al* may be related to the difference in brain regions (cortex versus nucleus accumbens) investigated or to the time of ethanol exposure. It was only for 14 d that rats were fed with ethanal[20], whereas in the present study, rats drinked ethanol solution for one month. It has been shown that chronic ethanol exposure impaired phosphorylation of CREB and CRE-binding activity in rat striatum, which were associated with a significant reduction in two CREB-modulated factors, proenkephalin and c-fos expression[21]. Taken together, these results suggest the possibility that the decreased expression of CREB-dependent genes in the nucleus accumbens may be involved in the phenomenon of alcohol dependence.

As summarized in the introduction, there are numerous clinical, biochemical, pharmacological, and behavioral studies showing interaction between ethanol and opiates. This interaction of ethanol and the endogenous opiate system in nucleus accumbens could occur, at least in part, directly *via* activation of opioid receptor or indirectly by realeasing endogenous opiate peptides from the nucleus accumbens neuronal processes known to contain enkephalin and perhaps dynorphin or β-endorphin as well[1]. The present study showed that long-term naloxone treatment did not affect the expression of CREB and p-CREB in the nucleus accumbens of control rats, but it antagonized the down-regulation of p-CREB in the nucleus accumbens at the time of exposure to ethanol, which suggested that the inhibitory actions of ethanol on the phosphorylation of CREB in the nucleus accumbens may involve the activation of opiate receptor. Opiod peptide could inhibit the translocation and phosphorylation of CREB in rheumatoid arthritis synovial cells and naloxone could reverse the inhibitory effect of the opioid peptides[22], which also supported that the activation of opiate receptor had an inhibitory action on the phosphorylation of CREB. However, the molecular mechanism by which naloxone blocked the down-regulation of the phosphorylation of CREB in the nucleus accumbens at the time of exposure to ethanol is sill unknown. Previous studies have demonstrated that naloxone increasing cytoplasmic Ca^{2+} in smooth muscle cells preincubated with morphine may be associated with phosphorylation of CREB enhancement[23]. So, it is suspected that blockade of the down-regulation of p-CREB by naloxone at the time of exposure to ethanol may be due to an increase in the activity of CaM kinase or PKA. It is also possible that naloxone treatment increases the expression of CREB mRNA in the nucleus accumbens. Further studies are needed to explore these possibilities.

In the present study, chronic ethanol exposure exerted an inhibitory action on the phosphorylation of CREB in the rat nucleus accumbens, and those changes did not recover toward control levels until 7 d of ethanol withdrawal. More importantly, chronic treament of rats with opiate antagonist (naloxone) in combination with ethanol treatment significantly blocks the decrease in phosphorylation of CREB in the nucleus accumbens at the time of exposure to ethanol. Since CREB has been shown to be involved in the long-term effects of other drugs of abuse, eg, morphine and cocaine[8-10], our results suggests that changes in the phosphorylation of CREB in the nucleus accumbens during chronic ethanol intake may be associated with the neuroadaptational mechanisms underlying alcohol dependence.

REFERENCES

1 Herz A. Endogenous opioid system and alcohol addiction. Psychopharmacology 1997; 129: 99-111.
2 Charness ME. Ethanol and opoid receptor signalling. Experientia 1989; 45: 418-28.
3 Sharpe AL, Samson HH. Effect of naloxone on appetitive and consummatory phases of ethanol self-administration. Alcohol Clin Exp Res 2001; 25: 1006-11.
4 Mason BJ, Salvato FR, Williams LD, Ritvo EC, Cultler RB. A double-blind, placebo-controlled study of oral nalmefene for alcohol dependence. Arch Gen Psychiatry 1999; 56: 719-24.
5 Koob GF, Bloom FE. Cellular and molecular mechanisms of drug dependence. Science 1988; 242: 715-23.
6 Mogenson GJ. Limbic-motor integration. Prog Physiol Phychol 1987; 12: 117-70.
7 Self DW, McClenahan AW, Beitner-Johnson D, Terwillger RZ, Nester EJ. Biochemical adaptations in the mesolimbic dopamine system in response to heroin self-administration. Synapse 1995; 21: 312-8.
8 Windnell K, Self DW, Lane SB, Russell DS, Vaidya VA,

Miserendind MJD, *et al*. Regulation of CREB expression: *in vivo* evidence for a functional role in morphine action in the nucleus accumbens. J Pharmacol Exp Ther 1996; 276: 306-15.
9 Carlezon Jr WA, Thome J, Olson V, Lane-Ladd SB, Brodkin ES, Hirol N, *et al*. Regulation of cocaine reward by CREB. Science 1998; 282: 2272-5.
10 Pliakas AM, Carlson RR, Neve RL, Konradi C, Nestler EJ, Carlezon WA Jr. Altered responsiveness to cocaine and increased immobility in the forced swim test associated with elevated cAMP response element-binding protein expression in nucleus accumbens. J Neurosci 2001; 21: 7397-403.
11 Montminy MR. Transcriptional regulation by cyclic AMP. Annu Rev Biochem 1997; 66: 802-22.
12 Silva AJ, Kongan JH, Frankland PW, Kidd S. CREB and memory. Annu Rev Neurosci 1998; 21: 127-48.
13 Sheng M, Thompson MA, Greenberg ME. CREB : a Ca^{2+}-regulated transcription factor phophorylated by calmodulin-dependent kinases. Science 1991; 252: 1428-30.
14 Moore AL, Waxhan MN, Dash PK. Neuronal activity increases the phosphorylation of the transcription factor cAMP response element-binding protein (CREB) in rat hippocampus and cortex. J Biol Chem 1996; 271: 14214-20.
15 Impey S, Obrietan K, Storm DR. Making new connections: Role of ERK/MAP kinase signaling in neuronal plasticity. Neuron 1999; 23: 11-4.
16 Turchan J, Przewlocka B, Toth G, Lason W, Borsodi A, Przewlocki R. The effect of repeated administration of morphine, cocaine and ethanol on mu and delta opioid receptor density in the nucleus accumbens and striarum of the rat. Neuroscience 1999; 91: 971-7.
17 Paxinos G, Watson C, editors. The rat brain in stereotaxic coordinates. 2nd ed. London: Academic Press; 1986.
18 Diamond I, Gordon AS. Cellular and molecular neuroscience of alcoholism. Physiol Rev 1997; 77: 1-20.
19 Ikemoto M, Osugi T, Wang XB. Decrease in CRE binding activity by chronic morphine administration in mouse brain. Neuroreport 1995; 6: 262-4.
20 Pandey SC, Roy A, Mittal N. Effects of chronic ethanol intake and its withdrawal on the expression and phosphorylation of the CREB gene transcription factor in rat cortex. J Pharmacol Exp Ther 2001; 296: 857-68.
21 Yang X, Horn K, Wand GS. Chronic ethanol exposure impairs phosphorylation of CREB and CRE-binding activity in rat striatum. Alcohol Clin Exp Res 1998; 22: 382-90.
22 Takeba Y, Suzuki N, Kaneke A, Asai T, Sakane T. Endorphin and enkephalin ameliorate excessive synovial cell functions in patients with rheumatoid arthritis. J Rheumatol 2001; 28: 2176-83.
23 Wang Y, Xu JG, Yu YQ, Wang HY, Jiang B, Li XY. Naloxone increases vascular responsiveness in chronic morphine treated rats and facilitates intracellular signaling in cultured vascular cells. Acta Pharmacol Sin 2001; 22: 881-6.