EFFECTS OF NALTREXONE ON THE ETHANOL-INDUCED CHANGES IN THE RAT CENTRAL DOPAMINERGIC SYSTEM

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Abstract — Aims: The opioid antagonist naltrexone may reduce ethanol reward, but the underlying neurochemical mechanisms has yet to be clarified. The afferent projections to the nucleus accumbens from the ventral tegmental area (VTA) provide a potential substrate by which endogenous opioids may modulate the dopaminergic rewarding effects of ethanol. We assessed mRNA levels of tyrosine hydroxylase (TH), a major regulatory enzyme in the dopamine synthesis and levels of dopamine and its metabolites after chronic ethanol administration with and without concomitant naltrexone. **Methods:** Sprague-Dawley rats were exposed to chronic ethanol consumption (5%, 4 weeks) with and without concomitant naltrexone administration. Levels of TH mRNA in the VTA and substantia nigra (SN) and dopamine and its metabolites in the striatum of the rats were measured by *in situ* hybridization and by high performance liquid chromatography, respectively. **Results:** Chronic ethanol consumption increased TH mRNA levels in the VTA, but did not cause any significant change in the SN. With naltrexone treatment, ethanol-induced increase in the TH mRNA level was reduced in the VTA. Chronic ethanol consumption did not cause any change in the levels of dopamine and its metabolites in most brain regions. Only in the striatum, ethanol consumption with naltrexone treatment significantly increases the dopamine level. **Conclusion:** This finding supports the presence of interactions of opioid and dopaminergic systems in the VTA in mediating ethanol reward, and thus naltrexone attenuates the rewarding properties of ethanol by interfering with the ethanol-induced stimulation of the mesolimbic dopaminergic pathway.

INTRODUCTION

A number of clinical studies have shown that the opioid antagonist naltrexone is a pharmacologically effective adjunct for reducing relapse in alcoholics (O'Malley *et al.*, 1992, 1995; Volpicelli *et al.*, 1992, 1995; Swift *et al.*, 1994, 1995; O'Brien *et al.*, 1996). There is considerable evidence demonstrating that ethanol rewarding stimuli enhance the activity of the endogenous opioid system (Froehlich *et al.*, 1991; Gianoculakis and de Waele, 1994, 1996; Herz *et al.*, 1997). Thus, it has been hypothesized that ethanol reward is mediated, at least in part, by an increase in opioid activity (Koob, 1992; Self and Nestler, 1995). In support of this hypothesis, there have been several studies reporting that naltrexone reduced ethanol reward or reinforcement in the animal system (Volpicelli *et al.*, 1992; Kranzler *et al.*, 1997; Middaugh *et al.*, 1999; Williams and Woods, 1999; Middaugh and Bandy, 2000).

Although the neurochemical mechanisms underlying the attenuation of ethanol intake by opioid receptor antagonists have yet to be clearly elucidated, some interaction with the dopaminergic neurotransmission system seems to be involved. The mesolimbic dopamine pathway that projects from the ventral tegmental area (VTA) to the nucleus accumbens (NA) has been implicated as a major site for the reinforcing actions of many addictive drugs including ethanol (Wise, 1978; Wise and Bozarth, 1981; Wise and Rompre, 1989; Koob *et al.*, 1992, 1998; Di Chiara, 1995; Everitt and Wolf, 2002; Kelley and Berridge, 2002; Weiss and Porrino, 2002). Systemic ethanol administration increases the dopamine concentration in the NA (Yoshimoto *et al.*, 1991; Blromqvist *et al.*, 1993; Mocsary and Bradberry, 1996; Yim *et al.*, 1998). Weiss *et al.* (1990, 1993) found that the oral administration of ethanol increases extracellular dopamine in the NA, which provides more direct evidence for the role of dopamine in the ethanol reward (Weiss *et al.*, 1990, 1993). In addition, both the NA and VTA are rich in opioid peptides and receptors (Wamsley *et al.*, 1980; Lewis *et al.*, 1983; Dilts and Kalivas, 1989, 1990). The afferent projections to the NA from the VTA provide a potential substrate by which endogenous opioids may modulate the dopaminergic and rewarding effects of ethanol (Khachaturian *et al.*, 1993; De waele *et al.*, 1995). In fact, the non-selective opioid antagonist naltrexone has been reported to inhibit the rise in extracellular dopamine concentrations elicited by the reverse microdialysis of ethanol (Benjamin *et al.*, 1993; Gonzales and Weiss, 1998).

Tyrosine hydroxylase (TH) is a major regulatory, rate-limiting enzyme in the synthesis of dopamine. Since the enzyme activity and protein levels are determined in dissected tissue homogenates of relatively small volume areas such as the catecholaminergic cell bodies and their projections, minor changes in the enzyme activity and the number of active enzyme molecules cannot be detected clearly. For this reason, an *in situ* hybridization technique has been applied to gain a semi-quantitative measure of enzyme mRNA in individual cells or cell clusters. Thus, assessing the levels of TH mRNA by *in situ* hybridization following chronic ethanol consumption with and without concomitant naltrexone administration might shed light on the mechanisms underlying the effect of naltrexone on the ethanol reinforcement system.

The aim of this study is to determine whether the effect of chronic ethanol to increase TH expression depends upon an opioid-mediated reward mechanism that is attenuated by naltrexone. We sought to examine changes in TH mRNA level

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by *in situ* hybridization in the VTA and substantia nigra (SN) and changes in the levels of dopamine and its metabolites by high performance liquid chromatography (HPLC), following regular ethanol consumption for 4 weeks with and without concomitant naltrexone administration.

METHODS

Animals

Sprague-Dawley male rats weighing 150 g, obtained from Hyocang Lab Animal (Daegu, Korea), were maintained on either 5% v/v ethanol admixed with a nutritionally complete liquid diet (High Protein-Ethanol Bio-Serv, Frenchtown, NJ) *ad libitum* or isocaloric sucrose in the liquid diet (for the control animals) for 4 weeks prior to the experiments. The group I animals were continuously maintained on the isocaloric sucrose diet, while the group II animals were maintained on the 5% v/v ethanol diet for the duration of the experimental period. The groups III animals were intraperitoneally (i.p.) injected every morning with naltrexone (1 mg/kg) and were exposed to a 5% v/v ethanol diet *ad libitum*. The handling of the animals done under the guidelines of the current laws governing animal experimentation in the United Kingdom.

In situ *hybridization*

Labelling of RNA probe with digoxigenin-11-UTP (DIG-11-UTP) was performed according to the manufacturer's recommendation. TH cDNA cloned into pBluescriptSK+ (Stratagene, La Jolla, CA) was given from Dr Kyong-Tai Kim (Pohang University of Science and Technology, Chae and Kim, 1995) After being linearized with PstI, the template cDNA was gel-purified and 1 µg of purified cDNA was used for *in vitro* transcription (corresponding to the antisense strand of 1.0 kb) with T7 polymerase and digoxigenin-labelled UTP according to the manufacturer's instructions (Dig RNA labeling kit; Boehringer Mannheim, Germany). The sense-stranded cRNA probe was synthesized from the same template cDNA but linearized with KpnI and driven by T3 polymerase. The transcription reactions were set up by mixing the following items at room temperature (RT): 8 µl H_2O-DEPC, 5 µl linearized DNA (1 g), 2 µl 10× transcription buffer, 2 µl NTP labelling mixture (10 mM ATP, CTP, GTP, 6.5 mM UTP, 3.5 mM DIG-11-UTP), 1 µl RNase inhibitor (20 U/µl), 2 µl RNA polymerase (T7 or SP6, 20 U/µl). The reaction was performed at 37°C for 2 h and subsequently stopped by degrading the plasmid with 2 µl DNase (RNase free, 10 U/µl) for 15 min at 37°C. cRNA fragments were precipitated with 1/10 vol. of 4 M LiCl and 2.5 vol. of ethanol at −80°C for at least 1 h. The precipitated cRNA was recovered in 100 µl H_2O-DEPC.

Rats were anaesthetized with pentobarbital (75 mg/kg, i.p.) and transcardially perfused with ice-cold PBS (pH 7.4) followed by ice-cold 4% paraformaldehyde in PBS. The brain was postfixed in the same fixative for 2 h and then cryoprotected in 30% sucrose-PBS overnight. They were then frozen by immersion in isopentane cooled at −80°C and stored at −80°C until use. Serial tissue sections were cut on a cryostat (10–20 µm) and thaw-mounted on clean RNase free slides (DAKO BioTek Solution, USA). Before acetylation, sections were fixed with 4% paraformaldehyde in PBS for 10 min. The acetylation was carried out in 0.25% (v/v) acetic anhydride in 0.1 M triethanolamine-HCl (pH 8.0) at RT for 10 min. After ethanol dehydration, sections were hybridized in a buffer containing 50% deionized formamide, 300 mM NaCl, 1× Denhardt's solution, 50 mM Tris-Cl pH 8.0, 2 mM EDTA, 10% dextran sulphate, 0.25 mg/ml yeast tRNA and with 200 ng/ml of either antisense or sense cRNA probe. The hybridization was performed overnight at 58°C in a chamber humidified with 50% deionzed formamide/4× SSC. After RNase A (20 µg/ml, AMRESCO, USA) treatment at RT, non-specifically hybridized probe was washed away through several post-hybridization steps in a shaking water bath starting in 2× SSC and ending with a high-stringency washing in 0.1× SSC at 60°C. A final wash in 0.5× SSC was done at RT. DIG-labelled hybrids were immunologically detected using DIG Nucleic Acid Detection Kit (Boehringer Mannheim, Germany) as recommended by the manufacturer. Briefly, the slides were incubated with anti-digoxigenin Fab-fragments (1:500) and then stained with a freshly prepared solution of 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate (NBT-BCIP). After 24 h, the reaction was stopped in 10 mM Tris-Cl pH 8.0, 1 mM EDTA and coverslipped in Kaiser's glycerol gelatin. Slides were examined on microscope and the amount of TH mRNA was analysed by a Image-Pro Plus program (Media Cybernetics) using optical density.

HPLC analysis of dopamine and its metabolites

Each isolated brain region (cerebral cortex, striatum, hypothalamus, hippocampus, midbrain, and pons and medulla oblongata) was exposed to microwaves for 1 s to stop the enzymatic reaction of the dopaminergic enzyme system. The dopamine concentrations were determined by micropore reverse-phase HPLC. We used a 20 µl sample, and a mobile phase [2.5 mM 1-octane sulfate, 17:83 of methanol:0.1 M KH_2PO_4 (pH 3.2)] that included 10 µM EDTA was pumped through the C18 column (4.6 × 150 mm, Nakalai) at 0.9 ml/min. We used the pump of model 307 (Gilson, France) and electrochemical detector (Toa company, Japan) for this study. The total detection time was 25 min. The applied potential was 0.75 V and the sensitivity was maintained at 16 nA.

Statistical analyses

The significance of the differences in the expression levels of the TH mRNA and the levels of dopamine and its metabolites between the different treatment groups was determined by ANOVA and the Scheffe test was used for the comparisons among the means.

RESULTS

Chronic ethanol consumption (5%, 4 weeks) increased the TH mRNA level in the VTA (*t* test, *t* = 2.499, *P* = 0.20), but did not show any significant change in the TH mRNA level in the SN. The administration of naltrexone concomitant with chronic ethanol consumption reduced the increase in the TH mRNA level in the VTA (Table 1). We found, using *in situ* hybridization technique, an increase in TH mRNA level in the VTA by ethanol consumption and a partial reverse by concomitant naltrexone administration (Fig. 1).

The levels of dopamine and its metabolites were measured in several brain regions including the cerebral cortex, striatum, hypothalamus, hippocampus, midbrain, and hindbrain (pons and medulla oblongata). Chronic ethanol consumption for 4 weeks did not cause any significant changes in the levels of dopamine and its metabolites in the most of the regions examined. Only in the striatum, the administration of naltrexone concomitant with ethanol consumption significantly increases the levels of dopamine (Table 2).

DISCUSSION

There is considerable preclinical/clinical evidence for the interaction of opioid and dopaminergic systems in the VTA and NA in mediating ethanol reward (Froehlich *et al*., 1991; Gianoculakis and de Waele, 1994; Gianoculakis, 1996; Herz *et al*., 1997; Gonzales and Weiss, 1998). In support of this evidence, the opioid antagonist naltrexone has been found to reduce ethanol reward or reinforcement in the animal system. However, the detailed neurochemical mechanism underlying this effect has not been clearly elucidated. In this study, we found that chronic ethanol treatment increased TH mRNA expression in the VTA. More interestingly, naltrexone administration concomitant with chronic ethanol consumption reduced this ethanol-induced increase in TH mRNA expression. Since the mesolimbic dopaminergic pathway projecting from the VTA to NA is involved in mediating reward-related behaviours (Wise and Rompre, 1989; Koob *et al*., 1992; Di Chiara *et al*., 1995), an increase in the TH mRNA level induced by chronic ethanol consumption may constitute new evidence for the implication of the dopamine reward system in the ethanol reinforcement. In this study, we found that naltrexone administration reduced an increase in the TH mRNA level induced by ethanol consumption.

Neural projections from the SN are directed towards the striatum, and pairs of these from the VTA area towards the NA. Since chronic ethanol consumption did not cause any significant change in the TH mRNA level in the SN, the levels of dopamine and its metabolites in the striatum were expected to be unchanged. On the other hand, because chronic ethanol consumption caused an increase in the TH mRNA level in the VTA, the levels of dopamine and its metabolites in the NA were expected to be increased. However, no significant difference in the levels of dopamine and its metabolites was found between the control and ethanol-treated group in most of the brain regions including the striatum.

Since the NA is a very small part attached to the striatum, we did not separate the NA from the striatum. In other words, the effects of ethanol on the dopamine metabolism in the NA could not be specifically examined. The lack of such separation is a

Table 1. Changes (optical density) in TH mRNA level induced by chronic ethanol consumption with or without concomitant naltrexone administration

	VTA	SN
Control	0.64 ± 0.03	0.75 ± 0.08
Ethanol	0.75 ± 0.08[a]	0.71 ± 0.08
Ethanol + naltrexone	0.63 ± 0.04[b]	0.67 ± 0.06

Control diet group were continuously maintained on an isocaloric sucrose diet, and a 5% v/v ethanol diet was administered to the ethanol diet group. The naltrexone + ethanol diet group were intraperitoneally injected every morning with naltrexone (1 mg/kg) and were exposed to a 5% v/v ethanol diet *ad libitum*.
[a]Control diet vs ethanol diet (*P* < 0.05).
[b]Ethanol diet vs naltrexone + ethanol (*P* < 0.05). These values are mean ± SD; *n* = 4 animals per group.
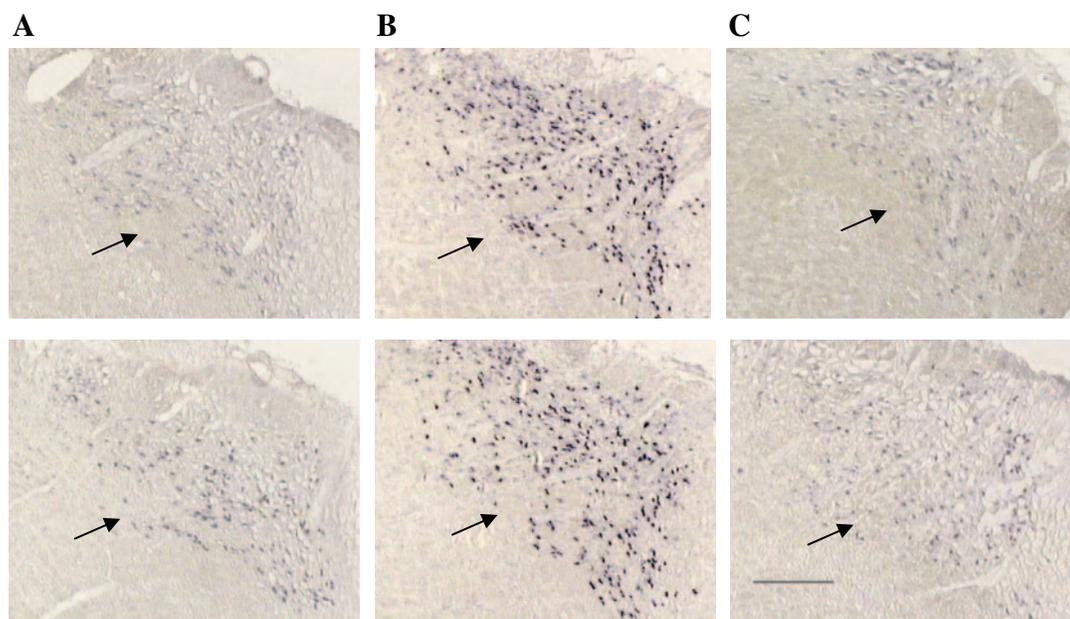

Fig. 1. TH mRNA expression in the VTA (indicated by arrowheads). Ethanol treatment for 4 weeks increased TH expression and concomitantly administered naltrexone reversed the increased expression in the VTA. A, B, and C present the pair-fed control, ethanol treated, and naltrexone + ethanol treated groups, respectively.

Table 2. The mean levels (ng/µl) of dopamine and its metabolites in various brain regions in rats treated with chronic ethanol consumption with or without concomitant naltrexone

Region	Dopamine	DOPAC	HVA
Cerebral cortex			
Control	0.34 ± 0.1	0.04 ± 0.01	0.02 ± 0.01
Ethanol	0.42 ± 0.06	0.06 ± 0.04	0.03 ± 0.01
Ethanol + naltrexone	0.41 ± 0.06	0.04 ± 0.01	0.03 ± 0.01
Striatum			
Control	4.76 ± 0.64	1.06 ± 0.28	0.90 ± 0.22
Ethanol	4.84 ± 0.54	1.25 ± 0.95	0.87 ± 0.25
Ethanol + naltrexone	7.22[a] ± 2.58	1.42 ± 0.57	1.16 ± 0.51
Midbrain			
Control	0.39 ± 0.07	0.12 ± 0.03	0.05 ± 0.08
Ethanol	0.45 ± 0.3	0.10 ± 0.06	0.04 ± 0.03
Ethanol + naltrexone	0.55 ± 0.22	0.11 ± 0.04	0.06 ± 0.06

The control diet group was continuously maintained on the isocaloric sucrose diet, and 5% v/v ethanol diet was administered to ethanol diet group. Naltrexone + ethanol diet group was intraperitoneally injected every morning with naltrexone (1 mg/kg) and were exposed to 5% v/v ethanol diet *ad libitum*. DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanilic acid.
[a]Ethanol + naltrexone vs ethanol and control diet group ($P < 0.05$). These values are mean ± SD; n = 10 animals per group.

limitation in the current data; hence, thus we will employ some methods to separate the NA from the striatum in future.

However, in the striatum only, the administration of naltrexone concomitant with ethanol consumption significantly increased the dopamine level whereas it did not cause any changes in the levels of its metabolites. The increase in the dopamine level may, thus, be due to increased dopamine turnover. Since the chronic ethanol consumption without naltrexone treatment did not induce any increase in the dopamine level in the striatum, we can interpret that chronic naltrexone might contribute to the increase in the dopamine level, regardless of chronic ethanol treatment. This suggests that the increased dopamine level induced by chronic naltrexone treatment in our striatal sample might arise from an increase in the dopamine synthesis in the VTA–NA system. As a matter of fact, we should admit that changes in TH mRNA and dopamine levels were not evaluated in a naltrexone-alone-treated group in this study. Thus, it might be the spurious result, without comparison with a naltrexone-alone-treated group, that the increase in the dopamine level in the striatum during chronic ethanol consumption is totally due to chronic naltrexone treatment. However, Middaugh *et al.* (2003) have recently found that extracellular dopamine levels of naltrexone-alone-treated rats are not significantly different from those of the control group. Thus, it seems that the increased dopamine level induced by chronic naltrexone treatment might arise from an increase in the dopamine synthesis in the VTA–NA system, which will be further studied in the near future.

Previous reports have suggested that the ethanol-induced increase in dialysate dopamine level in the NA is attenuated by naltrexone treatment (Benjamin *et al.*, 1993; Gonzales and Weiss, 1998). These reports only investigated the effects of a single pretreatment with naltrexone on acute ethanol treatment in the NA. In these reports, dopamine release was antagonized by acute naltrexone administration via the mu receptor in the striatum. However, in our report, the increased level of dopamine observed in the striatum following chronic ethanol administration with naltrexone treatment is considered to be a direct effect of chronic naltrexone on the dopaminergic system.

In conclusion, the current study investigated the action of naltrexone on decreasing alcohol consumption. The antagonistic effect of naltrexone against the change in the TH mRNA level after chronic ethanol consumption supports the previous hypothesis that naltrexone attenuates the rewarding properties of ethanol by interfering with the ethanol-induced stimulation of the mesolimbic dopaminergic pathway. Another preliminary finding that chronic naltrexone administration causes an increase in the striatal dopamine level requires further investigation with an appropriately designed trial.

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