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Effects of short-term ethanol ingestion on the expression of neurotrophins and their receptors: No changes in the expression of the glial-derived neurotrophic factor in the mouse hippocampus

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Abstract

Alcohol ingestion has adverse effects on the central nervous system (CNS). The hippocampus is one of the target sites of ethanol neurotoxicity. We hypothesized that short-term ethanol exposure alters the expression of neurotrophins and their receptors, leading to functional disruption in the CNS. Male BALB/C mice were fed a liquid diet containing 5% (v/v) ethanol. Pair-fed control mice were maintained on an identical liquid diet, except that ethanol was isocalorically substituted with sucrose. The hippocampus of mice exhibiting stages 1-2 of ethanol intoxication signs were used in the present study. Short-term ethanol exposure did not alter the mRNA expression of neurotrophin ligand/receptor (nerve growth factor [NGF]/TrkA and brain-derived neurotrophic factor [BDNF]/TrkB) systems in the hippocampus. Similarly, the expression of the glial-derived neurotrophic factor (GDNF), which is known to be a first-acting agent against ethanol neurotoxicity, and its receptor GFRα1 was not affected by short-term ethanol exposure. The mechanisms involved in the hippocampal neurotrophin responses against ethanol neurotoxicity remain unknown. However, our findings could provide a basis for further studies on the possible alterations in the expression of various neurotrophins related to hippocampal functions.

Introduction

Alcohol ingestion has various adverse effects on the mind and body, depending on the dose ingested. Of these effects, impairment of the central nervous system (CNS) is one of the most serious alcohol-related conditions. Impairment in learning, memory, and cognitive functions are well-known signs related to alcohol consumption in humans and laboratory animals. Such impairment can be noted even in the absence of obvious organic lesions in the CNS, such as those associated with Wernicke-Korsakoff's syndrome. In fact, basic and clinical studies have revealed that ethanol exposure alters neurotrophin expression, neuronal excitability, or synaptic transmission, without causing any histological alterations in brain regions such as the hippocampus, cerebral cortex, and cerebellum, which are closely related to functions of cognition, learning, and memory [1-3].

Neurotrophins are known to play key roles in normal brain functions, neural regulation, and the differentiation and survival of neurons in specific brains regions. The neurotrophin family includes various trophic factors such as the nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin (NT)-3, and NT-4/5. These factors initiate

signaling by binding to receptors with high (TrkA and TrkB) or low (p75) affinity. The high-affinity receptor isoforms TrkA and TrkB preferentially bind to NGF and BDNF, respectively [4-6]. Moreover, the uptake and internalization of neurotrophins are predominantly mediated by the high-affinity receptors, not the low-affinity ones [7, 8], and these processes seem sufficient for neurotrophins to elicit biological effects such as enhanced neuronal survival and neurite outgrowth [9-11].

Many studies have examined the effects of acute or chronic ethanol exposure on the responsiveness of various neurotrophic factor ligand/receptor systems in the CNS [12]. Surprisingly, no reports have addressed the effects of short-term exposure to alcohol on the brain. Moreover, we have previously demonstrated that short-term ethanol exposure for 3–5 days can disrupt neuron-astrocyte interactions in the hippocampus of mice [13]. More recently, we reported that long-term (19 weeks) ethanol exposure decreased the mRNA expression of oligodendrocyte-myelin glycoprotein (OMgp) but did not affect that of BDNF or the glial-derived neurotrophic factor (GDNF) in the rat hippocampus [14]. The results of our previous studies on the hippocampus [13, 14] raise the question of whether short-term ethanol exposure affects the mRNA expression of neurotrophins in the hippocampus, which is profoundly involved in learning and memory functions. These hippocamal functions are known to be disturbed by alcohol ingestion. In the present study, therefore, we evaluated the effects of short-term ethanol exposure on the hippocampus with respect to alterations in the neurotrophin ligand/receptor system by using real-time reverse transcription-polymerase chain reaction (RT-PCR).

Materials and Methods

Animals and ethanol administration

Adult (7–8 weeks old, male) BALB/C mice (SLC Japan, Shizuoka), weighing 22–25 g, were used in the present study. The animals were housed in separate cages in a room with strictly controlled temperature (21–23 °C) and humidity (50–65%) conditions. Lighting was programmed according to a regular light/dark (12 h/12 h) cycle. The ethanol exposure paradigm used in the present study was identical to the one we have previously described [13]. In brief, the mice were divided into two groups—the ethanol-fed group and the pair-fed control group. Both groups were allowed to acclimatize to the housing environment and were fed a normal liquid diet for 7 days prior to ethanol administration. Subsequently, the ethanol-fed group (n = 5) was provided unrestricted access to a liquid diet (Oriental Yeast, Tokyo, Japan) containing 5% (v/v) ethanol (99.5% ethyl alcohol; Wako, Osaka, Japan) as the sole fluid source. The pair-fed control group (n = 5) was maintained on an identical liquid diet, except that ethanol was isocalorically substituted with sucrose. The mice that exhibited stages 1-2 of ethanol intoxication signs, as per the classification proposed by Freund [15], were selected for use in subsequent experiments. Freund [15] classified intoxication into four stages characterized by hyperreactivity and tremor (stage 1); episodes of rapid tail beating, a slow broad-based gait, rapid backward movements (retropulsion), stereotype or repetitive movement (stage 2); generalized tonic convulsions (stage 3); and death during a convulsion (stage 4). This study was carried out in compliance with the guidelines for the experimental use and care of laboratory animals, issued by the European Communities Council Directive of November 24, 1986 (86/609/EEC), and was approved by the Kagawa University Animal Ethics Committee.

Real-time RT-PCR analysis

The mice showing ethanol intoxication signs of stages 1-2 were anesthetized with sodium pentobarbital (60 mg/kg, intraperitoneally) and subjected to intracardiac perfusion with medical-grade physiological saline. Blood was collected from the left ventricle prior to the perfusion, and its ethanol concentration was determined by gas chromatography (Shimadzu GC-8A, Tokyo, Japan). The brains were sectioned into 1-mm-thick slices in the horizontal plane by using a vibratome. The hippocampus was dissected from the brain in chilled physiological saline, with the aid of a dissection microscope. These specimens were processed for RT-PCR by using RNAlater (Ambion, Austin, USA).

Total RNA was isolated from the processed hippocampal slices by using TRIzol reagent (Invitrogen, Carlsbad, USA), according to the manufacturer's instructions. cDNA synthesis and quantitative detection were performed using a LightCycler rapid thermal cycler system (Roche Diagnostics, Lewes, UK) and the LightCycler-FastStart DNA Master SYBR Green I mix (Roche Diagnostics). The forward and reverse primers used in the present study are shown in Table 1. β-actin was used as an internal control. To confirm the specificity of the amplification, the PCR products obtained with each primer pair were subjected to melting-curve analysis and subsequent sequencing. To prevent genomic contamination, the PCR products that were amplified from cDNA by using each primer were electrophoresed on 2% agarose gel and stained with ethidium bromide. Similarly, the PCR products obtained without reverse transcription were electrophoresed and used as a negative control. The quantification data were analyzed using the LightCycler analysis

software. The mRNA expression of each gene was determined as the ratio of this expression to that of the housekeeping gene β-actin. Each sample was analyzed in duplicate to ensure consistent results.

Statistic analysis

Data are presented as the mean ± standard error of the mean (S.E.M.). All statistical analyses were carried out by Student's *t*-test, using the SigmaStat software (Systat, version 3.0).

Results

Blood ethanol concentration

The mean ± S.E.M. blood ethanol concentration in mice exhibiting signs of ethanol intoxication of stages 1-2 was 1.63 ± 0.06 mg/mL (n = 5).

Body and brain weights

The weights of the body and brain of the mice that were exposed to ethanol and of the pair-fed control mice are shown in Table 2. The body weight was significantly lower in the ethanol-treated mice than in the control animals. However, the brain weight did not significantly differ between the two groups.

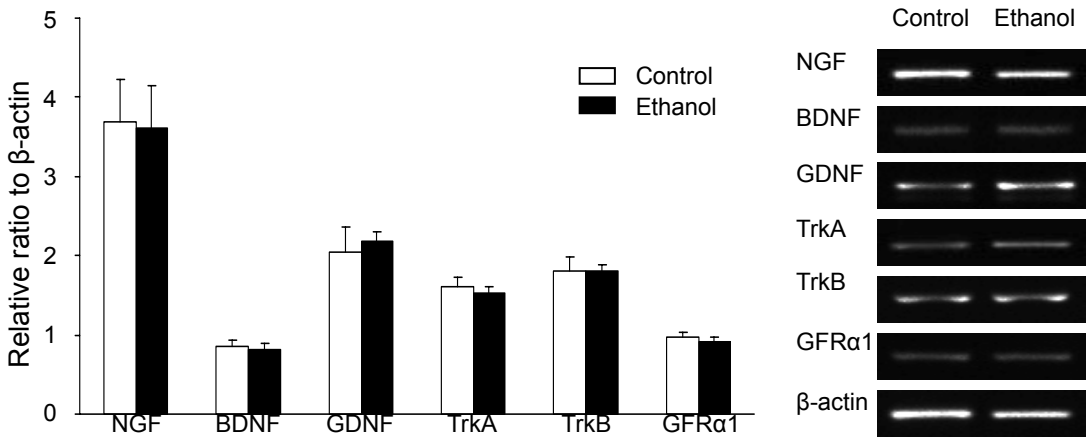

Figure 1: *Relative ratio of the mRNA expression of various neurotrophin ligands to that of their receptors in the hippocampus of the control and ethanol-treated mice. Representative results of agarose gel electrophoresis of the PCR products are shown; this electrophoresis was performed to verify the specificity of the amplification. Data are shown as the mean ± S.E.M. (n=5) for duplicate measurements.*

Table 1: Forward and reverse primers used for real-time RT-PCR

gene	primer sequence	PCR product (base pairs)	gene accession number	position
NGF	5'-CAGACCCGGAACATCACTGTA-3'	134	NM_013609	5': 628-605
	5'-CCATGGGCCTGGAAGTCTAG-3'			5': 494-513
BDNF	5'-GGTATCCAAAGGCCAACTGA-3'	182	NM_007540	5': 1180-1199
	5'-CTTATGAATCGCCAGCCAAT-3'			5': 1362-1343
GDNF	5'-CCCGAAGATTATCCTGACCA-3'	238	NM_010275.2	5': 512- 531
	5'- ACTGACTTGGGTTTGGGCTA-3'			5': 293-309
TrkA	5'-GAGGTCTCTGTCCAAGTCAGCG-3'	345	NM_85214.1	5': 890-911
	5'-GTCGTTAGTGTCCACTGGCGA-3'			5': 1293-1256
TrkB	5'-CGCCCTGTGAGCTGAACTCTG-3'	174	NM_008745	5': 2256-2232
	5'-CTGCTTCTCAGCTGCCTGACC-3'			5': 2082-2102
GFRα1	5'-TGTCTTTCTGATAATGATTACGGA-3'	167	NM_010279.2	5': 1358-1381
	5'-CTACGATGTTTCTGCCAATGATA-3'			5': 1525-1503
β-actin	5'-AGCCATGTACGTAGCCATCC-3'	250	NM_007393	5': 720-700
	5'-TTTGATGTCACGCACGATTT-3'			5': 470-489

Table 2: Mean ± S.E.M. body and brain weights of control and ethanol-fed mice

	n	Body weight (g)	Brain weight (g)
Control	5	24.70 ± 0.47	0.48 ± 0.01
Ethanol-fed	5	20.52 ± 0.13 *	0.49 ± 0.01

n, number of animals examined
** p<0.001 vs control group (Student's t-test)*

Real-time RT-PCR analysis

The mean ± S.E.M. ratio of the mRNA expression of various neurotrophin ligands and their receptors to that of β-actin, and representative results of agarose gel electrophoresis of these PCR products in the pair-fed control and ethanol-treated mice is shown in Figure 1. Quantitative real-time RT-PCR analysis revealed no significant difference between the two animal groups in terms of the expression of neurotrophin ligand/receptor systems.

As in our previous studies involving real-time RT-PCR [14, 16, 17], we used certain experimental procedures to verify whether specific amplification had been accomplished. Melting-curve analyses of the PCR products amplified from the target and β-actin genes revealed a single and sharp transition, confirming that a single PCR product was present; further, primer-dimer formation rarely occurred within the number of PCR cycles required for quantification. Additionally, agarose gel electrophoresis of the PCR products revealed a single band. When reverse transcription was omitted, no amplification products were formed.

Discussion

This study revealed that short-term ethanol exposure did not alter the expression of neurotrophin ligand/receptor systems, i.e., NGF/TrkA, BDNF/TrkB, and GDNF/ GFRα1, in the hippocampus of mice, even though the animals exhibited signs of ethanol intoxication. Recent studies indicate that neurotrophins are among the key factors profoundly associated with psychiatric conditions such as depression, schizophrenia, and bipolar disorder [18-22]. Indeed, injection of the epidermal growth factor in laboratory animals is reported to cause various behavioral abnormalities such as changes in motor activity and a deficit in sensorimotor gating; these abnormalities can be reversed by treatment with antipsychotic drugs, which normalize the neurotrophin levels in the CNS [23]. The postmortem brain of individuals who have encountered a stressful experience exhibits reduced neurotrophin levels; such reduced levels can have a negative effect on neuroplasticity through a reduction in the expression of BDNF [22]. Taken together, these findings indicate that neurotrophins are not only essential for the maintenance of normal neural functions and activities but are also involved in psychiatric and mental acquisition.

Accumulating evidence from many recent studies indicates that GDNF is profoundly involved in both ethanol-induced neuronal damage and the development of alcoholism [24-27]. Ethanol is known to activate an intracellular cell death-associated pathway involving c-jun N-terminal-kinase (JNK) and mitogen-activated protein (MAP) kinase, which are selectively associated with apoptosis. McAlhany et al. [27] reported that the exogenous administration of GDNF can attenuate both ethanol-induced apoptosis and the activation of the JNK cascade in cultured cells. The development of an alcohol addiction is strongly associated with the expression of GDNF in the ventral tegmental area (VTA) [24, 25]. Further, Carnicella et al. [24] described GDNF as a "fast-acting" selective agent that reduces an individual's craving for alcohol; their findings suggested that upregulation of the GDNF pathway may be a potent and valuable therapeutic strategy for combating alcohol addiction. On the basis of these reports, we speculate that ethanol consumption activates the effects of GDNF against ethanol neurotoxicity. In this regard, the present report is the first to examine whether the hippocampal GDNF/GFRα1 system is recruited even after short-term ethanol ingestion. However, in our present study, the mRNA expression of neither the GDNF ligand nor its receptor GFRα1 was significantly altered in the mouse hippocampus. GDNF is reported to protect neurons from ethanol-induced damage [26]. We hypothesized that ethanol exposure for even a short duration may, to some extent, affect the expression levels of GDNF and/or GFRα1 because glial cells are activated by various extrinsic insults. We do not know exactly why our results did not support our hypothesis. However, the following explanations are plausible: First, the responsiveness of the GDNF/GFRα1 system varies in the hippocampus and VTA. Second, the studies by Carnicella's group [24, 25] were performed on animals that were completely addicted to alcohol; the ethanol exposure paradigm in these previous studies was in sharp contrast to our paradigm involving ethanol exposure for 3–5 days. Further studies are required to determine the reasons for the discrepancy between our hypothesis and our results.

By using an ethanol exposure paradigm identical to the one used in the present study, we previously found that hippocampal neuron-astrocyte interactions are disrupted in mice with more severe intoxication (stages 2-3) [13]. On the basis of this finding, our primary hypothesis is that neurotrophin ligand/receptor systems in the hippocampus are likely to be disrupted in mice exhibiting signs of intoxication. Contrary to our hypothesis, the ethanol-treated mice did not exhibit any changes in the neurotrophin ligand/receptor expression in the hippocampus when compared with the control mice, although the animals exhibited ethanol intoxication signs (stages 1-2). We do not know the exact reasons for this finding but speculate that it may be attributable to the discrepancy between the protein and mRNA expression levels of neurotrophin ligand/receptors. In fact, McAlhany et al. [27] reported that ethanol inhibits the release of the GDNF protein from astrocytes but does not affect the mRNA expression of this factor. Similarly, ethanol exposure is reported to cause an imbalance between the GDNF mRNA and protein expressions [28-30]. Furthermore, increased mRNA expression in the CNS following ethanol exposure may be induced via enhancement of the transcript stability and/or synthesis or via a reduction in transcript degradation [28, 29]. Therefore, further studies involving tests such as western blotting should be conducted to evaluate the underlying mechanism at a protein level. Another plausible factor associated with ethanol exposure is the extent of intoxication. The discrepancy between the protein and mRNA expression of various neurotrophins is likely to depend on the extent of ethanol intoxication, and this raises the question of whether such a discrepancy can arise at more severe levels of ethanol intoxication. In the present study, we examined mice with relatively less severe ethanol intoxication (stages 1-2). However, if we were to examine animals exhibiting more severe signs of intoxication, e.g., those corresponding to stages 3-4, we may have obtained different results for each parameter considered in the present study. Further research would be required to address this issue.

To our knowledge, this is the first study to examine the effects of short-term ethanol exposure on neurotrophin expression. No previous report has described these effects, although numerous reports regarding the effects of acute or chronic ethanol exposure and developmental exposure are available. Therefore, we discuss here the effects of chronic ethanol exposure on neurotrophin expression in order to evaluate the responsiveness of various neurotrophins to ethanol neurotoxicity. In laboratory animals, prolonged ethanol exposure is known to affect the neurotrophin levels in various brain regions [1, 14, 31-34]. In fact, ethanol exposure for 6 months reduces the NGF level in the cerebral cortex, whereas exposure for a shorter duration does not affect the levels [31, 33]. Further, ethanol exposure for 6 months reduces the NGF level in the hippocampus [33]. The change in the BDNF level in response to ethanol exposure differs from that in the NGF level. We have previously reported that ethanol exposure for 19 weeks does not significantly alter BDNF mRNA expression in the hippocampus [14]. In contrast, Miller [32] reported that exposure for 24 weeks reduces BDNF expression in the hippocampus but increases that in the cerebral cortex. Taken together, these findings suggest that changes in the neurotrophin levels in response to ethanol exposure depend on the duration of ethanol ingestion or the brain regions examined.

In conclusion, this is the first report to directly examine neurotrophin ligand/receptor systems, namely, NGF/TrkA, BDNF/TrkB, and GDNF/GFRα1, in the mouse hippocampus after short-term ethanol exposure. We found that mice exhibit ethanol intoxication signs after short-term exposure, although no significant changes may be noted in the mRNA expression of various neurotrophin ligands and their receptors. The mRNA expression of both GDNF, which is known to be a first-acting agent against ethanol exposure, and its receptor GFRα1 remained unchanged after short-term ethanol exposure. The precise mechanisms underlying the neurotrophin response to ethanol neurotoxicity and the functional changes that may consequently arise in the CNS remain unknown.

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