Chronic Ethanol-Induced Glial Fibrillary Acidic Protein (GFAP) Immunoreactivity: An Immunocytochemical Observation in Various Regions of Adult Rat Brain

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CHRONIC ETHANOL-INDUCED GLIAL FIBRILLARY ACIDIC PROTEIN (GFAP) IMMUNOREACTIVITY: AN IMMUNOCYTOCHEMICAL OBSERVATION IN VARIOUS REGIONS OF ADULT RAT BRAIN

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In the present study, the effects of chronic ethanol (ETOH) treatment on the glial fibrillary acidic protein (GFAP) immunoreactivity was investigated in adult rat brains. ETOH were administered as increasing concentrations of 2.4%–7.2% (v/v) gradually for 21 days. Immunocytochemistry revealed that chronic-ETOH treatment increased synthesis of GFAP. The increase in the diameter and the number of GFAP (+) cells were statistically significant compared with the control group (p < .05). An increase of GFAP immunoreactivity was evident in various white matter and gray matter structures. We concluded that functional astrocytic cells responded to chronic ETOH exposure by increasing the synthesis of GFAP.

**Keywords** adult rat brain, chronic ethanol treatment, glial fibrillary acidic protein (GFAP)

**INTRODUCTION**

In the central nervous system (CNS), chronic ethanol (ETOH) consumption causes more neurological disorders than any other drug, toxin, or environmental agent. (Charness, Simon, & Greenberg, 1989). Neuronal death and gliosis have been reported in the brains of chronic human alcoholics (Harper, Kril, & Daly, 1987). It is hypothesized that chronic ETOH abuse causes the degeneration of myelin and axons (De la Monte, 1988). Metabolism of ETOH results in the production of the toxin acetaldehyde in liver (Vidal et al., 1998) and brain (Rintala et al., 2000), the accumulation of hydrogen peroxide (H2O2), and the propagation of free radicals (Bautista & Spitzer, 1992; Ishi, Kurso, & Kato, 1997). The neurodegenerative effects of ETOH are mediated by glutamate excitotoxic effects and changes in the calcium levels (Michaelis, 1990).

Studies have examined the effect of short-term ETOH with the exposure duration of 4–5 days (Satriotomo et al., 1999, 2000) and long-term effects or chronic ETOH exposure in specific areas (Franke, 1995; Madeira et al., 1997; Rintala et al., 2001) and in fetal tissue cultures of brain with exposure duration of 4–8 weeks (Davies & Ross, 1991, Renau-Piqueras et al., 1989). It is obvious that various investigators use short/long-term or acute/chronic ETOH exposure terminology. There is no clear-cut definition of short-term versus long-term duration. However, there are studies that show diversity effects between short/long-term ETOH exposure. After long-term ETOH exposure in male rats a dose-dependent decrease in glial fibrillary acidic protein (GFAP) immunoreactivity was found in the rat cerebellar cortical tissue (Rintala et al., 2001). ETOH consumption over a period of 4 weeks caused an increase in total GFAP immunoreactivity of the astrocytes; however, decrease of the total GFAP immunoreactivity was measured in the dorsal hippocampus after
36 weeks of ETOH treatment (Franke, Kittner, Berger, Wirkner, & Schramek, 1997). Therefore, it is important to note that time and dose response effect is important when determining the structural and functional changes.

Astrocytes are one of the important cells in the CNS which are involved in many functions including stimulation of neurite outgrowth, synaptogenesis, regulation of transmitters and growth factors, and provides nutrient support to neurons (Hansson & Rönback, 1996; Norenberg, 1994) including neurotransmitter uptake (Kimelberg & Katz, 1985), synthesis, and secretion of neurotrophic factors (Franke et al., 1997). GFAP is a specific marker for astrocytes (Hansson, Ronnback, Lowenthal, & Noppe, 1985; Trimmer, Reier, Oh, & Eng, 1982) and has been used to examine astrocyte proliferation and hypertrophy in response to various injuries (Katz, Iacovitti, & Reis, 1990; Kennedy & Mukerji, 1986). Effects of prolonged ETOH exposure on GFAP-containing cortical astrocytes has been studied in the primary cultures (Renau-Piqueras et al., 1989) showing that ETOH-exposed astrocytes failed to develop processes and also these cells showed less GFAP than astrocytes without ETOH. Increased GFAP immunoreactivity was shown in the suprachiasmatic nucleus of the hypothalamus (Satriotomo et al., 1999), and in the hippocampus (Satriotomo et al., 2000) of mice in short-term ETOH exposure. Although pathology affects both neuronal and glial cells, effects on glia are more dramatic than on neurons (Miguel-Hidalgo & Rajowska, 2003). Therefore, the present study was undertaken to investigate the effects of chronic ETOH on various adult brain regions using GFAP immunoreactivity.

**MATERIALS AND METHODS**

**Animals**

The experiments reported in this study have been carried out in accordance with the Declaration of Helsinki. Ethical approval was granted by the Kocaeli University ethics committee (Kocaeli, Turkey). Adult male Wistar rats (220–260 g at the beginning of experiments) were used. They were placed in a quiet and temperature- and humidity-controlled room (23 ± 1°C and 60 ± 5% respectively) in which 12–12hr light–dark cycle was maintained (0800–2000 hr light). Rats were individually housed in plexiglass cages.

**Administration of Ethanol**

The rats (n = 10) were presented the modified liquid diet (MLD), as previously described (Uzbek & Kayaalp, 1995), without ETOH for 5 days. Then ETOH,
2.4%, was added for 3 days. The ETOH was increased to 4.8% for 3 days. Finally, ETOH (7.2% v/v) was given to rats during 16 days. MLD was prepared and presented at fixed time daily (10:00 a.m.). The final composition of MLD with ETOH was as follows: low fat cow milk 925 ml, ETOH 75 ml (95.6%), vitamin A 5,000 IU, and sucrose 17 g. This mixture supplies 1000.7 kcal/L. Control rats \( (n = 10) \) were fed with an isocaloric MLD containing sucrose as a caloric substitute for ETOH. The weight of the rats was recorded daily and ETOH intake was measured and expressed as grams per kilogram per day. Blood alcohol concentrations (BAC) were determined by headspace gas chromatography method (Kumar & Gow, 1994). On the 21st day, before removing ETOH from diet, we took blood samples from three rats by intracardiac puncture under very weak ether anesthesia.

Daily ETOH consumption of the rats was in the range of 12.3–18.2 g/kg. BACs were 293.6 ± 5.2 mg/dl (mean ± standard deviation (SD)). The body weight of rats drinking ETOH in MLD was 3.8% less than the initial weight at the end of ETOH exposure of 21 days. The administration technique of ETOH as part of a total liquid diet is obviously preferable in the animal models for the development of ETOH tolerance and dependence (Erden et al., 1999).

**Immunohistochemistry**

Each rat was deeply anesthesized with ether and transcardially perfused with saline and then 4% paraformaldehyde in 0.1 M phosphate buffer. Fixed brains were removed and post-fixed overnight in the same fixative. The brains were embedded in paraffin. Serial brain sections (10 \( \mu \text{m} \)) were cut, deparaffinized, and rehydrated. Immunohistochemistry was performed using the avidin-biotin-peroxidase method. The rehydrated sections were pretreated with 3% H\(_2\)O\(_2\) for 10 min to eliminate endogenous peroxidase activity. Sections were then washed in PBS-Triton X 100 (Tx). In order to eliminate the nonspecific binding, sections were pretreated with normal rabbit serum. Sections were incubated in polyclonal rabbit antibody directed against GFAP (predelute, lot: 80642168, Zymed, San Francisco, CA, USA) for 24 hr at 4\( ^{\circ} \)C in a humidified chamber. Following washing in PBS-Tx, biotinylated anti-IgG secondary antibodies (100 \( \mu \text{L} \), Histostain plus kits, Zymed) were applied for 15 min at room temperature. Following a wash in PBS-Tx, streptavidin-peroxidase conjugate was applied to the sections for 15 min at room temperature. Excess conjugate was removed with a wash in Tris buffer, and the tissue was immunotreated with a solution of 0.6% H\(_2\)O\(_2\) and 0.02% dianminobenzidine (DAB) for 5 min at room temperature. Controls for the immunoreaction included omitting the
primary antibody and replacing it with nonimmune serum. Immunopositivity was examined by light microscopy (BX50F-3; Olympus, Tokyo, Japan).

Quantitative and Statistical Analysis

GFAP immunoreactive cells were quantified in 2.5 mm² fields of coronal brain sections in the immunoreactive areas of 10 rats of both groups with an X 40 objective using an ocular micrometer system (Olympus). Quantification and measurements of diameters of immunostained GFAP (+) cells in the distinct brain areas were done by three investigators through microscopic observation in a blinded fashion. Similar levels of brain sections were maintained between the ETOH-exposed and control brains according to the atlas of Paxinos and Watson (1994). Quantification and measurements of GFAP (+) astrocytes were presented as means and SD. As for statistical analysis, Mann-Whitney U test and Student’s t-test were used. \( p < .05 \) was interpreted as statistically significant.

RESULTS

Measurement of BAC

ETOH consumption and BACs were measured. Daily ETOH consumption by rats ranged from 12.3–18.2 g/kg. BAC were 293.6 ± 5.2 (mean ± SD).

GFAP Immunoreactivity

In the present study, GFAP immunoreactivity was observed in various brain regions (Figures 1–4) including corpus callosum (Figure 1), cerebral cortex (Figure 2), paraventricular nucleus (PVN) of the hypothalamus (Figure 3), medial basal hypothalamus (MBH), and internal layer of median eminence (Figure 4). In addition, GFAP immunoreactivity was observed in the internal capsule and globus pallidus, anterior comissure, and bed nucleus of stria terminalis, septal nucleus and caudate putamen surrounding the lateral ventricle, hippocampus, including CA1, CA2, CA3 subfields, and supraoptic tract (figures not shown). In chronic –ETOH-treated rats, there was a pronounced increase in the GFAP immunoreactivity (Figures 1–4(b)) compared with the controls (Figures 1–4(a)). Table 1 demonstrates the estimation of the number (mean ± SD) of GFAP immunoreactive astrocytes in various regions of the brain in ETOH-exposed and control rats. The statistics of the experimental and control groups revealed significant differences between two groups in the
examined areas of the brain. Significant increase in the number of GFAP (+) cells occurred in the examined brain regions of the ETOH-treated rats (Table 1). In the control group, the GFAP immunoreactive astrocytes were shown to be small and their processes were short. However, in the chronic ETOH-treated rats, GFAP immunoreactive astrocytes were observed to have hypertrophic bodies and long processes. The mean of astrocyte diameters was 4.28 ± 0.47 (mean ± SD) in the chronic –ETOH-treated group and 3.54 ± 0.70 in the control group (p < .05, 95% CI 0.62 to 0.83).

In astrocytes, GFAP immunoreactivity was confined to the cytoplasm surrounding the nucleus and in the processes as demonstrated in the cortex (Figure 2(b)). The immunocytochemical staining for GFAP was faint in the control group (Figures 1(a)–4(a)); however, in the sections of ETOH-treated rats the immunostaining was robust and fibrillary processes and their soma was
Figure 2. Photomicrographs of GFAP immunoreactivity in the control (a) and ETOH-treated (b) groups of the cerebral cortex (CX). Note the significant increase in density of GFAP (+) astrocytes in ETOH-treated (b) compared with the control (a) rats. Scale bar: 50 µm.

densely labeled (Figures 1(b)–4(b); see Figure 2(b) for magnified view). The hypertrophic astrocytes having relatively thick soma and processes were best visualized in the internal layer of the hypothalamic median eminence (Figure 4(b)) and in the cerebral cortex (Figure 2(b)) of the ETOH-treated rats.

DISCUSSION

The nervous system is one of the main targets of ETOH toxicity and it has been suggested that astrocytes may be important in their integrity, which is essential for the normal growth and functioning of neurons. Glial cells and their interactions with neurons play vital roles during the ontogeny of the nervous system and in the adult brain. ETOH-induced brain damage is a
significant problem that leads to permanent changes in the brain function, although abstinence does allow partial recovery of lost function (Crews, 1999).

The present study demonstrates that chronic ETOH exposure to the brain regions promotes GFAP synthesis in the astrocytic cells. The increase in GFAP demonstrated in the present study is correlated with earlier reports on animals treated with ETOH (Fletcher & Shain, 1993; Franke, 1995; Franke et al., 1997; Gonca et al., 2005; Goodlett, Leo, O’Callaghan, Mahoney, & West, 1993; Madeira et al., 1997; Miguel-Hidalgo, 2005, 2006; Renau-Piqueras et al., 1989; Satriotomo et al., 1999, 2000). However, the studies were slightly different, as some were performed on the short-term ETOH-treated mice (Satriotomo et al., 1999, 2000), some in developing rats (Fletcher & Shain, 1993; Goodlett et al., 1993; Miller & Potempa, 1990), and others in long-term ETOH-treated primary culture (Kane, Berry, Boop, & Davies, 1996; Renau-Piqueras et al.,

Figure 3. Photomicrographs of GFAP immunoreactivity in the control (a) and ETOH-treated (b) groups of the paraventricular nucleus (PVN) and third ventricle (3V). Note the significant increase in density of GFAP (+) astrocytes in ETOH-treated (b) compared with the control (a) rats. Scale bar: 25 µm.
Figure 4. Photomicrographs of GFAP immunoreactivity in the control (a) and ETOH-treated (b) groups of the medial basal hypothalamus (MBH), internal layer (il), and external layer (el) of median eminence (me). Note the significant increase in density of GFAP (+) astrocytes in ETOH-treated (b) compared with the control (a) rats. Scale bar: 25 µm.

1989). Long-term ETOH treatment studies were done with different treatment duration (Miguel-Hidalgo, 2005) and demonstrated only in specific areas such as hippocampus, suprachiasmatic nucleus, and prelimbic cortex (Franke, 1995; Franke et al., 1997; Madeira et al., 1997; Miguel-Hidalgo, 2005; Philips & Cragg, 1983). The duration of the ETOH exposure can significantly effect the GFAP expression. ETOH consumption over a period of 4 weeks caused an increase in the total GFAP immunoreactivity of the astrocytes; however, after long-term (3–36 weeks) ETOH treatment, downregulation of the total GFAP immunoreactive cells was observed in the dorsal hippocampus and prelimbic cortex (Franke et al., 1997; Miguel-Hidalgo, 2005). The present study clearly shows for the first time the presence of increased statement of GFAP immunoreactivity in new areas, such as corpus callosum, anterior commissure,
### Table 1. Number of GFAP immunoreactive astrocytes in various regions of the brain following chronic ETOH treatment. GFAP immunoreactive cells were counted in 2.5 mm² fields of coronal brain sections in 10 animals

<table>
<thead>
<tr>
<th>Brain fields</th>
<th>Control group (n = 10)</th>
<th>Experimental group (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Mean ± SD*</td>
</tr>
<tr>
<td>Corpus callosum</td>
<td>50.2 ± 7.9</td>
<td>782.4 ± 116.2</td>
</tr>
<tr>
<td>Internal capsul</td>
<td>188 ± 67.1</td>
<td>428.9 ± 66.2</td>
</tr>
<tr>
<td>Globus pallidus</td>
<td>376 ± 134.2</td>
<td>857.8 ± 132.5</td>
</tr>
<tr>
<td>Anterior commissure</td>
<td>10.9 ± 2.2</td>
<td>38.8 ± 14.0</td>
</tr>
<tr>
<td>Bed nucleus of stria terminalis</td>
<td>21.8 ± 4.4</td>
<td>120.0 ± 42</td>
</tr>
<tr>
<td>Septal nucleus and caudate</td>
<td>56.9 ± 9.9</td>
<td>602.3 ± 145.7</td>
</tr>
<tr>
<td>putamen surrounding the lateral ventricle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortex</td>
<td>182.0 ± 26.0</td>
<td>1207.2 ± 164.0</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>103.3 ± 21.7</td>
<td>770.4 ± 109.1</td>
</tr>
<tr>
<td>Optic tract</td>
<td>52.5 ± 15.3</td>
<td>180.6 ± 34.8</td>
</tr>
<tr>
<td>Paraventricular nucleus</td>
<td>16.5 ± 4.4</td>
<td>271.5 ± 106.4</td>
</tr>
<tr>
<td>Medial basal hypothalamus</td>
<td>99.1 ± 17.4</td>
<td>376.4 ± 72.6</td>
</tr>
<tr>
<td>Internal layer of median eminence</td>
<td>5.5 ± 1.4</td>
<td>18.6 ± 5.5</td>
</tr>
</tbody>
</table>

*Significantly different from control value, p < .001, n: number of animals.

internal capsule, globus pallidus, bed nucleus of stria terminalis, part of septal nucleus and caudate putamen surrounding the lateral ventricle, supraoptic tract, PVN, MBH, and internal layer of median eminence in the chronic –ETOH-treated adult rats. The above-mentioned areas may be important in contributing the effects of alcohol dependence.

It was demonstrated that chronic ETOH exposure as seen for chronic morphine and cocaine treatments, increased levels of tyrosine hydroxylase and GFAP immunoreactivity in ventral tegmental area. However, similar results were not seen in the substantia nigra or caudate putamen, components of the nigrostriatal dopamine system (Ortiz et al., 1995). Therefore, it can be noted that astrocyte in the white and in certain areas of the gray matter of the brain tissue has been affected by the ETOH neurotoxicity. From the present results it can be viewed that the BAC presented, in effect created this neurotoxicity and produced or elicited the astroglial reactions or astrogliosis. Although the previous studies have demonstrated the effects of ETOH (Altura & Altura, 1999; Philips & Cragg, 1983), the information about molecular mechanisms underlying the cytotoxicity is still lacking. Recent data indicate that ETOH-induced cellular loss of ionized magnesium is associated with cellular calcium overload and generation of oxygen-derived free radicals, which may be the
cause of ETOH-induced pathology of the brain (Altura & Altura, 1999). In addition, acetaldehyde is considered as a possible mediator of ETOH-induced neurotoxicity in vivo through formation of adducts with brain proteins and macromolecules. Acetaldehyde, by forming protein adducts with brain proteins, could contribute to the pathogenesis of alcoholic brain damage (Rintala et al., 2000; Rintala, 2002).

It has been shown that astrocytes have important roles in the CNS function including synthesis and/or secretion of neurotrophic factors (Furukawa, Furukawa, Satoyoshi, & Hayashi, 1986, 1987). It is not known whether there is a proliferation of astrocytes or just an increase in the intracytoplasmic synthesis of GFAP. This increase of the astrocytic GFAP may imply the response to the brain injury created by the toxic effect of the ETOH and therefore promoting the healing processes of the neurons (Mathewson & Berry, 1985).

After various brain injuries, astrocytes undergo changes that have characteristics of hypertrophy and hyperplasia (Duchen, 1984). In addition, ETOH exposure showed hypertrophy and increase in cell numbers of GFAP-producing astrocytes in the hippocampus (Satriotomo et al., 2000) and in the suprachiasmatic nucleus of the hypothalamus (Satriotomo et al., 1999) in the adult mice. Astrocytes, which were exposed to ETOH in the present study, showed hypertrophy in their cell bodies and expressed long and thick processes. The mean of astrocyt diameters was $4.28 \pm 0.47$ (mean ± SD) in the chronic –ETOH-treated group. Therefore a significant increase in the diameters of astrocytes was determined in the ETOH-treated animals compared to that of control group ($p < .01$). These cells clearly fit the criteria of reactive astrocytes (Norton, Aquino, Hozumi, Chiu, & Brosnan, 1992). The specific cellular and molecular mechanisms initiating the occurrence of astrogliosis are currently not known. Many in vitro studies have shown that long-term treated ETOH-induced impairment of growth or differentiation of astrocytes (Kennedy & Mukerji, 1986). Increased GFAP immunoreactivity in the glial cells surrounding the ventricles, particularly the lateral ventricle, suggests that ETOH may be associated with the damage of blood vessels in the choroid plexus. It has been demonstrated that high BACs can induce cerebrovascular rupture (Altura & Altura, 1984).

In the present study, GFAP immunoreactivity was also found to have increased in the corpus callosum, internal capsule, anterior commissure, and supraoptic tract. The increase in GFAP expression statement in the white matter may indicate a disruption or damage of myelin induced by ETOH exposure. These findings are in parallel with other studies performed in humans (De la Monte, 1988; Harper, Smith, & Kril, 1990; Raine, 1983). In addition, it
has been demonstrated in mice brains that chronic ETOH exposure alters the incorporation of unsaturated fatty acids into phospholipids of the axonal membrane, thus impairing cellular functions (Zhen, Barkai, & Hungund, 1997). The data presented in the present study also show that the astrocytes in the gray matter surrounding the neurons showed increase in the density in the ETOH-treated group. These astrocytes may take part of some neural activities occurring around them. It has been demonstrated that astrocytes interact with neurons and plays a crucial role in the synaptic activity (McCall et al., 1996).

A distinct increase in the GFAP positive cells was recognized in the hypothalamic PVN and internal layer of the median eminence. The combination of cyanamide (a potent inhibitor of aldehyde dehydrogenase) and low-dose ETOH resulted in a significant and maximal increase in c-fos mRNA in the PVN (Kinoshita et al., 2002). This could very well explain the direct neurotoxic effect of ETOH on hypothalamo–hypophysial axis neurons. Hypothalamus, including PVN and pituitary opioid peptides, is essential in addiction and in reward processes, which are affected by ETOH ingestion (Van Ree, 1986).

Increase in the number of GFAP immunoreactive astrocytes in hippocampal subfields in the present study is parallel with other studies (Franke, 1995; Satriotomo et al., 2000). In addition, colocalization of taurine and GFAP immunoreactive cells in rat hippocampus was increased by short-term ETOH exposure. This finding suggests that the expression of taurine immunoreactivity in reactive astrocytes after ETOH exposure may play an important role in neuroprotective process (Sakurai et al., 2003). It is stated that astrocytes are morphologically and functionally related to neurons, and astrocyte–neuron interactions provide strategic sites for the actions of many chemical compounds (Tagliaferro, Vega, Evrard, Ramos, & Brusco, 2002). In their study, the morphological alterations of glial cells and neurons in the hippocampus after long-term ETOH exposure and GFAP immunoreactivity were detected. After ETOH exposure, we observed an important astroglial reaction evidenced by the presence of GFAP (+) reactive astrocytes in the CA1 area of the hippocampus. The current study provides evidence that long-term ETOH exposure induces alterations in the neuronal cytoskeleton and astroglial reaction, which is a common response to brain injury and may promote functional recovery of the nervous system by the release of glial-derived trophic factors that promote cell survival and neurite growth.

In conclusion, the data presented in the present study have demonstrated that chronic ETOH exposure increased the synthesis of GFAP in astrocytes. These findings provide valuable evidence for the interactions between ETOH and astrocytes. Furthermore, the present findings suggest that astrocytes are...
target of ETOH toxicity and may inherent the neuropathological abnormalities observed after ETOH exposure.

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