Effects of alcohol administration during adulthood on parvalbumin and glial fibrillary acidic protein immunoreactivity in the rat cerebral cortex

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Abstract

The pathology of brain atrophy mediated by alcohol was investigated in all parts of the cerebral cortex (the frontal, parietal, temporal lobes and occipital cortex) by using two markers: parvalbumin (PV) and glial fibrillary acidic protein (GFAP). Three-month old male Wistar rats were divided into control (C) and alcohol-exposed groups. The control group received distilled water, whereas the alcohol-exposed groups received either a low dose (2 g/kg body wt) or a high dose (5 g/kg) of ethanol for periods of 21 days, 3 or 6 months. The brains of the animals were processed for immunohistochemistry using anti-parvalbumin and anti-GFAP antibodies and the number of PV immunoreactive (PV-ir) neurons and GFAP immunoreactive (GFAP-ir) astrocytes were counted per unit area. Results showed that all groups exposed to ethanol had significantly reduced numbers of PV-ir neurons in all parts of the cerebral cortex compared to those of the control group (p < 0.05). In contrast, the numbers of GFAP-ir astrocytes were increased in all parts of the cerebral cortex following the exposure to a high dose of ethanol after 21-days (but not a low dose) and both high and low doses of ethanol after 3-months or 6-months treatment compared to those of age-matched control groups (p < 0.05). This indicated that in young rats (21-days), PV-ir neurons in all cerebral cortex areas seemed to be more sensitive to alcohol than GFAP-ir astrocytes. Moreover, the change in densities of both PV-ir neurons and GFAP-ir astrocytes became more apparent after exposure to prolonged and high doses of ethanol. The decrease of PV-ir neurons and the increase of GFAP-ir astrocytes indicated that alcohol may induce pathology in broad areas of the cerebral cortex. This may explain the underlying mechanism of brain atrophy and other impairments found in alcoholics. For investigations of the effects of alcohol on mediating brain pathology, we recommend the use of the two markers (PV and GFAP).

Keywords:
Brain atrophy
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Rat

Introduction

Chronic alcohol abuse has been shown to induce brain atrophy. The degree of brain atrophy is associated with the rate and amount of alcohol consumed over a lifetime (Harding et al., 1996). Autopsy studies have demonstrated that patients with a history of chronic alcohol consumption have smaller, lighter, more shrunken brains than non-alcoholic adults of the same age and gender (Rosenbloom et al., 1995). Both post-mortem and in vivo imaging studies of brain morphology have shown abnormal reduced brain volumes of gray and white matter across multiple regions (Kril et al., 1997). The cerebral cortex is one of the important targets shown to be damaged in chronic alcoholics (Kril et al., 1997).

There are many reports on the signs and symptoms of brain damage in alcohol users such as: impairment of cognition (memory and thinking), difficulty in walking, blurred vision, slurred speech, slowed reaction times, impaired judgment, blunted effect, poor insight, social withdrawal, reduced motivation, distractibility and deficits in attention and impulse control (Parsons, 1987, 1996; Oscar-Berman and Hunter, 1993; Sullivan et al., 2000). All these are indications of the toxic effects of alcohol on many areas of the brain, especially the cerebral cortex.

Studies using positron emission tomography (PET) and single photon emission computed tomography (SPECT) have revealed decreased blood flow and metabolic rates in certain brain regions of heavy drinkers compared with those of non-alcoholics (Volkow et al., 1992; Wang et al., 1993). These may reflect a decrease in the number or size of neurons or a reduction in the density of communication sites between adjacent neurons. According to the Kril et al. (1997), the superior frontal cortex of the brains from...
alcoholics shows a significant neuronal loss. However, there was no information on the effects of alcohol on other parts of the cerebral cortex and no information on the other types of cell in the brain such as glial cells. In addition, the time of onset of neuronal loss in alcoholic brains remains unknown. Therefore, this study aimed to investigate the effects of alcohol administration on one type of neurons and glial cells in three different ages of rats.

Parvalbumin (PV) is a calcium-binding protein of the EF-hand family (Heizmann, 1984). This protein is widely distributed in the cerebral cortex (Celio, 1986) and is found in the non-pyramidal GABAergic neurons (Celio, 1986; Bergmann et al., 1991). GABAergic neurons play an important role in inhibitory neurotransmission (Celio, 1986; Bergmann et al., 1991), thus, alterations to the PV could severely compromise inhibitory neurotransmission. PV has been suggested to protect neurons from hyperexcitability by modulating the activity of calcium-dependent K+ channels and by sequestering intracellular calcium. Alcohol has been shown to decrease the number of PV immunoreactive (PV-ir) neurons in the adult hippocampus and cingulate cortex of rats (Vongvatcharanon et al., 2009a), and the cingulate cortex of prenatal (Moore et al., 1998) and neonatal rats (Mitchell et al., 2000). Therefore, PV has been used in this study as a neuronal marker.

Astrocytes are another type of brain glial cells used as a marker to investigate brain damage (Miguel-Hidalgo et al., 2002; Gonzalez et al., 2006; Vongvatcharanon et al., 2009a). This is because astrocytes have a supportive role in brain functions including neurotransmitter uptake (Danbolt, 2001) and the synthesis and secretion of neurotrophic factors (Müller et al., 1994). An increase of glial fibrillary acidic protein (GFAP), the glial-specific cell marker (Eng et al., 1992; Norton et al., 1992), has been found after brain injury. In addition, alcohol was shown to induce an increase of GFAP immunoreactive (GFAP-ir) astrocytes (Eng et al., 1992). Thus, GFAP-ir has been used in this study as a marker for astrocytes.

There is evidence that the degree of brain damage is correlated with the duration and amount of alcohol consumed over a lifetime (Harding et al., 1996). Therefore, this study aimed to investigate the effects of dose and duration of alcohol administration on the PV-ir neurons and GFAP-ir astrocytes in all parts of the cerebral cortex (the frontal, parietal, temporal and occipital cortex) of adult rat brains. The findings from this study may help explain the pathology of cerebral cortex atrophy and how it can be related to the observed impairments in alcoholic patients.

Materials and methods

Three month old male Wistar rats (weighing 200–250 g) were obtained from the Southern Laboratory Animal Facility, Prince of Songkla University, Thailand. The rats were maintained at 22 °C with a 12/12 dark/light cycle (light on at 06:00 am). Standard commercial food pellets and filtered tap water were available ad libitum. The experimental protocols described in this study were approved and guided by the Animal Ethics Committee of the Prince of Songkla University. The ethanol exposure protocol used in this study is the same as previously described (Vongvatcharanon et al., 2009a, b). Briefly, the rats were randomly divided into 2 groups: control (C) and alcohol groups. The control group was fed orally using a ball-tipped gavage with distilled water and the alcohol group was divided into 6 further groups and fed orally with either a low dose (LD, 2 g/kg, 20% w/v) or a high dose of ethanol (HD, 5 g/kg, 40% w/v) once daily for 21 days (21d, subacute) or 3 months (3 m, subchronic) or 6 months (6 m, chronic) (10 rats in each treatment group). The mean blood ethanol concentration during exposure to the low dose of ethanol was 32 ± 17.81 mg/dl and during exposure to the high dose of ethanol was 148 ± 34.65 mg/dl (Vongvatcharanon et al., 2009a, b).

At the end of the experimental treatment, rats were deeply anesthetized by intraperitoneal injection of 75 mg/kg pentobarbital sodium (Sigma-Aldrich, St. Louis, MO). The rats were perfused transcardially with 4% paraformaldehyde in phosphate buffer (PBS), pH 7.25. The brains were removed and post-fixed with 4% paraformaldehyde in PBS overnight at room temperature. Subsequently, the brains were cryoprotected by equilibration with 30% sucrose in PBS until they sank and were rapidly frozen. Serial frozen sections 40 µm-thick were cut coronally using a cryostat (Leica CM 1850, Leica Microsystems, Nussloch, Germany) and processed for immunohistochemistry.

Immunohistochemistry

The immunohistochemical method has been described previously (Vongvatcharanon et al., 2009a). Briefly, the sections were incubated in the following solutions and between each of the steps the sections were rinsed with PBS: (1) 10% normal horse serum (Vector Labs, Burlingame, CA) in PBS with 0.2% Triton-X 100 (J.T Baker Inc., Phillipsburg, NJ) for 30 min; (2) anti-parvalbumin mouse antibody (1:200 dilution, Sigma-Aldrich, St. Louis, MO) overnight at 4 °C; (3) Texas Red anti-mouse IgG (1:200 dilution, Vector Labs, Burlingame, CA) for 1 h at room temperature. Following all histochemical procedures, the sections received a final rinse with PBS, mounted with Vectashield (Vector Labs, Burlingame, CA), coverslipped and sealed with nail polish.

The adjacent series of sections from each control and alcohol-treated animal were immunostained in parallel using the anti-GFAP mouse antibody (1:200 dilution, Chemicon, Temecula, CA) and the same immunohistochemical protocol as described above.

Control sections, omitting the primary antibody, were routinely processed to ensure that any observed staining was due to parvalbumin or GFAP. These controls did not show any labeling. The morphology of PV-ir neurons and GFAP-ir astrocytes were studied with a BX 50 fluorescence microscope (Olympus, Tokyo, Japan).

Numbers of PV-ir neurons and GFAP-ir astrocytes

Each area of cerebral cortex (frontal, parietal, temporal lobes and occipital cortex) was identified using a rat brain atlas (Paxinos and Watson, 2007). Quantification of the number of PV-ir neurons and GFAP-ir astrocytes in each part of the cerebral cortex was achieved using previously described methods (Vongvatcharanon et al., 2009a). Briefly, every 15th section was selected and coded such that all subsequent analyses were carried out blind. About 10 sections from each animal of each study (counting of PV-ir neurons and GFAP-ir astrocytes) were used in this study. Micrographs from selected sections were captured by a digital camera (DP50, Olympus, Japan). The numbers of PV-ir neurons and GFAP-ir astrocytes in each micrograph were counted and the area of each section examined was measured using image analysis software (Olympus, Japan). The results were expressed as numbers of PV-ir neurons and GFAP-ir astrocytes per mm².

Results are expressed as a mean ± standard error of mean. The statistical evaluation of the data was performed using One-way ANOVA and the least significant difference test for post-hoc analyses to determine the significance between means. Differences among means were considered significant when p < 0.05.
Results

Parvalbumin immunoreactive (PV-ir) neurons and GFAP-ir astrocytes in the cerebral cortex

The frontal, parietal, temporal and occipital cortex regions were identified in the coronal sections of rat brain (Fig. 1A–D). The distribution of PV-ir neurons and GFAP-ir astrocytes was determined in the frontal cortex (Fig. 2A and B), parietal cortex, temporal cortex and occipital cortex (data not shown). A qualitative reduction of PV-ir neurons was observed in all areas of the cerebral cortex of the animals exposed to both the low and high dosage of ethanol for 6-months when compared with the controls (Fig. 3A–C). In contrast, a qualitative increase of GFAP-ir astrocytes was identified in these same brain areas of the animals exposed to both the low and high doses of ethanol after 6-months when compared with the controls (Fig. 4A–C).

Numbers of parvalbumin immunoreactive (PV-ir) neurons in the cerebral cortex

The effects of alcohol administered for 21 days, 3 months and 6 months on the numbers of PV-ir neurons in various cortical brain areas were investigated. In the frontal cortex, all three periods of alcohol treatment showed significant decreases in the numbers of PV-ir neurons induced by both low and high ethanol doses. When considering the number of PV-ir neurons in the control rats treated for 21 days as a percentage of the control, it was found that the numbers of PV-ir neurons decreased with age from 100 to approximately 90% and 46% in the 3 and 6 month groups, respectively, as shown in Table 1. In this area, the percentage change was more profound in older rats treated with either low or high doses. The most extreme decrease was seen in the high dose group treated for 6 months. Similar patterns of change were also observed in the cortical areas of the parietal, temporal and occipital cortex.

Fig. 1. (A) Photomicrograph of a coronal section showing the frontal cortex (Fr) and the cingulate cortex (Cg). Bar=40 μm. (B) Photomicrograph of a coronal section showing the frontal cortex (Fr), the retrosplenial agranular cortex (RSA), the retrosplenial granular cortex (RSG) and the hippocampus (Hip). Bar=40 μm. (C) Photomicrograph of a coronal section showing the temporal cortex (Tel), the parietal cortex (Par) and the hippocampus (Hip). Bar=40 μm. (D) Photomicrograph of a coronal section showing the occipital cortex (Oc), the retrosplenial agranular cortex (RSA) and the hippocampus (Hip). Bar=40 μm.
occipital regions (Table 1). These findings indicate that the number of PV-ir neurons decreased with age and in a dose-dependent manner of alcohol treatment.

**Numbers of GFAP-ir astrocytes in the cerebral cortex**

The effects of alcohol were also detected in an alteration of the numbers of GFAP-ir astrocytes in the cerebral cortices of these three age groups. In the frontal cortex of rats treated for 21 days, the alcohol treatment increased the numbers of GFAP-ir astrocytes in a dose-dependent manner to 114.69% and 161.70% of the control in low dose and high dose groups, respectively. After prolonged durations (3 and 6 months), the increase was progressive with a higher magnitude especially in the 6-month group treated with high dose alcohol. This group had approximately twice as many GFAP-ir astrocytes compared to the control group (Table 2). Similar increases were detected in the parietal, temporal and occipital cortices in comparison to the control after 21 days of treatment (Table 2). In general, there was an even increase in GFAP-ir astrocytes in all cortex areas after alcohol treatment.

**Discussion**

Our data have shown that in all groups exposed to a low or high dose of ethanol for 21 days, 3 months and 6 months, the number of PV-ir neurons was reduced in all parts of the cerebral cortex (the frontal, parietal, temporal and occipital cortex). This indicated that even after exposure to a low dose of ethanol for only 21 days, there was a significant reduction of PV-ir neurons in all parts of the cerebral cortex. These data are consistent with our previous studies that showed a significant reduction of PV-ir neurons in the hippocampus and cingulate cortex of rats using the same dose and duration (Vongvattharanon et al., 2009a). Thus, drinking alcohol for only a short period does induce neuronal...
damage. These data may reflect the adverse effects of alcohol in humans. In addition, ethanol has been shown to induce a decrease of PV-ir neurons in the cingulate cortex of the prenatal and neonatal rat brain (Moore et al., 1998; Mitchell et al., 2000). This indicates that ethanol exposure, not only destroys PV-ir neurons in prenatal and neonatal rats, but also in the adult. When compared to the control rats, after 21 days treatment, the numbers of PV-ir neurons were progressively reduced to about 90% and 45% in the 3 and 6 month groups, respectively. It means that neurons decrease in number by natural processes. These data confirm the age-related loss of neurons in the cortex of male and female rats (Yates et al., 2008). In addition, the loss might be aggravated by other factors including long-term toxicity.

The decrease of PV-ir neurons is due to the toxicity of alcohol that mediates neuronal death or reduces the PV levels in neurons. According to Goodlett et al. (2005) alcohol-induced neuronal death works via oxidative stress or activation of caspase-3, however, a decrease of PV was also found in adult animals following prenatal ethanol exposure (Mitchell et al., 2000). The decrease of PV has been suggested to induce neuronal loss. This is due to PV functioning as a buffer for excess calcium at presynaptic nerve terminals after a rapid series of action potentials (Heizmann, 1984) and excessively high levels of intracellular Ca^{2+} that are known to induce cell death. Thus, it is possible that an ethanol-mediated decrease of PV level reduces the ability of neurons to buffer Ca^{2+} resulting in the initiation of cell death (Moore et al., 1998). PV is an important component of GABAergic neurons that play a crucial role in inhibitory neurotransmission (Celio, 1986; Bergmann et al., 1991). Therefore, a reduction of PV may interfere with inhibitory neurotransmission in the cerebral cortex. The reduction of PV-ir neurons could represent one type of neuronal death. Whether or not this change could lead to permanent damage depends on many factors such as exposure to lead (Sharifi et al., 2002) or mercury (Sorensen et al., 2000). The level of brain damage may be estimated from the structural changes of the brain. In addition, the change in the number of glial cells may indicate the severity of the brain damage. Thus, it was necessary to confirm whether the damage is reversible by the detection of glial cells.

Our data have also demonstrated the effects of the same dose and duration of exposure to ethanol on GFAP-ir astrocytes in all parts of the cerebral cortex (the frontal, parietal, temporal and occipital cortex). In the groups exposed to a low dose of ethanol for 21 days, no alterations of GFAP-ir astrocytes were detected in all parts of the cerebral cortex, however, after a high dose of ethanol for 21 days and in both groups exposed to ethanol for 3 months, an increase of GFAP-ir astrocytes was observed. In addition, a high dose of ethanol exposure seemed to cause an increase of GFAP-ir astrocytes greater than that seen after exposure to a low dose of ethanol. Furthermore, both groups exposed to ethanol for 6 months, showed an increase of GFAP-ir astrocytes in all parts of the cerebral cortex. This indicated that a progressive increase of GFAP-ir astrocytes was more obvious after prolonged and high dose of exposure to ethanol. An ethanol-induced increase of GFAP-ir astrocytes was also observed in the cingulate cortex and hippocampus of adult rat brain in our previous study using the same dose and duration of ethanol exposure (Vongvatcharanon et al., 2009a). It has been suggested that astrocytes play a crucial role in controlling glutamatergic activity (Hertz and Zielke, 2004) as astrocytes synthesize glutamate (Shank et al., 1985, Yu et al., 1983) and take up most of the synaptically released glutamate that terminates transmitter activity and returns glutamate to neurons via the glutamate-glutamine cycle (Danbolt, 2001). Thus, an increase of GFAP-ir astrocytes may interfere with normal excitatory neurotransmission in the cerebral cortex.

By using the two markers (for PV-ir neurons and GFAP-ir astrocytes), our study has demonstrated that ethanol exposure induced a reduction of PV-ir neurons and an increase of GFAP-ir astrocytes in all parts of the cerebral cortex, and identifies the pathology of the cerebral cortex, especially after prolonged and high dose exposure to ethanol. In young rats after 21 days exposure, PV-ir neurons in all cerebral cortex areas seemed to be more sensitive to alcohol than the GFAP-ir astrocytes. This is because either low or high doses of alcohol reduced the number of

![Fig. 4. Representative photomicrographs showing GFAP-ir astrocytes (arrows) in sections from the frontal cortex of a control animal (A) and from a group exposed to the low dose (B) and the high dose (C) of ethanol for 6-months. Bar=400 μm.](image-url)
PV-ir neurons, but only the high dose increased the GFAP-ir astrocytes. The reduced number of PV-ir neurons may lead to an interference of the inhibitory neurotransmission in the cerebral cortex. In contrast, the increase of GFAP-ir astrocytes may result in interference to excitatory neurotransmission in the cerebral cortex. This indicates that ethanol exposure may alter both inhibitory and excitatory neurotransmission in the cerebral cortex.

It has been proposed that ethanol binds to postsynaptic gamma-aminobutyric acid (GABA) receptors to facilitate its action leading to enhanced inhibition and ultimately generating sedation. In addition, ethanol has been demonstrated to inhibit excitatory neurons by decreasing the activity of the N-methyl-D-aspartate (NMDA, glutamate subtype) receptors (McIntosh and Chick, 2004). However, long-term exposure to ethanol has been shown to result in down-regulation of the GABA receptors (Bayard et al., 2004). Our study has demonstrated that long-term exposure to ethanol induced a decrease of PV-ir neurons, which are GABAergic neurons. Therefore, we propose that a decrease of PV-ir neurons is one of the factors that induces down-regulation of the GABA receptors after long-term ethanol use. In addition, long-term exposure to ethanol has been shown to result in up-regulation of GFAP receptors (Bayard et al., 2004). The up-regulation of NMDA receptors may be due to an increase in the numbers of receptors or NMDA receptor-containing neurons. In contrast, our study has demonstrated that long-term exposure to ethanol induced an increase of GFAP-ir astrocytes. The increase of astrocytes may result in an increase of the glutamate level and accounting for the up-regulation of the NMDA receptors. The up-regulation of NMDA receptors has been suggested to be an important cause of tolerance, an adaptive change, so that increasing amounts of alcohol is required to produce an effect. However, we also suggest that, owing to the decrease of PV-ir neurons i.e. the GABAergic neurons, a higher dose of alcohol is needed to produce enhanced inhibition. In addition, due to the increase of GFAP-ir astrocytes, a higher dose of alcohol is required to diminish the excitatory action of glutamate.

Our study has demonstrated alcohol-induced pathology in all parts of the cerebral cortex (the frontal, parietal, temporal lobes and occipital cortex). As each part of the cerebral cortex is involved in different functions this may help to explain the various different impairments observed in alcoholics. The impairment of memory is one of the symptoms of cognitive dysfunction in alcoholics. It has been shown that the hippocampus plays an important role in short-term memory and short-term memory is transferred into long-term memory by conveying the information to each of the various parts of the cerebral cortex such as the frontal, parietal, temporal and occipital cortex (Purves et al., 2001). According to the study of White (2003), it has been shown that alcohol primarily interferes with the transfer of information from short-term to long-term storage. Our data have demonstrated that exposure to alcohol-induced pathology in all parts of the cerebral cortex. This explains the ability of alcohol to impair the transfer of information from short-term to long-term storage and subsequent long-term memory impairment.

Concerning the technique we have used to investigate the effect of alcohol administration during adulthood on the pathology of the brain, according to Franke et al. (1997), ethanol treatment (10% v/v) over a period of 4 weeks caused no significant change in the total number of neurons or the number of damaged pyramidal and granular cells in the hippocampus when compared with the control group. However, significant changes in the total number of neurons or the number of damaged pyramidal and granular cells were found in prolonged ethanol treatment over 12 weeks. In contrast our data obtained by using the PV immunoreactivity technique detected alterations in the numbers of PV-ir neurons even after 21 days exposure to ethanol. Thus the PV immunoreactivity technique is more sensitive than the technique used by Franke et al. (1997). In addition, the alteration of PV-ir neuron numbers could reflect alterations to NMDA receptors. The alterations observed regarding the NMDA receptors could not determine whether they were due to an alteration of the NMDA receptors of each neuron or alterations to the GABAergic neurons. Therefore, the PV immunoreactivity technique may in this case provide a better explanation. However, by using two markers (PV-ir neurons and GFAP-ir astrocytes) to investigate the effects of alcohol exposure on brain pathology, the explanations about how alcohol mediated the pathology of the brain and impairment of brain functions has become clearer. Thus to investigate the effect of alcohol exposure on brain pathology, the two markers have been used.
This study was performed during the adolescence period of rats. The reduced number of PV-ir neurons seemed to be compensated by the extra growth of glial cells. The increase of glial astrocytes might provide a supportive function in the brain, which is not likely to be a cognitive function. This compensation should at least fill the space of the neuronal loss at this age. However, the compensation mechanism was not expected to be tolerated in the case of alcoholism after prolonged periods or with heavier drinking. There might be a serious loss of other types of cortical brain cells apart from PV-ir neurons that could result in a total reduction of cortical brain mass. In addition, the cerebral cortex is a large component of the mammalian brain including rats. Reduction of the overall cortex could be one important factor resulting in brain atrophy.

Overall, alcohol administration reduced the numbers of PV-ir neurons in the cortex. The increase of GFAP-ir astrocytes might be one of the processes that compensate for the loss of brain cells. However, apart from the effects on the PV-ir neurons, there are many other neuronal cell types including calbindin, calretinin-ir neurons that have also been found to degenerate (Kril et al., 1997). In contrast, the GFAP-ir astrocytes appear to be one of the few cell types that regenerate during exposure to toxic substances. Thus, it is assumed that alcohol leads to a total greater cell loss than the proliferation.

We conclude that exposure to alcohol mediated a reduction of PV-ir neurons and an increase of GFAP-ir astrocytes in all parts of the cerebral cortex. Both markers (PV-ir neurons and GFAP-ir astrocytes) indicated that pathology occurred in all parts of the cerebral cortex. The pathology in all parts of the cerebral cortex could help explain the several observed impairments in alcoholics.

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W. Udomuksorn et al. / acta histochemica 113 (2011) 283–289