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Neuropathology of Aging and Long-term Ethanol Consumption

Morphological and Immunohistochemical Studies on Rat Cerebellum, Locus Coeruleus and Frontal Cortex

■

ACADEMIC DISSERTATION

To be presented, with the permission of
the Faculty of Medicine of the University of Tampere,
for public discussion in the main auditorium of Building K,
Medical School of the University of Tampere,
Teiskontie 35, Tampere, on February 1st, 2002, at 13 o'clock.

Acta Universitatis Tamperensis 851
University of Tampere
Tampere 2002

ACADEMIC DISSERTATION
University of Tampere, School of Public Health
National Public Health Institute, Helsinki
Finland

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Cover design by
Juha Siro

Printed dissertation
Acta Universitatis Tamperensis 851
ISBN 951-44-5269-0
ISSN 1455-1616

Electronic dissertation
Acta Electronica Universitatis Tamperensis 155
ISBN 951-44-5270-4
ISSN 1456-954X
http://acta.uta.fi

Tampereen yliopistopaino Oy Juvenes Print
Tampere 2002

To Marjo, Laura, Juho, Emmi and 'Lyyli'

Contents

List of original publications

The thesis is based on the following original publications, which are referred to in the text by their Roman numerals.

I Rintala J, Jaatinen P, Lu W, Sarviharju M, Eriksson CJP, Laippala P, Kiianmaa K and Hervonen A (1997): Effects of lifelong ethanol consumption on cerebellar layer volumes in AA and ANA rats. Alcohol Clin Exp Res 21:311–317.

II Lu W, Jaatinen P, Rintala J, Sarviharju M, Kiianmaa K and Hervonen A (1997): Effects of lifelong ethanol consumption on rat locus coeruleus. Alcohol Alcohol 32:463–470.

III Rintala J, Jaatinen P, Lu W, Sarviharju M, Eriksson P, Kiianmaa K and Hervonen A (1998): Lifelong ethanol consumption and loss of locus coeruleus neurons in AA and ANA rats. Alcohol 16:243–248.

IV Rintala J, Jaatinen P, Kiianmaa K, Riikonen J, Kemppainen O, Sarviharju M and Hervonen A (2001): Dose-dependent decrease in glial fibrillary acidic protein-immunoreactivity in rat cerebellum after lifelong ethanol consumption. Alcohol 23:1–8.

V Rintala J, Jaatinen P, Parkkila S, Sarviharju M, Kiianmaa K, Hervonen A and Niemelä O (2000): Evidence of acetaldehyde-protein adduct formation in rat brain after lifelong consumption of ethanol. Alcohol Alcohol 35:458–463.

Abbreviations

AA = Alko Alcohol (alcohol-preferring line of rats)
ANA = Alko, NonAlcohol (alcohol-avoiding line of rats)
APA = acetaldehyde protein adduct
BEC = blood ethanol concentration
BSA = bovine serum albumin
CNS = central nervous system
CYP450IIE1 = ethanol-inducible cytochrome P450
EtOH = ethanol
GABA = γ-aminobutyric acid
GFAP = glial fibrillary acidic protein
HE = hepatic encephalopathy
-IR = immunoreactivity
LC = locus coeruleus
NMDA = N-methyl-D-aspartate
PBS = phosphate buffered saline

Abstract

The damaging effect of long-term ethanol consumption on brain has been demonstrated in both human alcoholics and experimental animals, but whether chronic ethanol consumption accelerates aging of the brain, as suggested by Courville (1955), is not agreed upon. In addition, the mechanisms by which chronic ethanol causes brain damage are largely unknown. The present series of studies was designed to address these issues by using an experimental design in which alcohol-preferring AA (Alko, Alcohol) and alcohol-avoiding ANA (Alko, NonAlcohol) rats were given ethanol (10–12%, vol./vol.) from 3 months up to 24 months of age, i.e., lifelong ethanol exposure. Brain areas, such as cerebellum, brain stem including nucleus locus coeruleus and frontal cortex were processed for stereology and quantitative immunohistochemistry in order to compare the changes induced by lifelong ethanol consumption with the age-matched old controls ('ethanol effect') and alterations of the old controls to the young controls ('age effect'). In addition, possible interactions of age and ethanol on brain structure could be evaluated, as well.

Lifelong ethanol consumption produced no cerebellar atrophy in the AA male or female rats or in the ANA male rats, but in the molecular and granular layer of folium II there was a consistent tendency (15%) towards decreased volumes for the ethanol-exposed ANA female rats. However, a significant 26–30% loss of locus coeruleus neurons in the AA and ANA female rats was found after 21 months consumption of ethanol. Bergmann glial cells were also found to be vulnerable to chronic moderate-to-heavy ethanol consumption as the glial fibrillary acidic immunoreactivity (GFAP-IR) decreased markedly and dose-dependently in the molecular layer for the AA female rats. In the cerebellum aging produced different and partly opposite changes compared with chronic ethanol. For example, there was a consistent age-associated increase in the volume of the granular and white matter layers compared with the 'no change' results for ethanol. Similarly, GFAP-IR increased with age in the granular layer of the AA male rats compared with a slight attenuation of GFAP-IR due to lifelong ethanol consumption. In addition, contrary to ethanol-induced decrease there was no age-related alteration in the number of locus coeruleus neurons in the AA and ANA rats of either gender. Acetaldehyde, a toxic metabolite of ethanol, could play a role in the pathogenesis of brain damage induced by chronic ethanol because detectable amounts of acetaldehyde-protein adducts were found by immunohistochemistry in the frontal cortex, frontal white matter and cerebellar cortex of some, but not all, AA and ANA rats following lifelong ethanol consumption.

In summary, no support for the hypothesis of additive influence of chronic ethanol exposure with aging on brain morphology was found in these studies. In addition, locus coeruleus neurons in the brain stem and GFAP-immunopositive Bergmann glial fibers in the molecular layer of cerebellum were found to be the most vulnerable structures of adult rat brain to lifelong ethanol consumption. Acetaldehyde, by forming protein adducts with brain proteins, could contribute to the pathogenesis of alcoholic brain damage.

Introduction

Alcoholism is a chronic disease generally characterized by a loss of control over alcohol consumption and an inability to modify drinking habits in spite of harmful health consequences. Family, twin and adoption studies indicate a strong genetic predisposition to alcoholism (Ball and Murray 1994). However, other factors than genes, such as living environment and psychosocial background usually contribute to the development of alcoholism. It is well documented that long-term excessive alcohol consumption is associated with increased mortality and morbidity from diseases including liver cirrhosis, various cancers, and organic brain syndrome (Klatsky et al. 1992, Mann et al. 1992, Thun et al. 1997). In the central nervous system (CNS) chronic alcohol consumption causes more neurological disorders than any other drug, toxin or environmental agent (Charness et al. 1989). It has been estimated that 9% of alcohol-dependent individuals have clinically diagnosable organic brain syndrome and 50–70% of alcoholics display significant cognitive impairment (Eckardt and Martin 1986). Neuropathologically 42% of alcoholics show cerebellar atrophy post mortem (Torvik and Torp 1986).

Little attention, however, has been paid to the adverse effects of alcohol in the older individuals, whose alcoholism often goes unrecognized and untreated (Curtis et al. 1989, McInnes and Powell 1994). The prevalence of alcohol abuse and dependence tends to fall with age being between 2–4% among the elderly (>65 years of age) in the United States (Adams and Cox 1995). However, if heavy drinkers or problem drinkers are defined as persons drinking more than two drinks per day (Welte and Mirand 1994), the amount of heavy drinkers among the elderly is approximately 10% (Adams and Cox 1995). In Australia the estimates of heavy drinkers have been between 21–24% in men and 7–10% in women aged 60–80 years (Bucholz et al. 1998). Even among persons over 80 years of age nearly 12% of men and 2% of women were found to be heavy drinkers in the same study (Bucholz et al. 1998). If older nervous system is more vulnerable to chronic ethanol, as has been suggested (Pfefferbaum et al. 1992, Pfefferbaum et al. 1993, Sullivan et al. 1995, Pfefferbaum et al. 1997), it means that chronic alcohol consumption can cause severe neurological problems among a large group of elderly people.

Computerized tomography scans (CT) from alcoholics have shown brain atrophy comparable to 20 years older non-alcoholics (Cala and Mastaglia 1981, Carlen et al. 1981). Magnetic resonance images (MRI) obtained from the older alcoholics have shown more pronounced brain atrophy compared with the younger alcoholics suggesting age-ethanol interaction in brain morphology (Hayakawa et al. 1992, Pfefferbaum et al. 1992, Pfefferbaum et al. 1993, Sullivan et al. 1995, Pfefferbaum et al. 1997). If chronic ethanol accelerates the aging of the brain (e.g. premature aging of the brain hypothesis, Courville 1955), it could be used as a marker and model for aging in neurogerontological studies. However, the lack of well-controlled longitudinal follow-up studies as well as several confounding variables inherent to human studies, such as different life-history variables and other chronic diseases common in older people, might interfere with the results. Therefore, the need for controlled, experimental studies has been apparent (Wood 1980, Abel 1984). For this purpose, we designed a series of studies in which rats from two different lines, the alcohol-preferring AA line (Alko, Alcohol) and the alcohol-avoiding ANA line (Alko, NonAlcohol), were given ethanol as the only available fluid for all their adult life (21 months). The ethanol-exposed rats were compared with the old controls to see the effect of chronic ethanol on brain structures. Effect of aging or age-ethanol interaction was evaluated by including young controls in the studies. Similarly, female rats were included to find out possible gender differences in the age-related or ethanol-induced brain morphologies. Comparison between the AA and ANA rats was of interest, because these rat lines are known to differ genetically in terms of voluntary ethanol drinking, i.e., AA line drinking intoxicating amounts of ethanol in a free choice between ethanol and water, while the ANA line drinks only negligible amounts of ethanol (Eriksson 1968, Eriksson 1971, Eriksson and Rusi 1981), and in terms of ethanol metabolism, i.e., the ANA line producing higher amount of acetaldehyde, a toxic metabolite of ethanol (Eriksson and Sippel 1977). Thus additional information on the role of genetic susceptibility and acetaldehyde accumulation in the ethanol-induced brain damage could be obtained.

Review of the literature

1 Structural brain alterations following chronic ethanol consumption

1.1 Cerebellum

42% of human alcoholics show atrophic lesions of the midline cerebellar structures, the lesions being most marked in the anterior superior part of the vermis (Victor et al. 1959, Torvik and Torp 1986). In alcoholics with cerebellar damage, ataxia, impaired gait and dysarthria are the most common clinical signs (Victor et al. 1989). Cerebellar damage usually emerges gradually after several years of heavy alcohol consumption (Charness et al. 1989), but a more acute form of the disease has been reported in alcoholics with Wernicke's encephalopathy (Victor et al. 1989). The neuropathological features include atrophy of the molecular layer together with a loss of Purkinje and granule cells (Allsop and Turner 1966, Phillips et al. 1987, Karhunen et al. 1994). Furthermore, an atrophy of the vermal white matter has been found in ataxic alcoholics (Baker et al. 1999).

In rodents, the loss of granule cells and molecular layer interneurons appears to precede the degenerative changes of the Purkinje cells (Tavares and Paula-Barbosa 1982, Tavares et al. 1987), although a recent stereological study found no change in the number of granule cells or in the volume of granular layer after 40–48 weeks of ethanol exposure in rat (Tabbaa et al. 1999). In addition to neuron loss degenerative changes of the neuropil (dendrites, axons and synapses) may also contribute to cerebellar tissue shrinkage (Pentney 1982, Tavares et al. 1983, Ferrer et al. 1984, Pentney and Quiqley 1987, Tavares et al. 1987, Pentney 1995).

1.2 Hippocampus

Hippocampus, an integral brain area for learning and memory, has been suggested to be vulnerable to chronic ethanol (Bengochea and Gonzalo 1990).

For example, Bengochea and Gonzalo (1990) found significant pyramidal cell losses in the CA2, CA3 and CA4 areas of human hippocampus, but not in the CA1 or dentate gyrus. The volume of the hippocampal white matter, but not the grey matter, appears to be reduced in alcoholics (Harding et al. 1997). Furthermore, there was no difference in the pyramidal cell counts between amnesic or nonamnesic alcoholics and the controls (Harding et al. 1997). Interestingly, a volumetric MRI study in alcoholics showed volume reduction only in the right side of the hippocampus (Laakso et al. 2000).

The effect of chronic ethanol on rodent hippocampus has shown more consistent results. For example, there was a 16% loss of pyramidal cells in the dorsal hippocampus after five months of ethanol exposure in rats (Walker et al. 1980, Walker et al. 1981). Similarly, Paula-Barbosa et al. (1993) reported a 20–30% loss of pyramidal neurons in rat hippocampus after 18 month of ethanol consumption, in line with findings from mice showing 19% and 9% pyramidal cell losses in ventral and rostral hippocampus, respectively, after 9 months of ethanol consumption (Lescaudron and Verna 1985). In addition, dentate gyrus granule cells were damaged after 12 months of ethanol consumption in rats (Cadete-Leite et al. 1988b). Similarly, in the study by Paula-Barbosa et al. (1993) granule cell counts were 40% lower in the dentate gyrus compared with the controls after 4 months of ethanol exposure. Taken together, experimental studies indicate vulnerability of hippocampal neurons to chronic ethanol, but whether hippocampal neurons are lost in alcoholics or not remains to be clarified.

1.3 Cerebral white matter and cortex

Harper and colleaques (1985) have provided the most convincing results that the cerebral white matter volume is reduced in chronic alcoholics. The cerebral white matter volume decreased even in the absence of complications of alcoholism, such as cirrhosis of the liver or Wernicke-Korsakoff's syndrome, which in turn, further worsened the white matter shrinkage (Harper et al. 1985). The atrophy of the cerebral white matter in alcoholics has been confirmed by others both pathologically and neuroradiologically (de la Monte 1988, Pfefferbaum et al. 1992, Jensen and Pakkenberg 1993).

In addition to cerebral white matter shrinkage, the volume of the cerebral gray matter appears to be slightly reduced in alcoholics (de la Monte 1988, Jernigan et al. 1991). However, whether this reduction relates to cortical neuron loss or to neuronal atrophy is not clear, because some authors have reported selective neuron loss in the superior frontal lobe of the cerebral cortex (Harper et al. 1987, Kril et al. 1997), while another study (Jensen and Pakkenberg 1993) found no change in the total number of neurons in the frontal, temporal, parietal or occipital lobes of the neocortex.

1.4 Diencephalon

Diencephalon is a structure enclosing the third ventricle including the thalamus and the hypothalamus. As early as 120 years ago Carl Wernicke (Wernicke 1881) described a condition called 'acute superior haemorragic polio-encephalitis' in three patients with clinical features of tremor, abnormal gait, ataxia and ophthalmoplegia. Two patients had a history of heavy alcohol consumption and the third patient was a woman suffering from chronic vomiting due to pyloric stenosis. All patients had died within one week after admission to hospital and the post mortem findings showed petecchial hemorrhage around the third and fourth brain ventricles. Given the name of Wernicke's encephalopathy (Torvik 1985, Victor et al. 1989), this is one of the most common and serious complications of alcohol abuse with a mortality rate of 10–20% in the acute phase (Reuler et al. 1985). In addition to petecchial hemorrhage around the ventricles, neuropathological examination shows atrophy of the mamillary bodies, and occasionally lesions of the upper pons and medulla (Victor et al. 1989). In half of the cases, a selective loss of Purkinje cells in the anterior superior vermis can be found (Victor et al. 1989). 80% of alcoholics recovering from Wernicke's encephalopathy are suffering from a chronic condition of Korsakoff's syndrome clinically characterized by retro-grade and anterograde amnesia with relative preservation of alertness and other cognitive functions (Victor et al. 1989). Structural brain lesions in Korsakoff's syndrome include a loss of neurons in the medial thalamus and degenerative

changes in other diencephalic structures, such as nucleus basalis of Meynert (Arendt et al. 1983, Torvik 1985, Victor et al. 1989).

1.5 Other brain areas

Central pontine myelinolysis is a rare lesion, usually seen in chronic alcoholics, but also occuring in patients with several non-alcohol related disorders, such as liver disease, malnutrition, anorexia, burns, cancer, Addison's disease and severe electrolyte disturbances (Adams et al. 1959, Victor et al. 1989, Charness et al. 1989). Neuropathologically this condition is characterized by bilateral destruction of the white matter in the ventral pons, although 10% of cases have extrapontine lesions in the striatum, thalamus, cerebral white matter and cerebellum (Wright et al. 1979).

Locus coeruleus (LC), a dorsolaterally located noradrenergic nucleus in the pons, has also showed some alterations in chronic alcoholics. For example, in two alcoholics with Korsakoff's psychosis a 19% loss of LC neurons was found (Mayes et al. 1988) in contrast to no change in the morphology or number of LC neurons in four uncomplicated alcoholics or in nine alcoholics with Wernicke Korsakoff's syndrome (Halliday et al. 1992a). In rat LC, however, 17 weeks of heavy ethanol exposure, resulted in a decreased synapse-to-neuron ratio indicating that this region might be affected by chronic ethanol in animals, as well (Kjellström et al. 1993). Similarly to the LC, the neurons in the serotonergic raphe nuclei appear to be damaged by ethanol in alcoholics with up to 50% neuron losses in the dorsal and medial raphe nuclei (Halliday et al. 1992b).

Hepatic encephalopathy (HE) is a frequent complication seen in alcoholics with severe liver damage (Charness 1993), although other reasons than alcohol could cause this neurological disorder (Norenberg 1986). The clinical signs include personality changes, confusion, occasional seizures and, when severe, stupor and coma. The neuropathological changes typical of HE include astrocytic proliferation and hypertrophy in the basal ganglia, thalamus, red nucleus, pons and cerebellum (Victor et al. 1965, Norenberg 1986). The pathogenesis of HE is poorly understood, but the principal process involves

accumulation of gut-derived toxic substances, such as ammonium, in the CNS (Norenberg 1986).

In the spinal cord, degeneration of the posterior column secondary to peripheral neuropathy is quite a common finding in alcoholics (Victor et al. 1989) contrary to the Marchiafava-Bignami syndrome, which is an extremely rare neurological complication found in malnourished alcoholics. In the Marchiafava-Bignami syndrome neuropathological examination shows a demyelination and necrosis of the corpus callosum (Charness 1993).

2 Ethanol-induced brain tissue injury – possible molecular mechanisms

The ethanol molecule readily passes the blood-brain-barrier and equilibrates in the brain (Pohorecky and Brick 1988). In the brain ethanol acts on neuronal and glial membranes containing proteins and lipid molecules (Lovinger 1993, Joyce 1994, Peoples et al. 1996, Snyder 1996). Although the precise mechanisms for ethanol-induced brain lesions are not well known, it is widely agreed that thiamine deficiency with or without chronic ethanol can cause Wernicke-Korsakoff-type of lesions both in humans (Thomson et al. 1983, Lindboe and Loberg 1989, Victor et al. 1989, Butterworth et al. 1991, Butterworth et al. 1993, Butterworth 1995) and in rodents (Troncoso et al. 1981, Witt 1985, Laforenza et al. 1990, Langlais and Savage 1995). In human alcoholics, for example, treatment with high doses of thiamine can dramatically improve the symptoms and prognosis of Wernicke-Korsakoff patients (Victor et al. 1989). What is the pathogenesis of this syndrome and how can ethanol interfere with the metabolism of thiamine? In alcoholics the use and the availability of thiamine, an essential cofactor of three enzymes (α-ketoglutarate dehydrogenase, pyruvate dehydrogenase and transketolase) involved in glucose utilization and lipid synthesis (Cooper and Pincus 1979, Iwata 1982, Hakim and Pappius 1983), are compromised. For example, the amount of thiamine may be reduced before entering the brain due to inadequate dietary intake, reduced gastrointestinal absorbtion and decreased hepatic storage of thiamine (Hoyumpa 1980, Thomson et al. 1983, Gastaldi et al. 1989). In the brain, the use of thiamine,

which must be converted into an active form by thiamine pyrophosphokinase, is downregulated due to an inhibitory effect of ethanol on pyrophosphokinase enzyme activity (Laforenza et al. 1990). In addition, activities of enzymes that break down thiamine may be increased by ethanol, as well (LaForenza et al. 1990). All these effects of chronic ethanol on thiamine turnover and metabolism are likely to contribute to decreased glucose utilization and altered lipid metabolism in brain which, in turn, lead to Wernicke-Korsakoff-type of lesions both in humans and in experimental animals.

Withdrawal from ethanol causes neuronal hyperexcitability mediated by glutamate and other excitatory amino acids followed by calcium influx to cells (Lovinger 1993). Withdrawal from long-term ethanol consumption or chronic ethanol per se could enhance glutamate-induced excitotoxicity and calcium influx by increasing the number and binding sites of ligand-gated N-methyl-D-aspartate (NMDA) receptors (Michaelis et al. 1978, Gulya et al. 1991, Iorio et al. 1992, Iorio et al. 1993, Chandler et al. 1993, Lovinger 1993, Ahern et al. 1994, Hu and Ticku 1995). Chronic ethanol could also increase the amount of glutamate-mediated excitotoxicity by increasing the number and activity of voltage-dependent calcium channels thereby sensitizing neurons to excitotoxicity (Brennan et al. 1990). Decreased γ-aminobutyric acid-stimulated (GABA) chloride influx and activity of $GABA_A$ receptors (Harris and Allan 1989, Montpied et al. 1991) may also interfere with the normal balance between the glutamate-mediated excitation and GABA-mediated inhibition, resulting in glutamate-induced neurotoxicity (Sanna et al. 1993). Finally, excitotoxic mechanism of ethanol may be linked with thiamine-deficiency because high amount of glutamate is released from brains of thiamine-deficient rats even without ethanol exposure (Langlais 1995).

Ethanol is capable of producing oxygen free radicals, and of initiating lipid peroxidation in brain cell membranes (Montoliu et al. 1994). In the brain, ethanol-induced oxidative stress might be formed by imbalance between free radical formation and decreased antioxidant defence system (Guerri and Grisolia 1980, Ledig et al. 1981, Marlund et al. 1983, Rouach et al. 1987, Nordman et al. 1990, Montoliu et al. 1994). In rat cerebral cortex chronic ethanol increases nitric oxide synthase activity thereby promoting the production of nitric oxide, which in turn, can interact with superoxide radicals to form cytotoxic peroxynitrite

(Naassila et al. 1997, Xia et al. 1999). Especially in the glial cells ethanol could promote the production of cytotoxic cytokines, which could damage neuronal structures directly or indirectly through an autoimmune response mediated by microglial/macrophage cells or astrocytes (Singer et al. 1989, Lancaster 1993, Snyder 1996).

Ethanol neurotoxicity may be derived by its oxidative metabolite, acetaldehyde (Lindros 1978, Brien and Loomis 1983), which in the brain is formed by three ethanol-metabolizing enzymes, alcohol dehydrogenase (Bühler et al. 1983, Kerr et al. 1989), catalase (Aragon et al. 1992, Gill et al. 1992) and ethanol-inducible cytochrome P450 (CYP450IIE1) (Hansson et al. 1990). In rat brain chronic ethanol feeding increases the amount of CYP450IIEI (Anandatheerhavarada et al. 1993, Montoliu et al. 1995), which is a potent generator of reactive oxygen species (Persson et al. 1990).

The reactive oxygen free radicals or acetaldehyde could, for example, bind to neurotubulin (Jennett et al. 1980) disrupting the neurotubulin polymerization (McKinnon et al. 1987, Smith et al. 1989).

Non-oxidative metabolism of ethanol may cause excessive amounts of fatty acid ethyl esters (Bora and Lange 1993) or promote the production of proinflammatory eicosanoids by enhancing arachidonic acid selective phospholipase A_2 (Basavarajappa et al. 1997).

3 Chronic ethanol consumption and the aging brain

After absorption from gastrointestinal tract ethanol readily equilibrates to the water compartments of the body. As the fat content of body increases with age (Watson et al. 1980) the same dose of ethanol in grams per kilogram of body weight results in higher blood ethanol concentrations (BEC) among older individuals (Kalant 1996). In other words, the change in body water rather than age itself causes the higher BEC among the elderly (Egbert 1993). There is, however, evidence that at the same BEC the older nervous system could be more sensitive to ethanol. For example, in fatal accidental poisoning of ethanol the BEC correlated inversely with age (Poikolainen 1984). Impairment of fine motor movement was twice as high among the older persons compared with the

younger persons both having the same BEC (Vogel-Sprott and Barrett 1984). Interestingly, in women the verbal memory impairments by ethanol differed from that produced by aging, immediate recall of word sequences being decreased only by ethanol, and short-term memory being decreased both by ethanol and by age without significant interaction (Jones and Jones 1980). In addition, withdrawal from ethanol seems to produce more severe and longer lasting symptoms in older persons compared with younger persons (Liskow et al. 1989, Brower et al. 1994).

However, studies on the effects of chronic ethanol consumption on aging brain structures have provided inconsistent results. The first descriptions of the possible interactions of ethanol and aging on brain pathology were based on autopsy findings of alcoholics, which showed remarkably similar neuropathology to older non-alcoholics (Courville 1955). These findings led to the hypothesis of ethanol-induced accelerated brain aging (Courville 1955). Macroscopically there is a progressive decline in fresh brain weight after 45 years of age and by the age of 86 years the mean brain weight is 11% lower than the weight at 19 years of age (Dekaban 1978). Similarly, in alcoholics, the mean brain weight is slightly, but significantly reduced compared with the controls (Harper and Blumbergs 1982, Torvik et al. 1982, Harper et al. 1985). However, after 70 years of age the brain weight of alcoholics did not differ from the age-matched controls (Torvik et al. 1982, Lindboe and Loberg 1988). Similarly, in alcoholics the volume of cortical gray matter showed no change with respect to age (Harper et al. 1985). Evidence in favor of possible interaction of chronic ethanol and aging on brain come mainly from neuroimaging studies. Computerized tomography scans (CT) of alcoholics show a cortical brain atrophy comparable to 20 years older non-alcoholics (Cala and Mastaglia 1981, Carlen et al. 1981). Magnetic reconance images (MRI) from alcoholics display more pronounced brain abnormalities in the frontal cortex, white matter and hippocampus of older alcoholics compared with the younger alcoholics (Hayakawa et al. 1992, Pfefferbaum et al. 1992, Pfefferbaum et al. 1993, Sullivan et al. 1995, Pfefferbaum et al. 1997). Even after controlling for the differences in head size and lifetime ethanol consumption the brain alterations were more pronounced in the older alcoholics (Pfefferbaum et al. 1992, Sullivan et al. 1995, Pfefferbaum et al. 1997). The additive effect of ethanol on age-induced brain alterations might be more

prominent in the cerebral white matter as a recent stereological study showed that the degree of white matter shrinkage correlated significantly with age both in uncomplicated and in complicated alcoholics (Kril et al. 1997).

In rodent brain, there are only a few studies addressing the possible interaction of age and ethanol and the results have not been as consistent as in humans. For example, in rat cerebellum the age-related alterations in Purkinje cell dendrites have been different or even opposite from those induced by chronic ethanol exposure suggesting no age-ethanol interaction in the cerebellar cortex (Pentney and Quiqley 1987, Pentney and Quackenbush 1990). However, an additive effect of chronic ethanol on age-related lipofuscin accumulation, a typical finding in the aging neurons, has been clearly demonstrated (Tavares and Paula-Barbosa 1983, Tavares et al. 1985, Borges et al. 1986, Cadete-Leite et al. 1988a). Of separate brain areas this has been shown to occur in hippocampus (Borges et al. 1986), cerebellum (Tavares and Paula-Barbosa 1983, Tavares et al. 1985) and prefrontal cortex (Cadete-Leite et al. 1988a). In the older peripheral nervous system, such as superior cervical ganglion, chronic ethanol consumption markedly enhances the production of lipofuscin, as well (Jaatinen et al. 1992, Riikonen et al. 1999).

4 Effect of gender on ethanol-induced brain damage

It has been suggested that women could develop liver cirrhosis, pancreatitis or peripheral neuropathy with a lower daily ethanol intake and shorter drinking history than men (Morgan and Sherlock 1977, Krasner et al. 1977, Pares et al. 1986, Norton et al. 1987, Mezey et al. 1988). Similarly, the female brain is suggested to be more vulnerable to chronic ethanol (Jacobson 1986, Harper et al. 1990, Mann et al. 1992). For example, alcoholic women displayed equivalent levels of brain atrophy compared with age-matched alcoholic men with shorter drinking histories (Jacobson 1986, Mann et al. 1992). In addition, the volume of the right hippocampus reduced similarly in alcoholic men and women, relative to healthy age-matched controls, but women consumed ethanol only half as much as men (Agartz et al. 1999). This is in line with a study (Glenn 1993) showing equivalent cognitive dysfunction in alcoholic women compared with alcoholic

men after fewer years of ethanol consumption. The pattern of ethanol-induced brain injury may show sex-specific differences with respect to age; a recent study (Pfefferbaum et al. 2001) showed considerable atrophy of cortical gray matter and sulcal widening with age in alcoholic men in contrast to female alcoholics who displayed an age-related increase only in the volume of lateral ventricles. The only experimental study in addition to our own studies, so far, on the possible sex differences in ethanol-induced neuropathology, found no change in the frontal cortical or corpus callosal thicknesses between male and female rats (Savage et al. 2000).

The aims of the study

In the present studies rats from the alcohol preferring AA line and from the alcohol-avoiding ANA line (Eriksson 1968, Eriksson 1971) were exposed to ethanol for 21 months (lifelong ethanol exposure) in order to address the question whether long-term ethanol consumption accelerates the structural brain changes of aging brain (Courville 1955). More specifically the questions were:

1. Does lifelong ethanol consumption cause cerebellar atrophy in the AA or ANA rats?
2. Are there any age-related alterations in the volume of molecular, granular and central white matter layer of cerebellum in the AA and ANA rats? Are the possible age-related volume changes accelerated due to lifelong ethanol consumption?
3. What are the effects of aging and lifelong ethanol consumption on the number of LC neurons in the AA and ANA rats?
4. Does long-term ethanol consumption and/or aging alter the immuno-reactivity of an astrocyte-specific marker, glial fibrillary acidic protein (GFAP) in the molecular, granular and central white matter layers of cerebellum of the AA rats? Are there region specific differences in the GFAP vulnerability to lifelong ethanol? Are the changes in GFAP-IR induced by aging accelerated by lifelong ethanol?
5. Does lifelong ethanol consumption cause formation of acetaldehyde protein adducts (APA) in the brain of the AA and ANA rats?
6. Are there line (AA vs. ANA) or gender differences in the vulnerability of aging brain to chronic ethanol?

Material and methods

1 Animals and diets

A total of 97 rats of two age-groups (3- and 24-months-old) and of two rat lines, the alcohol-preferring AA line (Alko, Alcohol) and the alcohol-avoiding ANA line (Alko, NonAlcohol) were used. In studies I–IV female rats were included to find out possible gender differences in brain morphology. The total number and the grouping of the animals at the start of the experiment was as follows. Studies I and III: AA males: young controls (n=8), old controls (n=8), old EtOH (n=8) b) AA females: young controls (n=8), old controls (n=7), old EtOH (n=8) c) ANA males: young controls (n=10), old controls (n=8), old EtOH (n=8) d) ANA females: young controls (n=7), old controls (n=10), old EtOH (n=7). However, in studies II and IV only AA rats were used and in study V a male subgroup from the AA and ANA rats were processed for immunohisto-chemistry. The rats representing generations F_{65}, F_{67}, F_{69} and F_{71} were obtained from the Research Laboratories of former Alko Group Ltd., now part of the Department of Mental Health and Alcohol Research at the National Public Health Institute in Helsinki, Finland. The AA and ANA rats have originally been developed by selective outbreeding for high (AA line) and low (ANA line) levels of voluntary alcohol consumption, i.e., AA rats preferring alcohol and ANA rats preferring water in a free choice between alcohol and water (Eriksson 1968, Eriksson 1971, Eriksson and Rusi 1981). The rats were housed in stainless steel wire mesh cages in groups of four to five animals per cage. The conditions were kept constant with 12h/12h light/dark cycle at temperature of 20 ± 1°C and a relative humidity of 50 ± 5%. Food (RM1(E)SQC;SDS, Witham, England) was freely available for all animals.

In the present studies the ethanol-consuming (EtOH) animals had 12% (vol./vol.) ethanol as the only available fluid from 4 months up to 22 months of age. The old controls were maintained under the same conditions, except for having only water to drink. During the 3-week self-selection periods at 3 and 22 months of age the rats had a free choice between 10% (vol./vol.) ethanol and tap water, to measure the voluntary ethanol consumption. The consumption of ethanol (g absolute alcohol per kilogram of body weight) was monitored

monthly for a period of one week per cage between 4 and 22 months of age and the individual values for each rat housed in single cages were obtained over a one week period at 3 months and 22 months of age after which the rats were moved back into the group cages and the ethanol as sole source of fluid was continued up to one week before sacrifice, when the rats were withdrawn from ethanol.

2 Ethical considerations

Every effort was made to avoid unnecessary suffering of the animals. If the animal displayed signs of severe illness it was sacrificed. Prior to decapitation the animals were deeply anesthetized with sodium pentobarbital 60–120 mg/kg i.p. (Mebunat, Orion Corp., Turku, Finland). The research protocol has been approved by the Institutional Animal Care and Use Committee at the National Public Health Institute, Helsinki.

3 Histology of the brain and liver

Figure 1 shows the areas of interest from rat brain in the present studies. For histological studies the cerebellar vermis was separated from the cerebellar hemispheres and the right side of the brain stem including the locus coeruleus (LC) was cut sagittally from the rest of the brain. The LC proper, which is the major source of noradrenaline in brain (Dahlström and Fuxe 1968) is located in the dorsolateral corner of the fourth brain ventricle (Foote et al. 1983, Loughlin and Fallon 1985). Both vermis and brain stem were fixed in 4% para-formaldehyde in phosphate-buffered saline (PBS, pH 7.4) at 4 ^{O}C for 24 hours and cryoprotected in ascending sucrose series (10%, 20%, and 30% sucrose in PBS). Similarly, the right cerebellar hemisphere, the frontal cortex and liver were processed for the detection of acetaldehyde protein adducts (V).

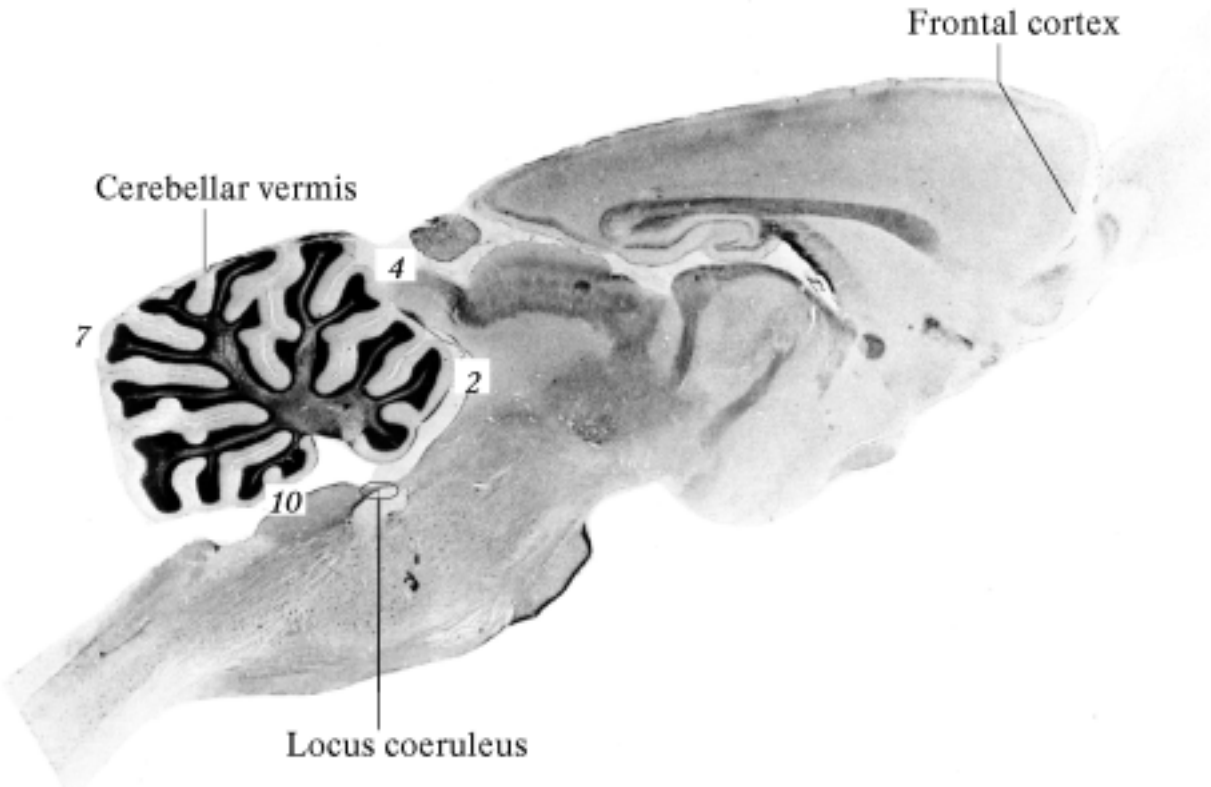

Figure 1. A sagittal section from a rat brain showing the areas of interest in the present studies. Numbers 2, 4, 7, 10 refer to different folia of the cerebellum

4 Morphometry of the cerebellum and the locus coeruleus (I–III)

For the morphometric analyses of the cerebellar layer volumes (I), the whole vermis was parasagittally cut in serial cryostat sections 6μm in thickness and stained with 0.05% cresyl violet acetate. According to the Cavalieri principle (Cavalieri 1966) the volume of each cerebellar layer (molecular, granular and white matter layers) in folia 2, 4, 7 and 10 as shown in figure 1 (Larsell 1952) was measured by using systematic sampling and a point-counting method (Gundersen and Jensen 1987). With a random start, every sixth series of 10 sections was collected on glass slides and one randomly selected section out of these 10 sections was viewed with the microscope (Olympus Vanox-T, Olympus Inc., Tokyo, Japan). A video camera (Hamamatsu Photonics, Hamamatsu City, Japan) provided the view to the video monitor (Panasonic, Matsushita Electric Industrial Co., Ltd., Japan). A systematic set of points was placed over the

video image, each point corresponding an area of 0.055 mm^2 [a(p)]. The sum of all points (∑P) hitting the molecular, granular and white matter layer in a section was counted by the image processor (ARGUS-10, Hamamatsu Photonics, Hamamatsu City, Japan) and the area for each folium over all sections was calculated from the formula (a) = ∑P x a(p). The total volumes of the molecular, granular and white matter layer for each of the four vermal folia was counted from the following formula:

$$V = a\,(m) \times t \times TSN$$

where a (m) is the mean section area and t is the section thickness and TSN is the total number of sections.

The total number of LC neurons (II–III) was measured by using the physical disector method (Gundersen et al. 1988, Pakkenberg and Gundersen 1988). The right side of the brain stem including the LC was processed for morphometry, because the number and distribution of the LC in rat brain is bilaterally symmetrical (Swanson 1976). Eight serial cryostat LC sections (8μm) were collected on glass slides. Six adjacent sections were stained with 0.05% cresyl violet and two other sections with tyrosine hydroxylase, the rate limiting enzyme in the cathecholamine synthesis, thus verifying the noradrenergic nature of LC neurons (Hsu et al. 1981). The volume (Vol) of the LC was measured similarly to that described above using the Cavalieri principle each grid point for the LC being equivalent to 0.0088mm^2. The numerical density Nv of the LC neurons was counted from all disector pairs over the entire LC. The neuronal nucleus was used as a counting unit. If the nucleus fell within a counting frame (2500μm^2) and touched the inclusion lines in a reference section, but not in the look-up section, it was counted. The total number of neurons N was calculated from the formula of N= Nv x Vol.

5 Immunohistochemistry and morphometry of glial fibrillary acidic protein (GFAP) in the cerebellum (IV)

GFAP is the predominant protein of astrocytic filaments (Eng et al. 1971), and it can be reliably detected by using immunohistochemistry (Bignami and Dahl 1974). The morphometry of GFAP-immunoreactive (GFAP-IR) structures was

performed from midvermis cryostat sections (6μm). The endogenous peroxidase activity of the sections was blocked by 0.25% hydrogen peroxide in methanol for 20 minutes followed by two washes in PBS. The sections were incubated with rabbit anti-cow GFAP antiserum (Dako, Glostrup, Denmark; dilution 1:2000) in PBS containing 1% bovine serum albumin (BSA) and 0.3% Triton X-100 (Sigma Chemical Co, St Louis, Montana, USA) for 24 hours at 4ºC. After 3 rinses in PBS for 10 minutes the sections were incubated in biotinylated anti-rabbit IgG in PBS (Vector Laboratories, Inc., Burlingame, California; USA; dilution 1:200) for 30 minutes at 37ºC and washed twice in PBS. The ABC protocol (Hsu et al. 1981) (Vectastain ABC kit, Vector) and 3,3'-diaminobenzidine reaction (DAB, Sigma) were used to detect GFAP-IR structures. The sections were mounted in Aquamount improved (BDH, Poole, England). All the samples were processed for immunohistochemistry simultaneously in order to minimize the variation in staining intensity. Measurements of GFAP-immunopositive structures were done from the molecular, granular and central white matter layers in folia 2, 4, 7 and 10 by using a well-established method (Shetty and Phillips 1992, Huang et al. 1996). Briefly, GFAP-IR was measured with an image-analyzer attached to the image processor (Argus-10, Hamamatsu Photonics, Hamamatsu City, Japan). The unspecific background staining was eliminated by selecting the optimum threshold values for GFAP-immunopositive structures. For GFAP-IR the highest values varied between 195–215 and the lowest value was 150. The final image was measured by using a computer assisted image-analyzer. A frame area of 40 000 pixels corresponding to area of 0.00805 mm^2 was projected over the final image and the proportion (% area) of GFAP-IR structures in a frame area were calculated. 10 measurements per layer, i.e. 90 measurements/animal for GFAP-IR structures were performed to obtain the mean value.

6 Antibody production and immunohistochemical method for the detection of acetaldehyde protein adducts (V)

Polyclonal antibodies against acetaldehyde-derived epitopes were raised in rabbits immunized by subcutaneous injections of acetaldehyde-BSA (Niemelä et al. 1991). After immunization the anti-acetaldehyde serum was collected and cross-adsorbed on human plasma protein-acetaldehyde conjugate attached to

sepharose 4B (Pharmacia, Uppsala, Sweden). The immunohistochemistry was used from brain sections containing cerebellum, frontal cortex and liver with following steps: a) incubation with 3% hydrogen peroxide for 5 min followed by rinsing in PBS; b) blocking with undiluted cow colostral whey (Hi-Col, Oulu, Finland) for 40 min followed by rinsing; c) incubation with an acetaldehyde adduct antiserum (1:200) in 1% BSA in PBS for 1 h followed by 3x10 min rinses in PBS; d) treatment with cow colostral whey for 40 min followed by rinses in PBS; e) incubation with biotinylated secondary antibody (goat antirabbit IgG, Dako, Glostrup, Denmark) 1:300 in 1% BSA-PBS for 1 h followed by 3x10 min washes with PBS; f) treatment with cow colostral whey for 5 min; g) incubation with peroxidase-conjugated streptavidin for 30 min followed by 3x5 min rinses in PBS; h) incubation in diaminobenzidine (DAB, Sigma) (9mg DAB in 15 ml PBS + 10µl of 30% hydrogen peroxide) for 2 min. The sections were mounted in Permount (Fisher Scientific, Fair Lawn, New Jersey, USA) and evaluated with a Leitz Aristoplan microscope (Wetzlar, Germany). The intensity of the reactions was evaluated as follows: 0=no reaction, (+)(=0.5), scanty reaction, + (=1) weak reaction, ++ (=2) moderate reaction, +++ (=3) strong reaction.

7 Statistical analysis

The results are expressed as mean (SEM), unless otherwise stated. The overall effects of aging and lifelong ethanol consumption (i.e. 'treatment') in each line of rat were first analysed by using a two-way ANOVA ('treatment' x sex). The post-hoc comparisons between different groups were further studied by Bonferroni-corrected unpaired *t*-tests. Ethanol intake was used as a covariate (= mean daily individual ethanol intake of one weeks period at 22 months of age) in order to take into account the line- and sex differences in the ethanol consumption. In the first study the body weight was used as covariate when comparing ethanol consuming female and male rats, because the volume of the vermis (= sum of folia 2, 4, 7, 10) of the old non-drinker rats correlated with the body weight ($r = + 0.433$, $p=0.039$). Pearsons correlation coefficients were used to see the possible dose-dependent effects of ethanol on brain parameters. In the fifth study, the group differences in the acetaldehyde-adduct immuno-

staining were compared by using a non-parametric Mann-Whitney or a Kruskal-Wallis tests. A tail probability of less than 0.05 indicated a statistically significant difference between group means. All the computation was carried out on a VAX/VMS computer with the BMDP statistical software (1993 version).

Results

1 General well-being, body weight and ethanol consumption (I–V)

The ethanol-exposed AA and ANA rats tolerated ethanol remarkably well during the 21-month exposure period and there was no difference in the survival rate between the ethanol-exposed rats and the old control rats of the same line. Rats dying before the end of the experiment were autopsied and the cause of death was recorded. Some of the autopsied ANA rats displayed renal pathology, such as polycystic kidneys, but this pathology was not likely to relate to ethanol, because the prevalence of polycystic kidneys did not differ between the ethanol-exposed and the control rats.

In both lines the weight of the female rats at 3 and 24 months of age was significantly lower than the weight of the corresponding male rats (Tables 1 and 2). In the AA line, the weight gain of the female control rats was lower than that of the male control rats (Table 1). However, the weight gain from 3 months up to 24 months of age between the controls and the ethanol-consuming rats of the same sex did not differ in the AA-line (Table 1) or in the ANA-line (Table 2). The female rats of both lines consumed significantly more alcohol (g absolute alcohol/kg body weight) than the male rats (Tables 1 and 2). There was no difference in the ethanol intake between the male rats of the AA and ANA line, but the female rats of the AA line consumed more ($p<0.05$) ethanol than the female rats of the ANA line. Ethanol constituted ca. 25–30% of the total calories in the ethanol-exposed AA rats and slightly less in the ethanol-exposed ANA rats, ca. 23–26% (Sarviharju et al. 2001). There was no difference in the total energy intake between the old controls and the ethanol-exposed AA or ANA rats of the same gender (Sarviharju et al. 2001).

Table 1. The mean body weights (g) at 3 and 24 months of age and the mean individual EtOH consumption (g alcohol/kg body weight/day) of the AA rats at 22 months of age.

Group	3 months	24 months	Weight gain	EtOH
AA males				
Young control (n=8)	310 ± 12	–	–	–
Old control (n=8)	313 ± 8	522 ± 32	210 ± 31	–
EtOH (n=8)	299 ± 18	498 ± 28	199 ± 26	3.6 ± 0.3
AA females				
Young control (n=8)	172 ± 8 ***	–	–	–
Old control (n=7)	211 ± 10***	314 ± 24***	103 ± 23*	–
EtOH (n=8)	210 ± 20***	314 ± 13***	104 ± 19	7.0 ± 0.6^{+++}

Values are mean ± SE. $^{+++}$ $p<0.001$ compared with the EtOH male rats. At 3 and 24 months of age the weights of the female rats were significantly (***, $p<0.001$) lower than the weights of the corresponding male rats. Similarly, the weight gain of the female old control rats was lower (*, $p<0.05$) than that of the male old control rats.

Table 2. The mean body weights (g) at 3 and 24 months of age and the mean individual EtOH consumption (g alcohol/kg body weight/day) of the ANA rats at 22 months of age.

Group	3 months	24 months	Weight gain	EtOH
ANA males				
Young control (n=10)	320 ± 18	–	–	–
Old control (n=8)	382 ± 9¶	582 ± 21	200 ± 21	–
EtOH (n=8)	381 ± 22	527 ± 26	146 ± 28	3.6 ± 0.4
ANA females				
Young control (n=7)	209 ± 6***	–	–	–
Old control (n=10)	231 ± 8¶***	364 ± 25***	133 ± 21	–
EtOH (n=7)	217 ± 11***	329 ± 22***	112 ± 30	5.4 ± 0.4^{++}

Values are mean ± SE. $^{++}$ $p<0.01$ compared with the EtOH male rats. ¶ $p<0.05$ compared with the young controls. At 3 and 24 months of age the weights of the female rats were significantly (***, $p<0.001$) lower than the weights of the corresponding male rats.

2 Summary of the main results

Table 3 on the next page gives a summary of the main results from the present studies.

3 Effect of aging and lifelong ethanol consumption on the cerebellar layer volumes (I)

In the AA and ANA rats of either gender there were no ethanol-induced volume alterations in the molecular, granular and central white matter layers compared with the old controls. However, a consistent tendency toward decreased volumes of the molecular (15%, ns.) and granular (15%, ns.) layers in folium 2 was found in the ethanol-exposed ANA females. Taking into consideration (ANCOVA with ethanol intake as a covariate) the higher ethanol intake of the AA females compared with the ANA females (7.0 vs. 5.4 g/kg/d) there was a significant decrease in the volume of the molecular (18%, $p<0.05$) and the granular layers (20%; $p<0.05$) of folium 2 in the ANA females, indicating a line difference in the vulnerability of the anterior superior cerebellum to long-term ethanol consumption. Similarly, the volume of the molecular layer in folium 2 was markedly lower ($p<0.05$ with ethanol intake and body weight as covariates) in the ethanol-exposed ANA females compared with the ethanol-exposed ANA males, indicating gender difference. No effect of age was found on the volume of the molecular layer. In the granular and white matter layers the volume tended to be higher among the older rats compared with the younger rats and the difference was significant (23%, $p<0.05$) in the granular layer of folium 4 for AA males and in the central white matter of folium 10 for ANA males (46%, $p<0.01$) and ANA females (48%, $p<0.05$). There was no interaction of age and ethanol on the volume of each cerebellar layer.

Table 3. The main results of the present studies. In the estimation of line and gender differences between the EtOH-exposed animals the different ethanol intake of the male and female rats was considered (ANCOVA).

	Age effect	Line difference (Age effect)	Gender difference (Age effect)	**Ethanol effect**	Line difference (Ethanol effect)	Gender difference (Ethanol effect)
Volumes of the cerebellar layers (I)	Increase in the granular layer of folium 4 (AA males only)	Yes (No change in the granular layer of folium 4 for the ANA males)	Yes (No change in the granular layer of folium 4 for the AA females)	Decrease in the molecular and granular layers of folium 2 (ANA females only)	Yes (ANA-line more vulnerable)	Yes (Females more vulnerable in the ANA-line)
	Increase in the white matter of folium 10 (ANA males and females)	Yes (No change in the white matter of folium 10 for the AA males and females)	No	No change in the white matter volumes	No	No
Number of LC neurons (II-III)	No	No	No	Significant neuron loss in the AA males and females and in the ANA females	No	No
GRAP-IR of cerebellum (IV)	Decrease in the molecular layer of folium 2,7 and 10 (AA males only)	Not studied	Yes (No change in the molecular layer of folium 2,7 and 10 for the AA females)	Decrease in the molecular layer of folium 7 (AA females only)	Not studied	No
	Increase in the granular layer of folium 10 (AA males only)	Not studied	Yes (No change in the granular layer of folium 10 for the AA females)	No change in the granular layer GFAP-IR	Not studied	No
Acetaldehyde adducts (V)	No adducts found	No adducts found	Not studied	Adducts found in 4/9 brains	No	Not studied

4 Effect of aging and lifelong ethanol consumption on the total number of LC neurons (II–III)

The number of LC neurons did not change with age in the AA or ANA rats of either gender, but as shown in figure 2, lifelong ethanol consumption caused a significant loss of LC neurons in the AA males (30%; $p<0.001$) and females (26%; $p<0.001$) compared with the respective old controls. In the ANA-line the ethanol-induced decrease in LC neuron number was significant in the females (30%; $p<0.01$), but not in the males (17%, ns.) (Figure 2). Taking into account (ANCOVA with ethanol as a covariate) the higher ethanol intake of the female rats there was no gender difference in the LC vulnerability to chronic ethanol in either line of rats. Neither was there any correlation between the average ethanol consumption and the number of LC neurons.

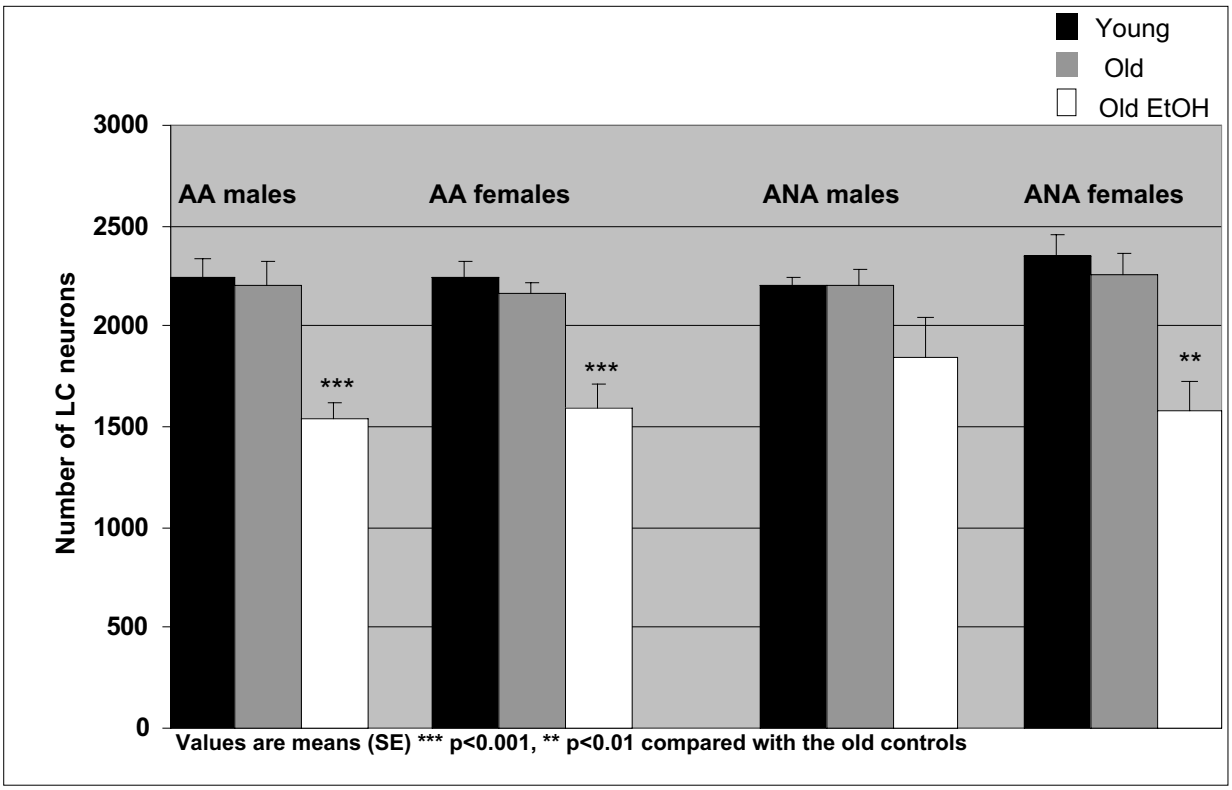

Figure 2. The total number of LC neurons in the AA and ANA rats.

5 Effect of aging and lifelong ethanol consumption on the cerebellar GFAP-immunoreactivity (IV)

In the male, but not in the female AA rats, an age-related decrease in GFAP-immunoreactivity (GFAP-IR) was measured in the molecular layer of folia 2 (18%, $p<0.05$), 7 (17%, $p<0.05$) and 10 (20%, $p<0.01$), and in turn, an age-related increase in the granular layer of folium 10 (21%, $p< 0.05$). No effect of age on GFAP-IR was measured in the central white matter. In the molecular layer, the moderate-to-high (i.e. average intake of 6.6 g/kg/d) ethanol consumption for 21 months caused a significant 28% ($p<0.01$) decrease in GFAP-IR of folium 7 in the female rats. Similarly, in the ethanol exposed female rats there was a marked 22% ($p<0.05$) decrease in GFAP-IR in the molecular layer, when the data on folia 2, 4, 7 and 10 were combined. Additionally this decrease correlated negatively ($r = –0.886$, $p<0.05$) with the daily ethanol consumption indicating a dose-dependent attenuation of GFAP-IR of the Bergmann glial fibers in the molecular layer. In the male rats an average daily ethanol intake of 3.2 g/kg had no effect on GFAP-IR in the molecular layer. Neither was there any effect of ethanol on the GFAP-IR in the granular or central white matter layers or interaction of age and ethanol on GFAP-IR in any of the three cerebellar layers.

6 Evidence of acetaldehyde protein adduct formation in the rat brain after lifelong consumption of ethanol (V)

An immunohistochemical staining using a specific antibody against acetaldehyde-modified protein epitopes was used to see the possible formation of acetaldehyde protein adducts (APA) in brain and liver during lifelong ethanol exposure. In 40% (2 out of 5) of the ethanol-exposed AA rats and in 50% (2 out of 4) of the ethanol-exposed ANA rats, an APA-immunopositive reaction was observed in various brain areas. The comparison of the staining intensities between the ethanol fed AA (0.75 ± 0.5) and ANA rats (0.94 ± 0.77) to the corresponding controls (AA; 0.30 ± 0.35, ANA; 0.22 ± 0.21) showed, however, no statistically significant group difference. Despite this, the qualitative observations of positive APA-IR in some of the ethanol-exposed rats need to

be described. In the molecular layer of the cerebellum APA-IR was localized in endothelial cells and interneurons and/or glial cells. Purkinje-cells and granule cells were devoid of staining. In the frontal cortex the highest APA-IR was found in the white matter, where round clusters of staining were found. In the gray matter area of the frontal cortex, longitudinal structures of APA-IR were found as well as some faintly stained large neurons in layers 4 and 5. The observed APA-IR was strongest among the ethanol-exposed ANA rat whose average daily intake of ethanol was only 2.6 g/kg. All the animals showing APA-IR in the brain displayed also a faint APA-IR in the liver. In the liver the staining was localized in centrilobular hepatocytes, sinusoids and capillary endothelium.

Discussion

1 Methodological aspects

All histological, immunohistochemical and morphometric procedures were done blindly, i.e., the person in charge did not know the group the animal belonged to. Thus, there is no bias due to this.

1.1 Animal model and ethanol consumption

The AA and ANA rats were originally developed for high and low levels of ethanol consumption in a free choice situation between ethanol and water (Eriksson 1968, Eriksson and Rusi 1981). For the present studies it was decided that ethanol would be given as the only available fluid, because otherwise the daily ethanol consumption of the ANA line would be less than 1 g/kg/day, which is very unlikely to cause any structural alterations in the brain. The reason why the animals were given 12% (vol./vol.) ethanol was based on the pilot studies, which indicated that 15% ethanol solution was too strong especially for the ANA rats and resulted in dehydration and higher mortality. However, the suitability of the ANA rats for these studies had some limitations, because some of the ANA rats died before the end of the experiment and displayed signs of renal pathology at autopsy. Therefore, we cannot exclude the possibility that the remaining rats used in the measurements were selected on the basis of a renal function, which could have had an effect on brain pathology.

During the forced ethanol exposure of the present studies AA rats consumed approximately the same quantities of ethanol as in a free choice situation (Eriksson 1968) indicating that in the AA line the preference for ethanol is very high. It has been shown that during voluntary ethanol consumption the mean daily BEC for the AA rats is only 7.8 mmol/l, which is well below the level considered to be intoxicating in rats (Aalto 1986). Therefore, the drinking of these animals may not be sufficient to produce major brain pathologies comparable to those found in human alcoholics, whose

drinking is more of a intoxication-withdrawal type. Taken together, our animal model does not necessarily resemble the typical human drinking behaviour, which must be taken into account when applying these results to human alcoholics.

The ANA rats consumed roughly the same quantities of ethanol as the AA rats during the 'forced' lifelong ethanol exposure, which is surprising considering the significant line difference when these rats are allowed to choose between water and ethanol. One possible explanation for such a high ethanol intake among the ANA rats could be that these rats simply drank ethanol in order to stay alive, i.e. to prevent dehydration.

The observed higher ethanol intakes among the female rats in both lines, could, in turn, relate to higher ethanol metabolism and clearance of ethanol from the blood and liver (Eriksson 1973). However, it has been shown that in the brain the activity of aldehyde dehydrogenase, which breaks down acetaldehyde, shows no difference between the AA and ANA rats or between male and female rats, respectively (Inoue et al. 1981), arguing against the role of different central acetaldehyde metabolism as a cause of gender difference in the drinking behaviour.

1.2 Morphometric methods

The quantitative measurements of the cerebellar layer volumes and the LC neurons numbers were performed by using systematic sampling (Gundersen and Jensen 1987) and disector methods (Sterio 1984, Gundersen 1986, Gundersen et al. 1988). These methods apply three-dimensional stereology to yield the total number of particles in contrast to the previous methods which employed a conventional two-dimensional probe, providing, for example, the number of profiles in a given area (areal density) (Coggeshall 1992). The latter method can be regarded as useful, if certain assumptions are justified. These assumptions are that measured particles are equal in size and shape and have similar probability of being counted in a section. Usually, however, this is not the case, because most often particle size varies significantly and the shape is not uniform. This results in overpresentation of bigger particles and under-

estimation of smaller particles. In the nervous system, for example, this means that neuronal atrophy is considered as neuron loss, because there are fewer neuronal profiles in a given area. This can be prevented by using a three-dimensional disector probe, which compares two physical or optical sections obtained at regular intervals in a frame of known area, as described earlier in the methods section. These well-validated methods are currently the methods of choice in neuroanatomy, because they are not biased by fixation artifacts, section orientation or section thickness on the slides usually found in the assumption-based methods (Sterio 1984, Gundersen and Jensen 1987, Gundersen et al. 1988, Pakkenberg and Gundersen 1988, West 1994).

In addition to the stereological methods, a quantitative immunohistochemical method was used in the measurement of the areal density of GFAP-IR structures in the cerebellar layers. Until recently, quantitative approach to estimate differences in the immunohistochemically stained sections has not been widely used, because of the difficulties in controlling the staining intensity, which usually arises from inconsistent tissue fixation and unequal distribution and penetration of antibodies. However, a recent study has shown (Huang et al. 1996) that with uniform tissue sampling and simultaneous immunohistochemical procedure, this method can provide reliable and accurate estimates of the antigenic concentrations in a section. Furthermore, computer-assisted image analysis greatly facilitates the measurements of regional variation in optical density (Huang et al. 1996). For the measurement of GFAP-IR structures in the cerebellum in the present study a method similar to that described by Shetty and Phillips (1992) was used. For tissue preparation, mid-vermis sections of the same thickness were cut by a microtome. Based on pilot experiments, an immunostaining which gave the well-contrasted GFAP-IR structures was chosen. Using this method, all the sections from midvermis were processed for immunohistochemistry simultaneously and were coded. The coefficient of error (SEM/mean) for the young controls in all cerebellar layers remained less than 10%, which indicates rather low standard errors.

2 Chronic ethanol effects on rat cerebellum – comparison with human studies

21 months of continuous consumption of ethanol in the AA and ANA rats of either gender did not cause a significant cerebellar atrophy, which is surprising considering the duration of the ethanol exposure. Only a covariate analysis taking into account the lower ethanol intake of the ANA female rats showed that there is a greater atrophy in folium 2 of the ANA female rats compared with the AA female rats. This suggests that the ANA line may be more prone to ethanol-induced damage in the cerebellar cortex than the AA line. The observed line difference in the cerebellar vulnerability to chronic ethanol exposure is not clear, but could be due to a genetic resistance of the AA line against ethanol-induced tissue injury developed during selection, i.e. selection favoring the highest drinking animals with the greatest resistance against tissue damage. Another explanation might be that the ANA-line produces locally higher acetaldehyde levels upon ethanol oxidation in the cerebellar cortex and results in atrophy. Interestingly, the ethanol-inducible CYP450IIE1 shows strong immunoreactivity in the molecular layer of cerebellum after chronic ethanol feeding (Hansson et al. 1990). Perhaps toxic levels of acetaldehyde in the cerebellar cortex of the ANA female rats could be formed by increased expression of CYP450IIE1.

A previous study with Sprague-Dawley rats has shown a 18% volume decrease in the molecular layer after 6 months of ethanol (9 g/kg/d) consumption and a 8% decrease in the volume of the granular layer after 3 months of ethanol consumption (Tavares et al. 1987) suggesting that the granular layer is most easily damaged due to chronic ethanol exposure in experimental animals. In human alcoholics, however, areal density measurements from the cerebellar vermis showed a 39% and 10% decrease in the molecular layer and granular layer, respectively, in folium IV (Phillips et al. 1987) indicating that the molecular layer is more affected in chronic alcoholics. In the ethanol-exposed ANA females with the average ethanol intake of 5.4 g/kg/d the marginal decrease in the volume of the molecular and granular layers seemed to be of the same magnitude, suggesting equal sensitivity of these layers to chronic ethanol in the ANA female rats. A recent experiment found, however,

no change in the total number of granule cells or in the volume of granular layer in the Fischer F344 rats after 10–12 months of ethanol consumption (Tabbaa et al. 1999) in line with the results from the AA and ANA male rats. Despite the differences in the cerebellar areas (whole cerebellum vs. vermis) and ethanol consumption in these studies the most likely explanation for the discrepancies arises from the different morphometric methods. For example, in the studies by Tabbaa et al. (1999) and the studies on the AA and ANA rats an unbiased stereological counting method was used, which provides more consistent and reliable results compared with the conventional counting methods. One should also be careful when comparing the results between human studies and animal studies. For example, in alcoholics the cerebellar damage is often worsened by malnutrition and liver cirrhosis (Harper et al. 1985), two factors usually absent from rats fed with chronic ethanol. Only when the rats are exposed to another hepatotoxin, such as carbon tetrachloride (Bosma et al. 1988) or high fat-diet (Tsukamoto et al. 1986) together with chronic ethanol, the liver damage can be induced. This assumption is in line with a recent stereological study, which showed a loss of Purkinje neurons and a decrease in the volume of the molecular layer and the white matter layer only in the alcoholics who displayed clinical signs of Wernicke's encephalopathy and decreased levels of thiamine in the blood (Baker et al. 1999). It is therefore suggested that in human alcoholics and in experimental animals chronic ethanol exposure per se does not produce significant cerebellar atrophy.

Purkinje cell dendrites and synapses may be the first and principal targets for ethanol-induced alterations in cerebellum as there are several reports showing alterations in the density of Purkinje cell dendritic network following long-term ethanol exposure (Pentney 1982, Tavares et al. 1983, Ferrer et al. 1984, Pentney and Quickley 1987, Tavares et al. 1987, Pentney 1995). For example, in the molecular layer ethanol interferes with the microtubules of the Purkinje cell dendrites. The number of microtubules was markedly decreased after 1 month of ethanol exposure in the adult rat cerebellar cortex (Paula-Barbosa and Tavares 1985). The only two studies, so far, on the effects of ethanol on the cerebellar white matter found a 23% decrease in the white matter area of folium IV in human alcoholics compared with the controls (Phillips et al. 1987) or a 42% reduction in the white matter of the vermis only in alcoholics having ataxia

(Baker et al. 1999). In the ethanol-exposed AA and ANA rats, the volume of the central white matter remained unchanged indicating that the central white matter of the cerebellum is not severely affected by chronic ethanol in adequately fed animals.

3 Chronic ethanol effects on rat locus coeruleus – comparison with human studies

The 26–30% decrease in the total number of LC neurons following lifelong ethanol consumption in the AA rats and in the ANA female rats indicates that the noradrenergic neurons in the rat brain are more easily damaged by chronic ethanol than the cerebellar cortex. In addition, adrenergic neurons of the rat central nervous system appear to be more vulnerable to chronic ethanol than the peripheral neurons, because lifelong ethanol exposure of the AA and ANA rats had no effect on the total number of neurons in the superior cervical ganglion (Riikonen et al. 1999). Previously, decreased synapse-to-neuron ratio was reported in rat LC after 4 months of ethanol exposure (Kjellström et al. 1993). On the basis of a recent study (Riihioja et al. 1999a) even a shorter ethanol exposure seems to damage the LC neurons, i.e. a five-week intermittent (4 days ethanol, 3 days withdrawal period) ethanol feeding resulted in a 24% neuron loss in young male Wistar rats. The loss of LC neurons in the ethanol-exposed AA males (30%) and females (26%) and ANA females (30%) following chronic ethanol consumption is in agreement with these experimental studies together with a human study reporting a 23% decrease in LC neurons in chronic alcoholics by using conventional counting methods (Arango et al. 1994). However, two earlier reports found no change in the number of LC neurons in alcoholics with or without Korsakoff's syndrome (Halliday et al. 1992a, Baker et al. 1994). The low number of control subjects and a different morphometric method in the latter studies are the probable causes for the 'no change' results. In contrast to the results obtained from the cerebellum (I), no line or sex differences in the vulnerability of LC neurons to chronic ethanol were found in the AA or ANA rats if the differences in ethanol intake were taken into account. This means that the male gender or the AA line does not protect LC neurons from the neurotoxicity of chronic ethanol.

The pathogenetic mechanism of LC vulnerability has not been established, but ethanol-induced overactivity of adrenergic neurons, enhanced auto-oxidation of catecholamines and production of cytotoxic oxygen free radicals and semiquinones have been suggested (Graham 1978). Aminergic neurons, such as the LC neurons may be more susceptible to acetaldehyde-induced neuronal damage because these neurons produce locally higher levels of acetaldehyde due to high catalase (Moreno et al. 1995, Zimatkin and Lindros 1996) and low aldehyde dehydrogenase activities (Zimatkin et al. 1992). In addition, the ependymal cells lining the fourth ventricle in close proximity to the LC may be more easily damaged by chronic ethanol because of the high catalase activity (Moreno et al. 1995) thereby disrupting the blood-brain-barrier. In alcoholics, reduced levels of noradrenaline have been detected in the hippocampus and the cingulate cortex, (Carlsson et al. 1980) areas with wide efferent projections from the LC (Grzanna and Molliver 1980, Foote et al. 1983). Therefore, the LC neuron loss induced by long-term ethanol may contribute to memory disorders or dementia in alcoholics, which is in line with several reports showing decreased numbers of LC neurons in Alzheimer's disease (Bondareff et al. 1983, Iversen et al. 1983, Marcyniuk et al. 1986, Chan-Palay and Asan 1989). Similarly, in depression and in depressive alcoholics the number of LC neurons has been shown to be reduced, as well (Arango et al. 1996).

4 Chronic ethanol effects on GFAP-IR in cerebellum

The GFAP-IR fibers located in the molecular layer of the cerebellar vermis decreased dose-dependently in the ethanol-exposed (>5.1 g/kg/day) AA female rats indicating that the Bergmann glial cells are vulnerable to long-term moderate-to-heavy ethanol consumption, at least in female rats. In the granular layer or in the central white matter no ethanol-induced alterations of GFAP-IR were found indicating that the GFA protein of fibrous astrocytes in the deeper cerebellar layers is not damaged by chronic ethanol. Previously, rat pups obtained from pregnant rats were exposed to 6.7% ethanol during gestation and a marked decrease in GFAP-IR in the cerebellar vermis on postnatal days 15 and 22 was found (Shetty and Phillips 1992). On postnatal day 15 GFAP-IR of the

Bergmann glial fibers was markedly lower in the molecular layer in the ethanol-exposed rats compared with the controls, but at the same time there was an ethanol-induced increase in GFAP-IR in the granular and white matter layers (Shetty and Phillips 1992) in line with our findings from adult rats. Interestingly, on postnatal day 22 the GFAP-IR differences of rat pups had disappeared indicating rapid corrections in GFAP-IR during the third postnatal week (Shetty and Phillips 1992). Studies from the adult rat hippocampus have also shown that exposure to 10% ethanol for 9 months causes a marked decrease in GFAP-IR (Franke 1995). Surprisingly, consumption of 5% ethanol over 9 months was not able to cause any change in GFAP-IR in the hippocampus, and in fact, consumption of 10% ethanol for 1–3 months caused an increase in GFAP-IR (Franke 1995). These studies indicate that the amount of GFAP protein can show rapid and even opposite alterations in the same brain areas depending on the duration and timing of the ethanol exposure. It is also suggested that in the adult brain long-term moderate-to-heavy ethanol consumption decreases the amount of GFA protein while a shorter (up to 3 months) exposure increases it. The mechanism by which ethanol interferes with the GFAP level in the Bergmann glia is not clear, but it may be due to a disruption of the transcriptional regulation of GFAP gene (Valles et al. 1997). In addition, higher levels of acetaldehyde, the toxic metabolite of ethanol, or reactive oxygen species could be formed locally by CYP450IIE1 or catalase (Hansson et al. 1990, Gill et al. 1992, Montoliu et al. 1995) thereby interfering with the transcriptional regulation of GFAP production or decreasing the stability of GFAP. As no effect of lifelong ethanol consumption on cerebellar atrophy was found in the AA rats of either gender (I), while there was a marked decrease in GFAP-IR in the Bergmann glial fibers of the female rats, it can be assumed that the glial changes precede the volumetric changes of cerebellum, but probably do not markedly contribute to it. Support for the assumption that glial cells are more easily damaged by chronic ethanol than neurons gives a recent stereological study showing a 37% decrease in the total number of glial cells, but not in the number of neurons, in the hippocampus of human alcoholics (Korbo 1999).

5 Effect of age on ethanol-induced neuropathology

An age-related decrease in the molecular layer volume has been reported (Dlugos and Pentney 1997). In the old AA and ANA rats the volumes of the molecular layer were slightly, but consistently lower than in the young rats. However, the differences did not reach statistical significance in any of the folia studied. Contrary to the molecular layer, there was a tendency towards age-related increase in the volume of the granular layer and in the central white matter. The age-related increase was significant in the granular layer of folium 4 for the AA males, and in the central white matter of folium 10 for the ANA males and females. Taken together, the volume changes in the granular layer and in the central white matter with aging were not similar to those seen with lifelong ethanol exposure. Although the volume of the molecular layer tended to decrease both with aging and due to ethanol, no significant interaction was found. Similarly to the results of the AA and ANA rats, morphological age-related alterations in the Purkinje-cell dendrites have been different from those seen in the ethanol-exposed animals. In the old, but not in the young Fischer F344 rats, the Purkinje cell dendritic arbors reduced in size due to chronic ethanol (Pentney 1982), but in the old control rats the mean length of terminal segments in Purkinje cell dendrites decreased contrary to the increase found in old ethanol-exposed rats (Pentney and Quiqley 1987, Pentney and Quacken-bush 1990, Pentney 1995). In addition, there was an irreversible age-related decrease in the synaptic density of the Purkinje cell dendrites in the old controls compared with the compensatory increase in dendrites after recovery or abstinence from long-term ethanol consumption in the ethanol-exposed rats (Dlugos and Pentney 1997).

The number of LC neurons showed no age-related decrease in the AA or ANA rats of either gender. Similarly, a recent study (Riihioja et al. 1999b) found no difference in the LC neuron number between old (29–30 months) and young (3–4 months) male Wistar rats. Previously, the effects of aging on the morphology and number of LC neurons in humans and in rodents have provided inconsistent results, some finding a marked neuron loss (Vijayshankar and Brody 1979, Mann 1983, Yoshinaga 1986, Marcyniuk et al. 1989) while others don't (Goldman and Coleman 1980, Mouton et al. 1994). Although subtle

changes in the experimental design can be found in these studies it is likely that the lack of an unbiased counting method contributes to most of the discrepancies. However, all the previous studies and the present ones using unbiased stereology found no change with age in the total number of LC neurons (Mouton et al. 1994, Riihioja et al. 1999b). It is therefore concluded that aging per se does not reduce the total number of LC neurons in humans or in experimental animals. Interestingly, 5 weeks of heavy intermittent ethanol feeding of young and old Wistar rats decreased the number of LC neurons only in the old rats (Riihioja et al. 1999b) indicating that withdrawal from ethanol is more deleterious to older than younger noradrenergic neurons.

In the male AA rats there was an age-related decrease in GFAP-IR in the molecular layer and an age-related increase in the granular layer indicating opposite age-related alterations in GFAP-IR in adjacent brain regions. The reason why female AA rats displayed no age-related alterations in GFAP-IR is not known, but it could be due to a regulatory role of sex steroid hormones on GFAP (Laping et al. 1994b). Of the three cerebellar layers the central white matter showed no age-related change in GFAP-IR in contrast with an earlier study (Sabbatini et al. 1999), which showed increased GFAP-IR in the cerebellar white matter of the aging (3, 12 and 24 months) male Wistar rats. However, an age-related accumulation of GFAP-IR in the granular layer similarly to the increase in GFAP-IR in the AA males, was found (Sabbatini et al. 1999). Most of the previous studies on human and rodent brain have shown that GFAP mRNA and protein content increases with age. In rodents an age-associated increase in GFAP-IR has been reported in the cerebellum, hippocampus, striatum, internal capsule, corpus callosum, optic tract, and in the supraoptic and cochlear nuclei (Bignami and Dahl 1974, Björklund et al. 1985, Goss et al. 1991, O'Callaghan and Miller 1991, Wagner et al. 1993, Linneman and Skarfelt 1994, Berciano et al. 1995, Jalenques et al. 1995, Kohama et al. 1995, Yoshida et al. 1996, Morgan et al. 1999, Sabbatini et al. 1999). In humans an age-related increase in GFAP-IR has been reported in the hippocampus, as well as in the frontal, temporal and entorhinal cortices (Nichols et al. 1993, David et al. 1997). Based on the results of the present study, it is suggested that the molecular layer of the cerebellum is the only brain area, so far, to show age-related attenuation of GFAP. Transcriptional control of the GFAP gene (inhibition or

stimulation) has been suggested to be the site of action in the age-related regulation of GFAP (Yoshida et al. 1996, Morgan et al. 1999), although no age-related change in the transcription rate was found in one study (Laping et al. 1994a). Interestingly, caloric restriction can attenuate the mRNA and protein synthesis of GFA in rats (Morgan et al. 1999). The attenuation of the GFAP-IR in the molecular layer of the ethanol-exposed AA female rats most likely results from the effects of ethanol and not from the differences in the caloric intake in these rats, because the total amount of calories did not differ between the ethanol-exposed and the control rats of either gender (Sarviharju et al. 2001). No additive effect of age and ethanol on the GFAP-IR was found in the cerebellar layers studied indicating that these changes occur independently from each other.

Taken together, the effect of aging on the cerebellar layer volumes, LC neuron numbers and GFAP-IR in the cerebellum was different from those induced by lifelong ethanol exposure. Therefore, these experimental data do not lend support to the neuroimaging studies in human alcoholics, which found a cumulative effect of long-term ethanol on age-related structural brain alterations (Hayakawa et al. 1992, Pfefferbaum et al. 1992, Pfefferbaum et al. 1993, Sullivan et al. 1995). In addition, the reversibility of white matter volume loss in abstinent alcoholics, but not in aged individuals argues against the additive effect of aging and chronic ethanol on brain (Carlen and Wilkinson 1987). There are, however, obvious limitations when comparing the results from human studies to the present studies in the AA and ANA rats. For example, in human studies the interactions of age and ethanol were mostly found in the anterior hippocampus (Sullivan et al. 1995) and the frontal lobes (Pfefferbaum et al. 1997), areas which were not included in our studies. In addition, the ethanol consumption of human alcoholics does not necessarily resemble the continuous even exposure to ethanol in the AA and ANA rats, i.e., in human alcoholics binge-type drinking and repeated intoxication-withdrawal cycles are more often seen, which, in turn, could have a more profound effect on CNS pathology in aged alcoholics.

6 Effect of gender on ethanol-induced neuropathology

In the ethanol-exposed AA and ANA rats there was no gender difference in the number of LC neurons. Neither was there any gender difference in the GFAP-IR of cerebellum in the AA rats taking into consideration the higher ethanol intake of the female rats. However, the volume decrease in the molecular layer of the ANA female rats was significant compared with the ANA male rats, indicating a gender difference in the vulnerability of cerebellar cortex to chronic ethanol. Because this gender difference was only observed in the ANA line but not in the AA line, it means that the different genetic background and ethanol metabolism of the ANA line is likely to contribute to ethanol sensitivity in cerebellum. Between the male and female Sprague-Dawley rats, however, a 20-week exposure to 20% ethanol resulted in the same degree of tissue shrinkage in the frontal cortical and corpus callosal thicknesses (Savage et al. 2000). Excluding the cerebellum of the ANA line, there seems to be no gender difference in the ethanol-induced neuropathology in experimental animals. This disagrees with several studies on human alcoholics showing greater vulnerability of the female brain to chronic ethanol exposure (Jacobson 1986, Harper et al. 1990, Mann et al. 1992, Agartz et al. 1999, Pfefferbaum et al. 2001). Although the reason for these contrasting results is not known, factors such as differences between species in: a) first pass metabolism of ethanol (Frezza et al. 1990, Smith et al. 1992), b) sensitivity of liver to ethanol-induced tissue damage (Harper et al. 1985, Tsukamoto et al. 1986), c) thiamine-related brain pathology (Baker et al. 1999, Tabbaa et al. 1999), could all account for a greater degree of tissue damage in brains of women alcoholics compared with female animals chronically exposed to ethanol.

7 Acetaldehyde protein adducts – implications for ethanol-induced neuropathology

One of the suggested mechanisms by which chronic ethanol exposure can cause neuronal degeneration is through the formation of acetaldehyde (Hunt 1996). Although acetaldehyde is 10 times more toxic than ethanol, there is as yet

no evidence that biologically relevant amounts of acetaldehyde can enter the brain from the circulation or be produced by ethanol metabolizing enzymes in situ (Lindros 1978). The lack of acetaldehyde in the CNS has been attributed to an efficient blood-brain-barrier to acetaldehyde or the low capacity of enzymes, such as alcohol dehydrogenase, catalase or CYP450IIE1 to metabolize ethanol in the brain (Lindros 1978, Tabakoff and Gelpke 1975, Eriksson and Sippel 1977, Coon and Koop 1987, Hansson et al. 1990). One of the possible mechanisms by which acetaldehyde could produce brain tissue alterations is through formation of covalent bonds with brain proteins, thereby producing acetaldehyde protein adducts (APA), which in turn, might trigger an autoimmune response similar to that found in peripheral tissues (Israel et al. 1986, Tuma and Klassen 1992). For example, acetaldehyde binding to various tissue proteins, such as albumin and erytrocyte proteins (Israel et al. 1986, Tuma et al. 1987, Hernandez-Munoz et al. 1989), lipoproteins (Wehr et al. 1993, Lin et al. 1995), and cytochrome enzymes (Behrens et al. 1988, French et al. 1993) has been previously demonstrated. Furthermore, APA formation has been previously detected by immunohistochemistry in the liver of ethanol-exposed rats (Lin et al. 1988, Lin et al. 1993, French et al. 1993, Nicholls et al. 1994), micropigs (Halsted et al. 1993, Niemelä et al. 1995) and human alcoholics (Niemelä et al. 1991, Holstege et al. 1994). Using an immunohistochemical method we were able to detect, for the first time, APA-IR structures in the brain of some (44%), but not all animals exposed to ethanol. Although the staining intensity did not differ significantly between the ethanol-exposed rats and the control rats, these results are suggestive for APA formation in the brain during long-term ethanol exposure. Distinctive APA-IR was found in the cerebellum, frontal cortex and liver. In the cerebellum the staining was found in the molecular layer probably localized in cerebellar interneurons, glial cells or capillary endothelium. Purkinje cell somata were devoid of staining together with the granule cells. In the frontal cortex, only a few large pyramidal neurons in layers 4 and 5 displayed faint immunopositive staining. The strongest APA-IR was seen, however, in the central white matter and in the deeper cortical layers of the frontal cortex. Although the exact site for the APA-IR structure in the frontal cortex could not be determined, it is suggested on the basis of the staining pattern that neuropil (axons and dendrites) of both gray and white matter are likely to be involved. In the liver,

APA-IR was evident in the centrilobular hepatocytes and in sinusoids and capillary walls, in line with several previous studies (Lin et al. 1988, Lin et al. 1993, French et al. 1993, Nichols et al. 1992, Nichols et al. 1994, Niemelä et al. 1991, Halsted et al. 1993, Holstege et al. 1994, Niemelä et al. 1994, Niemelä et al. 1995, Niemelä et al. 1998). In vitro assays with higher than physiological levels of acetaldehyde have shown that acetaldehyde binds to liver (Jennett et al. 1989) and brain microtubular proteins (McKinnon et al. 1987) and can inhibit microtubule polymerization (Smith et al. 1989). The exact site of acetaldehyde binding to these structures is not clear, but free ε-aminolysine or α-amino groups have been suggested (Tuma et al. 1987). By binding to microtubules acetaldehyde might interfere with the axo-dendritic transport of organelles, nutrients and neurotransmitters from the cell body to synapses or vice versa, eventually leading to neurodegenerative changes. For example, in the cerebellum acetaldehyde might bind to microtubules of Purkinje-cell dendrites and cause dendritic regression typically seen in the molecular layer of the cerebellum in animals exposed to ethanol (Paula-Barbosa and Tavares 1985, Pentney 1995). This is in line with our observation of positive APA-IR in the molecular layer of the cerebellum in AA and ANA rats. Astroglial damage by acetaldehyde could also contribute to brain pathology because acetaldehyde, but not ethanol, increases intracellular calcium levels by 155% in cultured astrocytes and causes marked DNA fragmentation (Holownia et al. 1999). Time-dependent increases in activities of catalase and antioxidant enzymes (super-oxide dismutase and glutathione peroxidase) together with increased levels of acetaldehyde have also been measured from cultured astrocytes exposed chronically to ethanol, indicating that acetaldehyde is related to enhanced production of oxygen free radicals (Eysseric et al. 2000). In future studies, acetaldehyde binding to GFAP *in vivo*, for example, would be important to show in order to get support for the observed decrease in GFAP-IR in the molecular layer of the cerebellum in the ethanol-exposed AA females. In addition, APA-IR structures should also be demonstrated from the brains of alcoholics in order to get further support for the role of acetaldehyde in the pathophysiology of alcoholic brain damage.

Summary

1. In the AA and ANA male rats, the 21-month exposure to 10–12% (vol./vol.) ethanol did not result in significant cerebellar atrophy. However, using the ethanol intake as a covariate, the female ANA rats displayed greater atrophy of folium 2 in the molecular and the granular layers compared with the AA female rats, indicating a line difference in the cerebellar vulnerability to lifelong ethanol consumption.
2. The effects of aging on the volume of cerebellar layers were different from those induced by lifelong ethanol. In the granular and central white matter layers the volumes tended to increase with age, whereas lifelong ethanol exposure had no effect on the volumes of these vermian layers.
3. Lifelong ethanol consumption caused a significant loss of LC neurons in the male (30%) and female (26%) AA rats and in the female (30%) ANA rats. Aging, however, had no effect on the total number of LC neurons in the AA and ANA rats of either gender. These results indicate that the LC of the AA and ANA rats is more easily damaged by chronic ethanol than the cerebellar cortex.
4. GFAP-IR of the molecular layer in folium 2, 7 and 10 decreased significantly with age in the male, but not in the female rats of the AA line. However, lifelong moderate-to-heavy (>5.1 g/kg/d) ethanol consumption caused a dose-dependent attenuation of GFAP-IR of the Bergmann glial fibers in the molecular layer of the AA female rats. No effect of ethanol was found in GFAP-IR in the granular and central white matter layers contrary to an age-related accumulation of GFAP-IR (only males) in these layers indicating different changes with age and ethanol on GFAP-IR in cerebellum. Bergmann glial fibers were found to be the most sensitive GFAP-IR structures of adult rat cerebellum to long term ethanol consumption.
5. Lifelong ethanol consumption produced APA in the gray and white matter areas of the frontal cortex and in the cerebellar cortex of some, but not all, ethanol-exposed AA and ANA rats. This is the first report to show that APA can be formed in the brain *in vivo* during long-term ethanol consumption.

6. The only line and gender difference in the ethanol-induced vulnerability of the aging CNS was found in the cerebellar cortex of the ANA females, where the volume of the molecular and granular layer in folium II decreased significantly compared with the AA females ('line difference') and was also lower in the molecular layer of folium II compared with the ANA males ('sex difference'). The ethanol-induced loss of LC was of similar magnitude in the AA and ANA rats of both genders using ethanol intake as a covariate. This implies no gender or line differences in the vulnerability of the LC to chronic ethanol. Although the GFAP-IR decreased significantly in the molecular layer of the ethanol-exposed AA females, but not in the AA males, the covariate analysis showed that this decrease was due to the higher ethanol intake of the female rats rather than the gender itself.

Conclusions

The morphological alterations of the rat brain induced by lifelong ethanol consumption differed considerably from those induced by aging, providing no support for the ethanol-induced premature aging hypothesis of the brain (Courville 1955). Therefore, chronic ethanol exposure does not seem to be a suitable experimental model for aging studies in the brain.

In the brains of the AA and ANA rats lifelong ethanol exposure resulted in a marked loss of the LC neurons, but caused no cerebellar atrophy indicating differential regional vulnerability of rat brain to chronic ethanol. In the cerebellum, however, the GFAP-IR decreased dose-dependently by moderate-to-heavy chronic ethanol consumption in the molecular layer indicating that the major protein constituent of the intermediate filament of the Bergmann glial cells is prone to be damaged due to chronic ethanol exposure.

Acetaldehyde protein adducts could be detected by immunohisto-chemistry in the brains of the ethanol-exposed AA and ANA rats. The strongest immunoreactivity was found in the frontal cortex and white matter as well as in the molecular layer of cerebellum, i.e. in areas where ethanol-induced degenerative changes are found in chronic alcoholics or in experimental animals chronically exposed to long-term ethanol. It is therefore suggested that acetaldehyde could be involved in the ethanol-induced neuropathology through formation of adducts with brain proteins.

Acknowledgements

This study was carried out at the School of Public Health and at the Medical School, University of Tampere during the years 1996–2001.

First of all, I express my sincere gratitude to Professor Antti Hervonen, M.D., Ph.D., Chief of the Laboratory of Gerontology, who encouraged me to the aging research and introduced the challenging field of brain studies. His quidance and support as a researcher and his example as a teacher have been of great importance to me. I am also deeply indebted to Antti for providing an opportunity to spend a two-year period (1998–2000) as a Visiting Fellow at the National Institutes of Health in Bethesda, Maryland.

I express my warmest thanks to Docent Pia Jaatinen, M.D., Ph.D., who patiently supervised and helped me through all the 'blocks on a road' to complete this thesis. Her expertise in the field of alcohol research and ability to solve all the technical problems of the laboratory work have been of crucial significance to the studies. In addition, her tireless and careful corrections of the manuscripts have been irreplaceable, for which I am very thankful. I also admire her silent observational disposition combined with her good sense of humor.

My special thanks are due to Professor Kalervo Kiianmaa, Ph.D., and Maija Sarviharju, M.A., for their work and collaboration in carrying through the 'lifelong' ethanol experiments with the AA and ANA rats at the Department of Mental Health and Alcohol Research, National Public Health Institute, Helsinki. I also want to thank Docent Peter Eriksson, Ph.D., from the same department for his comments and advice in preparing the manuscripts.

I wish to express my sincere thanks to Professor Onni Niemelä, M.D., Ph.D., and Professor Seppo Parkkila, M.D., Ph.D., for their work and collaboration in the study which provided evidence for the acetaldehyde-adduct formation in the brains of ethanol exposed animals.

Professor Kaija Seppä, M.D., Ph.D., and Docent Petri Hyytiä, Ph.D., are warmly thanked for the careful and prompt review of the thesis. Their suggestions and comments were of great help to me and considerably improved this thesis.

The work and help of Jarno Riikonen, M.D., and Lu Wei, M.D., who were also co-authors, is greatly appreciated. Olli Kajander, M.D., Ph.D., and Päivi Riihioja, M.D., are thanked for their help in collecting and preparing the brain samples. In addition, Jarno, Olli, Päivi and Lu are all thanked for their support and friendship.

I wish to express my deepest gratitude to Ms Oili Kemppainen for her hard work at the laboratory during these years. Without the skills and devotion of Oili to several of the laborious steps in the preparation of brains for morphometry and immunohistochemistry this thesis would have never been materialized. Her straighforward positive attitude towards work and life in general have been very inspiring and important.

The language of this study was revised by Esko Väyrynen, M.A., to whom I express my deep gratitude.

I want to express my sincere thanks to Professor Simo Oja, M.D., Ph.D., for providing an opportunity to work as a researcher for 5 months at the International Graduate School in Neuroscience at the Medical School of Tampere.

The assistance of the following persons is greatly acknowledged: Professor Pekka Laippala, Ph.D., for help in statistics, Mrs Heli Koivisto for help in technical completion of the manuscripts and in solving the computer problems, Ms Nina Mäkinen and Mrs Marita Hallila for assistance in the layout of this study, Mr Mark Ollikainen for doing the excel macro programs for the first study, Mrs Raili Lepistö and Mrs Kirsti Pinomäki for being at the office when and whatever needed. The help from Tomi Antikainen and Anja Kylkilahti is also appreciated.

I am also thankful for the happy moments and interesting discussions in our coffee room with several people including Marja Jylhä, Tapio Kirsi, Outi Jolanki, Kari Kuure, Kirsti Nitz, Ritva Rautiainen, Kirsi Mäkinen, Merja Junttila, Katja Rämö, Minna Koskinen, Minna Ylimys and Eija Pirttiniemi.

The staff and colleaques from the Section of Brain Physiology at the National Institutes of Health, National Institutes on Aging, Bethesda, Maryland, are warmly thanked for their friendship and collaboration during the year 1998-2000. Especially I want to mention Professor Stanley I Rapoport, M.D., Ph.D, Michael CJ Chang,, Ph.D., and Miguel A Contreras, Ph.D., for their supervision and quidance in the research area, which provided new information of the effects of mood-stabilizers on brain phospholipids. Their enthusiasm and dedication to research were inspiring and greatly deepened my knowledge on brain functioning.

I wish to express my deepest thanks to Docent Jari Parkkari, M.D., Ph.D., whose support and encouragement in finishing this study have been very important. His ability to put the science into the right perspective in everyday life makes him an exceptional friend, for which I am grateful. I want to express my sincere thanks to Arto Palmu, M.D., Ph.D., for his help in the final steps of this thesis. I also thank Arto for being an excellent friend and neighbour. Jari Hakomäki, M.D., Lauri Seinelä, M.D., and Mikael Lundström, B.Sc.(Econ.), are thanked for their encouragement and friendship on and off the golf-course.

The colleaques at the Department of General Practice, Medical School of Tampere, are thanked for their support.

I sincerely thank my parents Pirkko and Ossi for their love and support. They have always encouraged me in their own way to finish all the work that has been started.

I also want to thank my dear sister Jarja, and Timo, Anna, Lauri, Aino-mamma, Onni-setä, Helmi, Onni, Anne, Tapio, Maria, Sanna, Marita, Harri, Kirsi, Anu and Nina for all the happy and enjoyable moments together.

Finally and above all, I want to thank my dear wife Marjo and my children Laura, Juho, Emmi and "Lyyli" for their endless love, understanding and support. Everyday they remind me of what is important in life.

The financial support from the Emil Aaltonen Foundation, the Yrjö Jahnsson Foundation, the Finnish Foundation for Alcohol Studies, the Juho Vainio Foundation, the Pirkanmaa Fund of the Finnish Cultural Foundation, the Medical Research Fund of Tampere University Hospital, the Finnish Medical Foundation, and the Science Foundation of Tampere City are greatly acknowledged.

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Tampere, December 2001

Jyrki Rintala

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Original publications