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Ethanol-induced Alterations in the Nervous System of Rat

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ACADEMIC DISSERTATION

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LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following communications, which are referred to in the text by their Roman numerals I-V.

I. Sarviharju M, Riikonen J, Jaatinen P, Sinclair D, Hervonen A and Kiianmaa K (2004): Survival of AA and ANA rats during lifelong ethanol exposure. Alcohol Clin Exp Res 28:93-97.

II. Riikonen J, Jaatinen P, Sarviharju M, Kiianmaa K and Hervonen A (1999): Effects of lifelong ethanol consumption on rat sympathetic neurons. Alcohol 17:113-118.

III. Riikonen J, Jaatinen P, Karjala K, Rintala J, Pörsti I, Wu X, Eriksson CJP and Hervonen A (1999): Effects of continuous versus intermittent ethanol exposure on rat sympathetic neurons. Alcohol Clin Exp Res 23:1245-1250.

IV. Riikonen J, Jaatinen P, Rintala J, Pörsti I, Karjala K and Hervonen A (2002): Intermittent ethanol exposure increases the number of cerebellar microglia. Alcohol Alcohol 37:421-426.

V. Jaatinen P, Riikonen J, Riihioja P, Kajander O and Hervonen A (2003): Interaction of aging and intermittent ethanol exposure on brain cytochrome c oxidase activity levels. Alcohol 29:91-100.

ABBREVIATIONS

AA	Alko, Alcohol (alcohol-preferring rat line)
ADH	alcohol dehydrogenase
ALDH	aldehyde dehydrogenase
ANA	Alko, Non-Alcohol (alcohol-avoiding rat line)
ANOVA	analysis of variance
BBB	blood brain barrier
BEC	blood ethanol concentration
CNS	central nervous system
CO	cytochrome c oxidase
CYP2E1	cytochrome P450 2E1
FIF	formaldehyde-induced histofluorescence
GABA	gamma-aminobutyric acid
GFAP	glial fibrillary acidic protein
LC	locus coeruleus
MEOS	microsomal ethanol oxidizing system
MPF	medial prefrontal cortex
NMDA	*N*-methyl-D-asparate
ROS	reactive oxygen species
SCG	superior cervical ganglion
SPSS	Statistical Package for the Social Sciences
TH	tyrosine hydroxylase
TH-IR	tyrosine hydroxylase immunoreactivity

ABSTRACT

Epidemiological studies have demonstrated that heavy alcohol consumption is related to increased mortality and to increased occurrence of cancer. Moreover, alcoholics suffer from many neurological disturbances, but in humans differences in genetic and environmental factors make the impact of ethanol consumption *per se* difficult to estimate. In experimental studies the confounding factors can be controlled. Therefore in the present study three different experimental models for chronic alcohol consumption were used: Lifelong ethanol exposure (I, II), 5½-month intermittent versus continuous exposure (III, IV), and 5-week heavy ethanol intoxications followed by weekly ethanol withdrawals (V). The experimental animals were both genders of AA (Alko, Alcohol) and ANA (Alko, Non-Alcohol) rats (I, II), young male Wistar rats (III, IV), and young and old male Wistar rats (V). The effects of ethanol consumption on superior cervical ganglion (SCG) neurons and cerebellar microglia were studied. Cytochrome oxidase activities were measured in central nervous system areas known to be affected by chronic ethanol exposure, i.e. in the locus coeruleus, frontal cortex and cerebellum. Also the ethanol-related effects on the longevity and general health of the rats were studied.

Lifelong ethanol consumption did not alter the mortality of AA or ANA rats, but increased the occurrence of malignant neoplasms. However, the mortality of ANA rats was significantly higher compared to the AA rats. The ANA rats had a higher rate of kidney diseases than the AA rats, and their autopsy showed higher rates of benign tumors and cardiovascular pathology. Lifelong ethanol exposure caused increased lipopigmentation and tyrosine hydroxylase immunoreactivity in the SCG of male rats, but did not change the number of SCG neurons or the volume of the ganglia in either AA or ANA rats. However, 5½ months of intermittent ethanol consumption decreased the number of SCG neurons by 22% compared to the continuously ethanol-exposed group and by 28% compared to the water-consuming control group. The results suggest that intermittent ethanol exposure is more harmful to the SCG neurons than continuous ethanol exposure. The number of cerebellar microglia in folium II granular layer increased after 5½ months of intermittent ethanol exposure, but no microgliosis was found if ethanol exposure was continuous suggesting that microglia may be an early marker for ethanol-induced nervous system degeneration. Cytochrome oxidase activity was decreased after 5 weeks of repeated ethanol intoxications and withdrawals in young and aged rats. Increased number of microglia and decreased activity of cytochrome oxidase may result in an increased production of reactive oxygen species. The resulting oxidative stress may have a crucial role in ethanol-induced neuronal damage.

INTRODUCTION

Ethanol is globally the third most used addictive substance after nicotine and caffeine. The popularity of ethanol is mostly based on its euphoric effects. The annual ethanol consumption in Finland was 9.3 litres of 100% ethanol per person in 2002, and the consumption has been increasing during the last decades (Yearbook of Alcohol and Drug Statistics 2003). The pattern of alcohol consumption in Finland is typically weekend drinking, meaning that alcohol is mostly consumed during weekends (Fridays and Saturdays) causing ethanol withdrawal symptoms on the day after. Incidental moderate ethanol consumption is not likely to cause significant harm to the nervous system, but chronic heavy ethanol exposure leads to neuronal degeneration in humans, for example encephalopathy, cerebral atrophy, cerebellar atrophy, fetal alcohol syndrome and polyneuropathy (Charness 1993), although wide individual variation in susceptibility can be found. The acute effects of ethanol include euphoria, disinhibition and sedation, and confusion, coma and death when the doses are high. Chronic ethanol consumption may lead to impaired learning, memory and balance of the body (Martin et al. 1986, Viktor et al. 1989). In addition, the role of nutritional deficiencies is also crucial in the development of ethanol-induced morphological and functional central nervous system (CNS) changes (Charness 1993).

Alcoholism is described in the International Classification of Diseases (10^{th} revision, 1993) as a chronic disease with a strong and frequent need to use alcohol, and a poor control of alcohol consumption in spite of its remarkable negative social, occupational and health consequences. People who suffer from alcoholism need continuously greater doses of alcohol to achieve the wanted effects, and they develop significant withdrawal symptoms when blood ethanol concentration decreases. The use of alcohol is the main concern in his/her life (ICD-10, 1993). Although alcohol addiction has a strong genetic component, environmental factors are also crucial to the development of alcoholism. These genetic, environmental and sociodemographic aspects make the studies of alcoholism difficult (Dufour 1993). Alcoholism is also related to an increased rate of nutritional deficiencies and abuse of other substances (Andreasson 1998). The large genetic and environmental variation makes the effects of long-term ethanol consumption itself difficult, if not impossible to estimate in human populations.

Due to the difficulties included in observational human studies, experimental models have become important tools in alcohol research. In experimental animal studies many of the confounding parameters can be controlled, yielding more reliable results than those achieved in human studies. The disadvantage of animal models is that the common laboratory rats voluntarily consume only small amounts of ethanol. However, an alcohol preferring AA line of rats has been developed (Eriksson 1971) offering a model to study alcohol addiction and voluntary ethanol consumption in rats. The ANA line of rats, correspondingly, avoids ethanol and prefers water in a free choice situation. These two lines of rats were used also in this series of experiments, in addition to common laboratory rats.

REVIEW OF THE LITERATURE

1. Effects of ethanol consumption on mortality and morbidity

Alcohol consumption is related to 3-9% of deaths in the Western countries (Shultz et al. 1990, Pignon and Hill 1991, Yanez et al. 1993, Holman and English 1995, Cipriani et al. 1998, Mäkelä 1998, Single et al. 1999, Sjögren et al. 2000). In Finland the number of deaths directly related to alcohol consumption was 2431 in 2002. The main causes of death were alcohol-related illnesses (cardiomyopathy, cirrhosis of the liver, illness of the pancreas, ethanol intoxication, and alcohol dependence), and accidental or violent deaths under the influence of alcohol. Alcohol consumption is also an important background factor in suicides and homicides (Yearbook of Alcohol and Drug Statistics 2003). In addition, alcohol consumption increases the number of several other illnesses, and causes indirect deaths by increasing occurrence of malignant neoplasms, psychological diseases, neurological diseases, arrhythmias and gastrointestinal haemorrhages (Sjögren et al. 2000). In Finland alcohol abuse caused 33211 hospitalizations in 2002 (Yearbook of Alcohol and Drug Statistics 2003) and 6% of all deaths (Mäkelä 1998).

There is epidemiological evidence that light to moderate ethanol consumption decreases mortality (Single et al. 1999, Sjögren et al. 2000). This is mostly due to the preventive effect of ethanol on the occurrence of coronary heart disease (Mäkelä et al. 1997, Hart et al. 1999, Dawson 2000). Therefore, the correlation between alcohol consumption and mortality is a J-shaped curve: moderate drinkers have the lowest mortality, abstainers have a slightly higher risk of death, and heavy alcohol consumption increases mortality significantly. Because alcohol consumption reduces the mortality of ischaemic heart disease, the preventive effect is most evident in the aged population, whereas the harmful effects of ethanol are more prominent than the benefits among young people (Sjögren et al. 2000). In human studies there are several confounding factors making results unreliable, such as age, sex, ethnic background, education, body mass index, smoking, quality of nutrition, pattern of drinking, social support, psychopathology and medications (Andreasson 1998). However, when these parameters have been standardized in large follow-up studies, the mortality of

moderate drinkers has been significantly lower compared to the abstainers (Thun et al. 1997, Farchi et al. 2000, Gronbaek et al. 2000). Although some studies have suggested that different beverages would have similar effect on mortality (Marques-Vidal et al. 1996, Rimm et al. 1996), a large follow-up study (The Copenhagen City Heart Study) showed a significant benefit of wine on mortality (Gronbaek et al. 1995), stroke (Truelsen et al. 1998) and dementia (Truelsen et al. 2002) compared to the consumption of beer and spirits.

Moderate ethanol consumption decreases coronary heart disease and myocardial infarction compared to total abstinence (Mäkelä et al. 1997, McElduff and Dobson 1997, Hart et al. 1999, Dawson 2000). This is most probably due to an alcohol-induced increase in high-density lipoprotein concentration (Hein et al. 1996, Rimm et al. 1999) and due to advantageous changes in blood coagulation system (Ridker et al. 1994, Mennen et al. 1999). Increased alcohol consumption also correlates with increased insulin sensitivity (Kiechl et al. 1996) and with the occurrence of decreased coronary heart disease in adult-onset diabetics (Valmadrid et al. 1999). On the other hand, heavy alcohol consumption increases cardiovascular diseases by increasing the occurrence of arrhythmias (Koskinen et al. 1987, Koskinen and Kupari 1991, Sjögren et al. 2000) and cardiomyopathy (Sjögren et al. 2000). Also a Finnish study of 700 men who succumbed to a sudden cardiac death showed a U-shaped curve of left and right (n.s.) ventricular cavities of the heart with increasing ethanol consumption (Kajander et al. 2001).

In the liver, the accumulation of fat in hepatocytes is the most common and the earliest event associated with alcohol consumption (Lieber et al. 1965), occurring in half of the drinkers when the daily ethanol consumption is 40-80 g (Savolainen et al. 1993). Fatty liver is reversible if alcohol consumption is ceased, but may lead to alcoholic hepatitis or perivenular fibrosis if alcohol consumption continues (Worner and Lieber 1985). Both of these are considered precursors of cirrhosis, i.e., irreversible liver scarring leading to dysfunction (Lieber 2001). Alcoholic liver disease may cause portal hypertension, ascites, encephalopathy, gastrointestinal haemorrhage and peritonitis, and increase the risk of hepatocellular carcinoma (Lieber 2001). Kupffer cells are resident hepatic macrophages that are activated by different endotoxins. Chronic alcohol consumption increases blood endotoxin levels (Bode et al. 1987) and therefore Kupffer cells are activated during ethanol administration (Thurman 1998) and also during ethanol withdrawal (Bautista and Spitzer 1992). Activated Kupffer cells release proinflammatory cytokines and free radicals (Bautista and Spitzer 1992, 1999), and increase their cyclooxygenase-2 activity (Nanji et al. 1997). Ethanol exposure also increases the number of Kupffer cells (Shiratori et al. 1989). Kupffer cell proliferation and activation is suggested to have an important role in the development of alcoholic liver disease (Thurman 1998).

Alcohol consumption is the most common cause of acute pancreatitis in Finland (Jaakkola and Nordback 1993). If the acute pancreatitis is severe and ethanol exposure continues, the disease may develop into a chronic form (Kloppel 1999). The severity of acute pancreatitis correlates with the amount of recently consumed ethanol (Jaakkola et al. 1994). Ethanol-induced pancreatic diseases caused 2391 hospitalizations in 2002 (Yearbook of Alcohol and Drug Statistics 2003). Heavy ethanol consumption increases the risk of type 2 diabetes (Holbrook et al. 1990, de Vegt et al. 2002). However, low to moderate alcohol consumption decreases the occurrence of type 2 diabetes (Rimm et al. 1995, de Vegt et al. 2002) by increasing insulin sensitivity and decreasing insulin resistance and hyperinsulinaemia (Kiechl et al. 1996, Lazarus et al. 1997). Alcohol consumption plays also a significant role in the development of spermatogenic arrest and Sertoli cell only syndrome (Pajarinen et al. 1996, 1997).

Epidemiological studies have shown that chronic ethanol consumption is related to an increased occurrence of certain cancers and cancer-related deaths (IARC 1998, Bagnardi et al. 2001). *In vitro,* the growth and mitotic activity of human breast cancer cells increase with ethanol treatment (Izevbigie et al. 2002), and the influence of ethanol increases squamous carcinoma cells proliferation and decreases their differentation (Kornfehl et al. 1999). Previous studies made with experimental animals have shown that ethanol is carcinogenic in the colorectum (Seitz et al. 1985, Niwa et al. 1991), but does not increase the incidence of breast cancer in female rats (McDermott et al. 1992). Animal studies have suggested that ethanol *per se* is not carcinogenic, but may be cocarcinogenic or a tumor promoter (Takada et al. 1986, Seitz et al. 1998). The cocarcinogenic effect has also been found in human lymphoid cell lines (Hsu et al. 1991). A recent meta-analysis has suggested that, in humans, alcohol consumption increases the risk of cancer in the oral cavity, pharynx, oesophagus, larynx, stomach, colon, rectum, liver, breast and ovaries (Bagnardi et al. 2001). Local acetaldehyde accumulation is probably the reason for increased occurrence of cancers in gastrointestinal tract (Salaspuro 2003).

2. Ethanol metabolism

Orally administrated ethanol is mostly absorbed to the circulation from the proximal duodenum and smaller amounts (20-30%) are absorbed through the mucosa of stomach. After first-pass metabolism in the liver, ethanol is quickly distributed all over the body's water phase of all the organs without any binding to the plasma proteins, and penetrates the blood brain barrier (BBB) easily (Watson 1989). Ethanol is mostly metabolised in the liver to acetaldehyde by alcohol dehydrogenase (ADH), microsomal ethanol oxidizing system (MEOS)

and catalase enzymes (Riveros-Rosas et al. 1997). Although ADH is the most important enzyme in ethanol metabolism during irregular ethanol consumption, MEOS becomes more important during heavy ethanol intoxication and is induced also by chronic ethanol consumption (Badger et al. 1993, Ronis et al. 1993). The most important MEOS enzyme in ethanol metabolism is cytochrome P450 2E1 (CYP2E1). Under normal circumstances the significance of catalase in the ethanol metabolism is minimal because the availability of peroxides needed in the reaction is limited (Lieber 1994). Although ADH, CYP2E1 and catalase mostly act in the liver, they are also found in other tissues, e.g. CNS, testes, lungs, pancreas and kidneys (Riveros-Rosas et al. 1997). Acetaldehyde is further metabolised to acetate in the reaction mostly (more than 90%) catalysed by aldehyde dehydrogenase (ALDH), but also by aldehyde oxidase and CYP2E1 (Riveros-Rosas et al. 1997).

Ethanol is also metabolised in the epithelial cells of the digestive tract (Dong et al. 1996, Seitz et al. 1996, Seitz and Oneta 1998). In addition to the epithelial metabolism, many microbes of the gastrointestinal tract (mouth, stomach and large bowel) have ADH activity, but their capacity to metabolise acetaldehyde is poor (Salaspuro 1996, 1997). This may lead to the local accumulation of acetaldehyde in the saliva (Homann et al. 2000, 2001), gastric juice (Väkeväinen et al. 2000, 2002) and large bowel (Tillonen et al. 1999, 2000).

Even though ethanol is easily accessible to the CNS (Pohorecky and Brick 1988), acetaldehyde in low concentration does not penetrate the BBB (Sippel 1974, Tabakoff et al. 1976). On the contrary, acetaldehyde can be formed in the CNS in the reaction catalysed by ALD (Bühler et al. 1983, Kerr et al. 1989), CYP2E1 (Hansson et al. 1990), and catalase (Aragon et al. 1992, Gill et al. 1992) *in situ*. CYP2E1 is the main enzyme responsible for ethanol metabolism in the CNS during chronic ethanol consumption or high ethanol concentrations (Ravindranath et al. 1989, Cohen et al. 1980). Chronic ethanol consumption induces CYP2E1 in astrocytes and neurons (Anandatheerthavrada et al. 1993, Tindberg and Ingelman-Sundberg 1996), but in ethanol naive rats CYP2E1 is expressed only at low levels in the CNS (Tindberg and Ingelman-Sundber, 1996). When ethanol is metabolised via CYP2E1, reactive oxygen species (ROS) are generated as a by-product (Ekström et al. 1986, Persson et al. 1990). The oxidative damage caused by ROS may be a crucial mechanism in ethanol-induced CNS damage (Cohen and Werner 1993, West et al. 1994, Mantle and Preedy 1999), as well as in alcoholic liver disease (Ingelman-Sundberg et al. 1993). The distribution of ADH in CNS is not homogenous. In wide brain regions ADH is absent, but in hippocampus, cerebellum and cerebral cortex ADH is present, thus enabling local accumulation of acetaldehyde after ethanol exposure (Martinez et al. 2001). In the CNS the role of catalase in ethanol metabolism may be more important than in the liver (Cohen et al. 1980, Aragon et al. 1992).

3. Ethanol-induced functional effects on neurons

3.1. Cell membrane changes

The neuronal cell membrane, like other cell membranes, consists of two layers of phospholipids and protein molecules. The hydroxyl group of ethanol molecule can form hydrogen bonds with cell membrane phospholipids thus modifying the architecture of the cell membrane (Barry and Gawrish 1994). Ethanol also disturbs the function of cell membrane proteins by changing their conformation (Li et al. 1994, Lovinger 1997). In addition, high ethanol concentrations change the composition of lipids in cell membranes near the receptors (Nutt and Peters 1994, Tan and Weaver 1997). The cell membrane actively maintains resting membrane potential by ion pumping, and cell membrane of neurons also mediates nerve impulses. *In vitro* chronic ethanol exposure did not change the resting membrane potential or the action potential magnitude in hippocampal neurons (Durand and Carlen 1984). However, the action of one potassium channel (G protein-activated inwardly rectifying potassium channel) was enhanced by ethanol dose-dependently leading to decreased neuronal excitability (Lewohl et al. 1999). The activation of the potassium channel may be related to ethanol-induced analgesia (Ikeda et al. 2002).

3.2. Effects on CNS neuronal receptors

Ethanol has no specific receptor in the nervous system (Merikangas 1990), but it affects the function of many receptors and many neurotransmitter systems (for review see Faingold et al. 1998, Deitrich et al. 1989, Nevo and Hamon 1995, Tabakoff et al. 1996). The most important receptors affected by ethanol are *N*-Methyl-D-asparate (NMDA) and gamma-aminobutyric acid (GABA) receptors. Other receptors and ion channels are also modulated by ethanol: nicotinic acetylcholine, 5-hydroxytryptamine type 3 and glycine receptors are potentiated, and calcium channels are inhibited (Narahashi et al. 2001).

NMDA receptors are the main excitatory receptors of CNS. Their activation accelerates the influx of Ca^{2+}, and therefore increases intracellular concentration of Ca^{2+} ions predisposing neurons to excitotoxicity (MacDermott et al. 1986). Glutamate is a major excitatory neurotransmitter in the CNS and the main ligand of NMDA receptors (Nakanishi 1992). Acute ethanol exposure inhibits NMDA receptors (Hoffmann et al. 1989, Wirkner et al. 1999), but chronic ethanol consumption compensatorily up-regulates the number of NMDA-receptors (Chen et al. 1997). The ethanol-induced inhibition of NMDA receptors decreases the possibility of seizure (Danysz et al. 1992). During ethanol withdrawal the

inhibitory effect of ethanol on the NMDA receptors decreases and the amount of glutamate in CNS increases (Rosetti and Carboni 1995). This leads to a hyperexcitatory state in the CNS, which significantly increases susceptibility to ethanol withdrawal seizures (Grant et al. 1990), and could lead to the degeneration of neurons, e.g. by increasing the amount of intraneuronal calcium (Iorio et al. 1993, Davidson et al. 1995).

GABA is the major inhibitory neurotransmitter in the CNS. Acute ethanol intoxication increases $GABA_A$ receptor responses by potentiating Cl^- influx to neurons (Nestoros 1980, Allan and Harris 1987, Deitrich 1989, Mihic and Harris 1996), but chronic ethanol exposure only minimally increases $GABA_A$ receptor function, representing a possible mechanism for ethanol tolerance (Allan and Harris 1987). During ethanol withdrawal ethanol-induced $GABA_A$ receptor desensitisation increases the possibility of seizures (McQuilkin and Harris 1990). In summary, acute ethanol-exposure induces an inhibitory state in the CNS, causing e.g. sedation and motor impairment. When ethanol consumption becomes chronic, adaptation mechanisms restore the balance between excitatory and inhibitory impulses near to the normal level. Ethanol withdrawal causes a hyperexcitatory state in the CNS causing withdrawal symptoms, increased seizure susceptibility and, eventually, degeneration of neurons.

3.3. Effects on adrenergic receptors

Adrenergic receptors are classified into two major subtypes, α- and β-receptors, both of which are further divided into subtypes 1 and 2. It should be pointed out that β_3-and β_4-receptors have also been characterized. Noradrenaline is an agonist of all the adrenergic receptors, which are located both in the central and the peripheral nervous system, as well as in the peripheral target organs of the sympathetic nervous system. α_1- and β-receptors are post-synaptic excitatory receptors, whereas α_2-receptor is an inhibitory presynaptic autoreceptor. α_2-receptor activation decreases the release of noradrenaline. Although acute ethanol exposure does not affect adrenergic receptors (Hunt and Dalton 1981), long-term ethanol exposure decreases the density of β-receptors but does not affect the density of α-receptor (Muller et al. 1980, Rabin et al. 1980). However, ethanol withdrawal reduces the sensitivity of α_2-receptors, which increases sympathetic activity (Nutt et al. 1988, Hawley et al. 1994). Acute and chronic alcohol consumption, and particularly ethanol withdrawal increases the synthesis of noradrenaline in sympathetic neurons (Ahtee and Svartström-Frazer 1975, Jaatinen et al. 1993, Jaatinen and Hervonen 1994). In experimental animals decreased CNS adrenergic activity increases voluntary ethanol consumption (Aalto and Kiianmaa 1987, Hilakivi et al. 1987), while stimulation of the adrenergic system decreases it (Daost et al. 1987).

3.4. Effect on mitochondrial energy metabolism

Mitochondria are cytosolic organs that produce energy. The inner membrane of mitochondria is highly folded and contains enzymes for electron transport and oxidative phosphorylation. Neurons produce almost all of their energy via oxidative phosphorylation, and therefore the mitochondrial electron transport chain plays a critical role in the energy metabolism of neurons (Wong-Riley 1989). Cytochrome c oxidase (CO) is the terminal and rate-limiting enzyme in oxidative phosphorylation (Capaldi 1990), and CO activity correlates to the activity of neurons (Wong-Riley 1989). Decreased brain CO activity has been found during aging (Curti et al. 1990, Bowling et al. 1993,) and in several degenerative neurological diseases, such as Parkinson's disease and Alzheimer's disease (Beal 1992, Kish et al. 1992, Davis et al. 1997). Reduced mitochondrial energy metabolism has been suggested to induce functional impairment in the CNS and contribute to the neuronal death associated with aging and neurodegenerative diseases (Beal 1992, Bowling et al. 1993, Cottrell et al. 2001).

Alcohol consumption has been shown to reduce CO content and activity in the liver of experimental animals (Arai et al. 1984, Thayer and Cummings 1990, Puzziferri 2000). In the CNS, ethanol treatment has been shown to suppress the expression of CO mRNA level in the hippocampus of rat (Kim et al. 2001). After chronic ethanol consumption CO activities of whole brain homogenates have been reported to be unaltered (Thayer and Rottenberg 1992) or decreased in the adult rats (Marin-Garcia et al. 1995). *In utero* ethanol exposure did not change the brain CO activity of newborn rats (Marin-Garcia et al. 1996). However, the whole brain homogenates are not able to show local changes in the amount or activity of CO.

4. Chronic ethanol exposure and glial cells

4.1. Structural alterations

There are three major types of neuroglia in the CNS: astrocytes, oligodendrocytes and microglia. *Astrocytes* maintain CNS ion homeostasis and metabolise several neurotransmitters. They are spread throughout the CNS and e.g. form BBB. It has been suggested that astrocytes are more sensitive to ethanol-induced degeneration than neurons (Korbo 1999), and that the damage of astrocytes could lead to neuronal degeneration (Guerri and Renau-Piqueras 1997). Astrocytes contain glial fibrillary acidic protein (GFAP) filaments. Previously a 21-month ethanol-exposure has been shown to decrease GFAP

immunoreactivity in the cerebellum of female rats but not in male rats (Rintala et al. 2001). As the female rats consumed more ethanol than the males, the results suggested a dose-dependent decrease in GFAP rather than a factual gender difference (Rintala et al. 2001). GFAP immunoreactivity in rat hippocampus has been shown to increase after 1-3 months´ ethanol exposure and decrease after 9 months exposure (Franke 1995). However, no change in the number of Bergman astroglia was found in the molecular layer of cerebellum after a 10-month ethanol consumption (Dlugos and Pentney 2001). Therefore, it seems that the GFAP filaments of astroglia degenerate during long-term ethanol exposure, but the number of astrocytes does not decrease in the course of 10 months´ ethanol exposure. *In vitro* studies have shown that an acute exposure to physiological ethanol concentrations induces swelling of astrocytes (Kimelberg et al. 1993).

Each *oligodendrocyte* surrounds several axons in the CNS. They produce myelin in the CNS, like Schwann cells in peripheral neurons. Prenatal ethanol exposure disturbs the maturation of oligodendrocytes of mice pups probably causing impaired brain myelination and neuronal dysfunction (Ozer et al. 2000). Also prenatal ethanol feeding for 4 days has been shown to decrease the myelination of optic nerve fibers (Phillips 1989).

Microglia have been first described by Nissl (1891), and a silver staining method to distinguish microglia from other glial cells has been introduced by del Rio-Hortega (1919, 1932). It has been suggested that microglial cells are of mesodermal origin, distribute throughout the CNS during the brain development, and constitute the resident macrophages of the mature CNS (Hickey and Kimura 1988, Perry and Gordon 1988, 1989, Streit et al. 1988, Perry 1994). Microglia are divided into activated and resting microglia. The resting microglia have a small rounded cell body containing a nucleus, only a small volume of cytoplasm, and long branched processes (Murabe and Sano 1982, Oehmichen 1983, Streit and Graeber 1996). In contrast with the resting microglia, the activated microglia have large cell bodies and short processes resembling macrophages (Thomas 1990, Streit and Graeber 1996). In the developing CNS activated microglia are the major types of microglia, which phagocytosize the redundant apoptotic neurons (Cunningham 1982, Barres et al. 1992). During the course of CNS development the resting microglia increase in number and the activated microglia disappear. In the mature CNS almost all of the microglia are resting, but after a neuronal damage the resting microglia can change their phenotype and function, and become activated (Imamota and Leblond 1978, Streit et al. 1988). When the damage has been repaired, the activated microglia return to the resting form (Giulian and Baker 1986, Suzumura et al. 1990, 1991).

Microglia are activated and/or their number increases in several CNS diseases. In Alzheimer's disease the microglia are associated with senile plaques (Perlmutter et al. 1990). In brain ischemia activated microglia phagocytosize degenerated or dead neurons (Gehrmann et al. 1992). In multiple sclerosis

microglia may present CNS antigens to circulating T-cells (Banati and Graeber 1994). Also the main targets of HIV-1 infection in the CNS are microglia and macrophages (Price et al. 1988). Cerebral atrophy has been found to correlate strongly with the cortical proliferation of microglia in AIDS patients (Gelman 1993). The only previous study where the density of microglia has been measured after ethanol exposure showed that 40 weeks' treatment caused no microgliosis in the cerebellar cortex (Dlugos and Pentney 2001).

4.2. Functional alterations in microglia

There are only a few studies concerning the changes in microglial function during ethanol exposure. However, it has been shown that in cultured microglia ethanol exposure increases ROS (especially superoxide anion) production suggesting microglial activation (Colton et al. 1998). The activated microglia are able to phagocytosize and release secretory products (Banati et al 1993). The physiological functions of microglia are important: they regulate the proliferation of astrocytes, neuronal growth and angiogenesis, and eliminate degenerated myelin (Nakajima and Kohsaka 1993). In CNS injury and during several CNS diseases microglia phagocytosize degenerative tissue and release biologically active substances, such as growth factors, cytokines (Giulian et al. 1986), proteinases, lipid mediators and cytotoxic products (for review see Nakajima and Kohsaka 1993, Minghetti and Levi 1998), such as glutamate (Piani et al. 1991), nitric oxide and ROS (Giulian and Baker 1986, Colton and Gilbert 1987). Although the microglial functions are highly important to the well-being of CNS, the secretory products are also neurotoxic and may lead to the neuronal damage (Banati et al. 1993).

5. Ethanol-induced structural changes in the nervous system

5.1. Central nervous system

Alcohol abuse causes CNS damage indirectly by increasing the frequencies of head injuries, brain infections, seizures and metabolic disorders (e.g. electrolyte imbalance, hypoglycaemia and hepatic encephalopathy) (Charness 1993). In chronic alcoholism one of the main neuropathological manifestations is brain atrophy, which was found by using pneumoencephalography (Brewer and Perrett 1971), computer tomography (Carlen et al. 1978), magnetic resonance imaging

(Pfefferbaum et al. 1998) and pathological studies (Torvik et al. 1982). Alcoholics also have an increased pericerebral space (Harper and Kril 1985) and size of brain ventricles (Harper et al. 1985) as an evidence of brain shrinkage compared to non-alcoholics. The atrophy is mostly due to a reduced volume of cerebral white matter (Harper et al. 1985, de la Monte 1988, Jensen and Pakkenberg 1993), but the volume of cerebral grey matter is also slightly decreased (de la Monte 1988).

Although brain atrophy is clinically the most common finding in the CNS of alcoholics, the pathological changes in specific CNS regions are clinically more important. Hippocampus is related to cognition, short-term memory and learning, which may all be impaired after long-term alcohol consumption in humans (Tuck and Jackson 1991) and in experimental animals (Beracochea et al. 1986). In humans chronic alcoholism causes loss of hippocampal pyramidal cells (Bengoechea and Gonzalo 1990) and volume loss of the right hippocampus (Laakso et al. 2000). Harding et al. (1997) found that in humans ethanol consumption decreases the hippocampal white matter volume, but the number of hippocampal pyramidal neurons was, however, equal between alcoholics and non-alcoholics. In experimental animals chronic ethanol consumption is shown to decrease the number of pyramidal and dentate gyrus granular cells in the hippocampus (Walker et al. 1980, 1981, Cadete-Leite, 1988a, 1988b, 1989, Bengoechea and Gonzalo 1991, Paula-Barbosa et al. 1993, Lundqvist et al. 1994, 1995). Structural alterations in rodent hippocampus require at least 2 to 4 months of ethanol exposure to develop (Walker et al 1993).

Cerebellum is involved e.g. in the coordination of movements, controlling posture and maintaining the balance of body. The classical signs of alcoholics with cerebellar damage are ataxia and broad-based, incoordinated walking (Victor et al. 1989). In humans cerebellar atrophy has been reported after chronic alcohol consumption in autopsy (Victor et al. 1959, Torvik et al. 1982) and in computer tomographic studies (Cala et al, 1978). It has been suggested that chronic use of alcohol reduces the number of cerebellar Purkinje cells and granular neurons in humans (Torvik and Torp 1986, Phillips et al. 1987, Karhunen et al. 1994). On the other hand, a recent autopsy study showed neither cerebellar shrinkage nor loss of granular cells after long-term alcohol consumption, and the total number of Purkinje cells was decreased only in alcoholics with Wernicke's encephalopathy (Baker et al. 1999). In experimental animals, loss of Purkinje cells has been found after chronic (4-5 months) ethanol consumption followed by ethanol withdrawal (Walker et al. 1981, Phillips and Cragg 1984), and after 18 months of ethanol consumption (Tavares et al. 1987). However, the total number of granular cells and the volume of granular layer were unchanged after 40-48 weeks of ethanol treatment (Tabbaa et al. 1999). Also, after 21-month ethanol exposure the volumes of cerebellar vermis layers were unchanged, except in the ANA female rats whose molecular layers in

folium II were decreased compared to the male rats and AA female rats (Rintala et al. 1997).

Locus coeruleus (LC) is a group of adrenergic neurons located in the brain stem. It sends adrenergic innervation to almost every CNS region (Foote et al. 1983). The LC has a variety of functions such as the control of vigilance, selective attention, learning, and memory processes (Foote et al. 1983). Alcoholism causes a 23% loss of LC neurons compared to non-alcoholics (Arango et al. 1994), but also no-change results are reported (Halliday et al. 1992, Baker et al. 1994). In LC glutamate neurotransmission is high during ethanol withdrawal increasing the activity of rat LC neurons (Engberg and Hajos 1992). Lifelong ethanol exposure has been shown to reduce the number of LC neurons in both genders of alcohol preferring (AA) rats and female rats of the alcohol avoiding (ANA) line (Lu et al. 1997, Rintala et al. 1998). Loss of LC neurons has been found after 5 weeks of repeated heavy ethanol intoxications and withdrawals in aged rats but not in young rats (Riihioja et al. 1999b). Synapse-to-neuron ratio in the LC has also been shown to decrease after 17 weeks of heavy ethanol consumption (Kjellström et al. 1993). However, early postnatal ethanol exposure for 5 days during CNS growth spurt did not cause any loss of LC neurons even if the whole CNS weight decreased (Chen et al. 1999).

5.2. Peripheral nervous system

In alcoholic peripheral neuropathy long axons gradually degenerate, affecting mainly the nerves of lower limbs, but also the nerves of upper limbs. The symptoms of polyneuropathy are symmetric distal sensory, motor and autonomic dysfunctions (e.g. sensory loss, pain, muscle cramps, weakness of muscles, flushing of the skin and hair loss) (Viktor 1984). However, the alcoholic polyneuropathy is also often asymptomatic (Vittadini et al. 2001). The reported frequency of motor and sensory polyneuropathy in alcoholics varies from 12.5% to 48.6%, and the frequency is increased by high ethanol doses and long duration (over 10 years) of ethanol consumption (Beghi and Monticelli 1998, Wetterling et al. 1999, Vittadini et al. 2001). The frequency of polyneuropathy is also related to the method of study and to the diagnostic criteria. E.g. 33% of alcoholics, who suffer from severe polyneuropathy according to electroneurographic investigation, are asymptomatic (Vittadini et al. 2001). The consumption of wine may increase the incidence of peripheral neuropathy more than the consumption of other alcoholic beverages (Vittadini et al. 2001), which may be due to the lead content of wine.

5.3. Autonomic nervous system

Autonomic neuropathy was found in 36% of chronic alcoholics occuring most frequently in older individuals with alcoholic liver disease (Barter and Tanner 1987). Autonomic neuropathy is related to increased mortality (Novak and Viktor 1974, Johnson and Robinson 1988). Dysfunction of the autonomic nervous system may cause e.g. orthostatic hypotension (Abdel-Rahman and Wooles 1987) and impaired thermoregulation (Kalant and Le 1983).

Sympathetic overactivity is one reason for ethanol withdrawal symptoms such as anxiety, tremor, sweating, hypertension and tachycardia (Airaksinen and Peura 1987, Linnoila et al. 1987, Hawley et al. 1994). Both acute and chronic ethanol exposure increases plasma catecholamine concentration, which mostly originates from the adrenal medulla (Eisenhofer et al. 1983, Ireland et al. 1984, Howes and Reid 1985). During chronic ethanol consumption and withdrawal sympathetic activity increases in humans and in experimental animals (Pohorecky 1974, Ahtee and Svartström-Frazer 1975, Chan et al. 1985, Russ et al. 1991, Jaatinen et al. 1993, Jaatinen and Hervonen 1994). The concentration of noradrenaline in cerebrospinal fluid elevates in line with the severity of withdrawal symptoms (Hawley et al. 1985). Prolonged sympathetic overactivity may lead to an enhanced auto-oxidation of catecholamines, an increased production of free radicals, and finally to degeneration of sympathetic neurons (Graham 1978, Jaatinen et al. 1993, Jaatinen and Hervonen 1994). The prevalence of sympathetic neuropathy in alcoholics is 20% (Barter and Tanner 1987).

The superior cervical ganglion (SCG) is a peripheral sympathetic ganglion located in the carotid bifurcation. It innervates iris, heart, lacrimal and salivary glands, and blood vessels of head and neck. In rats it has a well-defined and relatively homogenous neuron population (Eränkö 1971, Burnstock and Costa 1975, Gabella 1976), and is therefore a simple model for studying the nervous system (Hervonen et al. 1986). In previous experimental studies neuronal vacuolation (Jaatinen et al. 1993), decreased neuronal packing density (Jaatinen et al. 1992, Jaatinen and Hervonen 1994) and increased lipopigmentation (Jaatinen et al. 1992) have been found in the SCG after long-term ethanol exposure.

AIMS OF THE STUDY

In humans chronic ethanol consumption has been related to an increased occurrence of several diseases (cancers, liver diseases, pancreatic diseases, etc.), but the impact of ethanol consumption *per se* is difficult to estimate. Variabilities in life history between humans are impossible to standardize, and the actual amount and duration of ethanol consumption cannot be accurately estimated in humans.

Several mechanisms leading to the ethanol-induced neuronal damage have been suggested in previous studies (e.g. oxidative stress, neuroreceptor alterations, excitotoxicity). There are also suggestions that ethanol withdrawal may be more harmful to the neurons than ethanol exposure itself, but in humans the pattern of drinking is difficult to verify. The effects of ethanol consumption on the aged and on females have not been thoroughly studied yet.

Therefore, the aim of the present study was to clarify the effects of long-term ethanol exposure on mortality and morbidity, and on the structure and function of rat nervous system. In the present experimental study the pattern of drinking and environmental factors could be controlled. More specifically the aims were:

1. To study the survival, morbidity and causes of death of alcohol-preferring (AA) and alcohol-avoiding (ANA) rats during lifelong ethanol exposure.

2. To find out whether there are gender or line (AA *vs.* ANA) differences in the sensitivity of peripheral sympathetic neurons to lifelong ethanol exposure.

3. To compare the effects of long-term intermittent *vs.* continuous ethanol exposure on peripheral sympathetic neurons.

4. To study the effects of long-term continuous and intermittent ethanol consumption on cerebellar microglia.

5. To analyse cytochrome c oxidase (CO) activities in locus coeruleus, prefrontal cortex and cerebellum after repeated ethanol intoxications and ethanol withdrawals in young and aged rats.

MATERIALS AND METHODS

1. Animals and experimental settings

Three different ethanol exposures were used in the present study. The summary of experimental settings is shown in Table 1. The details are described later in the text.

Table 1. *The summary of experimental settings.*

Study	Strain of rats	Sex	Age[1]	Exposures	Measurements
I (n=317)	AA and ANA	Male, female	24 mo (3 mo young controls)	*Ethanol groups:* 12% ethanol as the only available fluid for 21 months. *Controls:* tap water.	Survival and morbidity. Kidney histology.
II (n=81)	AA and ANA	Male, female	See above (I).	See above (I).	SCG histochemistry and morphometry.
III (n=27)	Wistar albino	Male	7½ mo	*Continuous*: 10% ethanol as the only available fluid for 5½ months. *Intermittent*: 10% ethanol as the only available fluid on Mon, Tue, Thu and Fri, and tap water on Wed, Sat and Sun. *Controls:* tap water.	SCG histochemistry and morphometry.
IV (n=18)	Wistar albino	Male	7½ mo	See above (III).	Volume and number of microglia in cerebellar vermis folia II and X.
V (n=48)	Wistar albino	Male	4 mo, 30 mo	*EtOH*: Intragastric feeding of 25% ethanol 3 times a day on Mon-Thu for 5 weeks, and tap water from Fri to Sun. *Sucrose*: Similar feeding with sucrose. *Controls*: tap water.	Cytochrome oxidase histochemistry in the medial prefrontal cortex, cerebellum and locus coeruleus.

[1]Age at the end of the ethanol exposure.

1.1.Lifelong ethanol exposure (I, II)

AA (Alko, Alcohol) and ANA (Alko, Non-Alcohol) rats are selectively outbred for their high and low voluntary alcohol consumption respectively (Eriksson 1968, 1971). In addition to their differences in ethanol preference, AA and ANA rats differ from each other in their behaviour, metabolism and neurochemistry (Sinclair et al. 1989, Tuominen et al. 1990, Kiianmaa et al. 1991, Korpi et al. 1991, Soini et al. 2002). In the present studies 317 (I) and 81 (II) AA and ANA rats of both genders from generations F65, F67 and F69 were used. The rats were housed in group cages (four to five animals per cage) in the Research Laboratories of Alko under standard conditions: a room temperature of 20 ± 1°C, a light-dark cycle of 12/12 h, and a relative humidity of 50 ± 5%. All the rats had food (RM1(E)SQC;SDS, Witham, England) *ad libitum*. Ethanol consumption, water consumption, food intake, and body weight were measured throughout the experiment (Sarviharju et al. 2001). At the age of 3 months the AA and ANA rats were divided into (AA and ANA) ethanol or (AA and ANA) water consuming groups. There were 3 weeks long self-selection periods in ethanol and water consuming groups for measuring the voluntary ethanol consumption at the beginning and at the end of the exposure. The rest of the time the ethanol-exposed groups had 12% (v/v) ethanol as only available fluid. The water consuming groups had tap water instead. The survival of the rats was carefully monitored up to 24 months of age. If a rat died or was killed due to severe symptoms of illness, it was autopsied and studied macroscopically, microscopically and microbiologically. The autopsy reports were analysed and significant findings were collected. At the age of 24 months the rats were killed and autopsied. The rats were gradually withdrawn from ethanol for one week before killing. At the same time the water consuming 3-month-old controls rats were killed for the microscopic examination.

1.2.Long-term intermittent and continuous ethanol exposure (III, IV)

27 (III) and 18 (IV) male Wistar albino rats were used in the present studies. Group cages (five animals per cage) and standard conditions (a room temperature of 23 ± 1°C, a light-dark cycle of 12/12 h, a relative humidity of 40 ± 5%) were maintained. All the rats had free access to standard rat food (Ewos R36, Ewos AB, Sweden). At the beginning of the experiment the 2-month-old rats were divided into three groups. One group had tap water (*control*), the second group had 10% (v/v) ethanol as the only available fluid (*continuous*), and the third group had 10% ethanol on Mon, Tue, Thu and Fri, and tap water for the rest of the days (*intermittent*) throughout the 5½ month experiment. This weekly schedule produced two ethanol withdrawals per week to the intermittently exposed rats. The weights of the animals and fluid consumption in each cage

were observed throughout the experiment. Individual fluid consumptions were measured during the 23rd week of the experiment.

1.3. Repeated ethanol intoxications and withdrawals (V)

24 young (3-4 months of age) and 24 old (29-30 months of age) male Wistar albino rats were used in the study. The rats were housed in individual cages under constant conditions with a room temperature of 22 ± 1°C, 13 h light/ 11 h dark cycle (lights between 08:00-21:00). The 3-4-month-old (*young*) and the 29-30-month-old (*old*) rats were divided into ethanol-fed (*EtOH*), sucrose-fed (*sucrose*) and tap water consuming (*control*) groups. The *EtOH* rats were given 25% (v/v) ethanol in 5% sucrose by intragastric intubations 3 times a day for 4 days (from Mon to Thu). After that (from Fri to Sun) there was a 3-day ethanol withdrawal period with tap water. As this weekly schema was repeated 5 times (5 ethanol intoxications and withdrawals), the duration of the experiment was 5 weeks. The given ethanol dose on each feeding session was individually adjusted according to the intoxication level of the rat, as described previously (Riihioja et al. 1999a, 1999b). The aim was to keep animals on intoxication level 3 (clearly impaired walking, impaired elevation of abdomen and pelvis) or 4 (slowed righting reflex, no elevation of abdomen and pelvis), when severity of intoxication was evaluated by using 7-level scale (Hemmingsen et al. 1979, Clemmesen et al. 1988). The *sucrose* rats were pair-fed with isocaloric sucrose three times a day. *EtOH* and *control* rats had always food available, and *sucrose* rats were also pair-fed according to the food consumption of *EtOH* rats. During the experiment the ethanol dose, food intake and body weight of the rats were daily monitored.

2. Ethical considerations

The local committee for animal research approved all the experimental protocols. During the experiments all unnecessary suffering of the animals was avoided. All animals were killed by decapitation under deep sodium pentobarbital (Mebunat®, Orion Corp. Turku, Finland) anaesthesia.

3. Preparation of tissues (I-V)

The left superior cervical ganglion (SCG) was removed immediately after decapitation and frozen in liquid nitrogen (II, III). The samples were further processed for formaldehyde-induced histofluorescence (FIF). Kidneys (I), medial prefrontal cortex (MPF) (V), cerebellar vermis (IV, V), and the right locus coeruleus (LC) (V) were fixed in 4% paraformaldehyde in phosphate-buffered saline, and incubated through ascending sucrose concentrations to avoid cryodamage. The samples were then sectioned at 8 μm (V), 10 μm (IV) or 20 μm (I) in a cryostat, and the sections were processed for histochemical demonstration of cytochrome oxidase (CO) (V), or tomato lectine histochemistry (IV). The kidneys were stained with hematoxylin-eosin (I).

4. Histological procedures

4.1. Formaldehyde-induced histofluorescence (II, III)

Noradrenaline is a catecholamine and the main neurotransmitter in sympathetic postganglionic nerve endings. Its production in postganglionic neuron somata is elevated when sympathetic activity increases. When noradrenaline containing neurons are exposed to formaldehyde vapour, they become fluorescent and are visible with fluorescence microscope, as demonstrated by Eränkö (1967). FIF intensity correlates with the catecholamine concentration of sympathetic neurons (Alho et al. 1983). In our standardized FIF method the samples stored in liquid nitrogen are freeze-dried under a vacuum of 10^{-4} Torr at -40°C for 7 days using phosphorous pentoxide as a water trap. Subsequently the samples are exposed to paraformaldehyde vapour at +60°C for 60 minutes and vacuum-embedded in paraffin. In the present studies the samples were cut at 8μm and the sections were embedded in nonfluorescent liquid paraffin. FIF intensities were measured by using fluorescence microscope (Olympus Vanox-T) and pseudo-colour video images (Hamamatsu ARGUS-10 image processor). The neurons, as well as other postmitotic cells, contain lipopigments, which is a lipogenic by-product of oxidative metabolism (Sohal and Brunk 1990). The accumulation of lipopigment during aging is characteristic of postmitotic cells (Sohal 1981). Lipopigments are autofluorescent, and therefore no additional staining methods were needed.

4.2. Tyrosine hydroxylase immunoreactivity (II, III)

Tyrosine hydroxylase (TH) is the rate-limiting enzyme of noradrenaline synthesis. Tyrosine hydroxylase immunoreactivity (TH-IR) can be used as semi-quantitative marker of sympathetic activity. FIF stained sections were further processed with TH immunohistochemistry as follows. Paraffin-embedded sections were deparaffinized with xylene and ethanol. Endogenous peroxidase activity was blocked by incubation with 0.3% H_2O_2 in methanol. The free-floating sections were incubated with TH antiserum (Eugene Tech. Int., Allendale, NJ), and after that in biotinylated goat anti-rabbit antibody (Vectastain® ABC kit, Vector Labs, Burlingame, CA) and in avidin-biotin-label complex (Vectastain® ABS kit). Diaminobenzidine (Sigma) was used as chromogen. TH-IR intensities were microscopically estimated blind to treatment.

4.3. Tomato lectin histochemistry (IV)

In the present study tomato (*lysopersicon esculentum*) lectin was used to stain cerebellar microlia. Lectins are proteins or glycoproteins of plant and animal origin (Goldstein and Hayes 1978, Alroy et al. 1988). They are isolated from many natural sources, including seeds, roots, bark, fungi, bacteria, seaweed, sponges, molluscs, fish eggs, body fluids of intervertebrates and lower vertebrates and from mammalian cell membranes. Even if their physiological function is unknown, they have a wide variety of applications *in vitro*. The ability of lectin to fasten specific sugar residues of complex glycoproteins makes them valuable for identification of different cell types in cytological and histological studies (Goldstein and Hayes 1978). Tomato lectin has an affinity for poly-N-acetyl lactosamine sugar residues (Nachbar et al. 1980, Zhu and Laine 1989), which results in binding to ameboid and ramified microglia, endothelial cells and ependyma without any binding to neurons or to other types of glial cells (Acarin et al. 1994).

The tomato lectin histochemistry (Acarin et al. 1994) was performed, as follows: The tissue was fixed in 4% paraformaldehyde for 24 h, and cryoprotected with ascending concentrations of sucrose. After that the tissue was frozen and sectioned in a cryostat, the sections were melted and rinsed in Tris-buffered saline. Endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol. After that the sections were incubated in biotinylated tomato lectin (Sigma, St Louis, MO; USA), and labelled with avidin peroxidase in Tris-buffered saline (1:30000). Diaminobenzidine was used to visualize the reaction products.

4.4. Cytochrome oxidase staining (V)

CO histochemistry is based on the ability of CO enzyme to transfer electrons. CO histochemistry was introduced by Seligman et al. (1968), and modified to be used in neural tissue by Wong-Riley (1979). In the reaction diaminobenzidine acts as an electron donor, and is oxidatively polymerised to a visible form of indamine polymer. Intensity of the staining and CO activity are closely correlated (r = 0.90) (Darriet et al. 1986). In the present study a slightly modified protocol of Wong-Riley was used for histochemical demonstration of CO, as follows. After the sections were rinsed in phosphate-buffered saline and pretreated in Tris buffer, the incubation in a solution containing 50 mg diaminobenzidine, 30 mg cytochrome c, and 4000 mg sucrose per 100 ml of 0.1 M phosphate buffer was carried out. The incubation times (optimised for each CNS area in a pilot series) were 2.5 h for the cerebellar vermis, 3.5 h for prefrontal cortex, and 5.0 h for LC at +37°C. Finally, the samples were rinsed and embedded in Aquamount®.

5. Morphometric measurements

5.1. Microscopy

Histological analyses were made with an Olympus Vanox-T microscope (Olympus Optical Co. Ltd., Japan). Ultraviolet light was used to visualize the FIF (II, III) and lipopigments (II). Filter combination V (excitation light wave-length 395-415 nm, emission light wave-length 455 nm and up) was used for analysing FIF, and filter combination G (excitation light wave-length 465-550 nm, emission light wave-length 590 nm and up) was used for the lipopigments. Visible light was used for the observation of the histochemical (CO, tomato lectin) and immunohistochemical (TH) stainings.

5.2. Morphometric analyses (II-IV)

A Hamamatsu ARGUS-10 image processor (Hamamatsu Photonics K.K., Japan) was used to aid the measurements (II-IV). The section thickness and the height of the optical disector were measured with a microcator (DT512N, Sony Precision Technology Inc., Japan).

5.2.1. Volume estimation (II-IV)

The volume of the analysed structure was measured by point-counting at a magnification of 50x. Every 12th (II, III) or 36th (IV) section was evaluated throughout the structure. Each grid point equalled an area of 55000 μm^2 [$a(p)$]. The sum of all the points (ΣP) hitting the measured structure was counted. The volumes were counted by using the Cavalieri priciple (Gundersen and Jensen, 1987):

$$V = t \times s \times a(p) \times \Sigma P$$

where t is the section thickness (8 μm (II, III) and 10 μm (IV)), and s is the frequency of measured sections. Because every 12th section analysed in the studies II and III and every 36th section in the study IV, the s was 12 and 36, respectively.

5.2.2. Stereological estimation of total particle number (II-IV)

Physical and optical disectors were used for counting SCG neurons and cerebellar microglia, respectively. The disector is a stereological probe, and by using the disector method it is possible to count objects without having to make any assumptions about their size, shape and orientation (Gundersen 1986, Sterio 1984). In the physical disector there are two separate sections: a reference section and a look-up section. The distance between these two sections (height of the disector) should be smaller than the smallest diameter of the measured object. All the objects within the disector frame of 10000 μm^2, and not touching the exclusion line were counted from the reference section at a magnification of 1000x (Gundersen 1977). Then objects found in the reference section but not in the look-up section were counted (Q). The first measured frame is selected randomly and after that the whole section is analysed in a stepwise manner. When optical disector is used, the reference section and the look-up section are two planes of focus in the same section. The most important advantage of the optical disector method is the time saving compared to the physical disector method. The numerical density (N_v) and the total number (N) of counted particles are estimated as follows:

$$N_v = \Sigma Q \; / \; (\Sigma A \times t)$$
$$N = N_v \times V$$

where ΣQ is the sum of calculated particles, ΣA is the sum of the disector frames, and t is the height of the disector (thickness of the section when physical disector is used).

5.2.3. Relative volume of lipopigment, neuropil and neurons (II)

Measurements were performed on a systematically, randomly selected set of sections, which were analyzed in a stepwise manner, with a random startpoint. Grid points hitting the ganglion, the neurons and the lipopigments were counted and the relative volume of intraneuronal lipopigment (% of neuronal volume) and the total volume of SCG neurons were calculated (Gundersen et al. 1988). The grid point area equalled 550 μm^2 at a magnification of 1000x.

5.3. Intensity of cytochrome oxidase histochemistry (V)

Adjacent sections to those used in CO histochemistry were stained with cresyl violet to help the identification of the different cortical layers, and the brain stem sections with TH immunohistochemistry, to identify the adrenergic neurons of the LC. The analysis of the prefrontal cortex was performed on the rostral pole of frontal area 2 (medial precentral area, medial agranular cortex (Uylings and van Eden, 1990)), and in the cerebellar vermis the analysis was focused on the anterior folia (I-II). In both of these cortical areas (cerebral and cerebellar) ethanol-induced structural and functional alterations have been previously found (Victor et al. 1959, Adams et al. 1993, Rintala et al. 1997, Fadda and Rossetti 1998). Comparisons were performed only between samples processed simultaneously, and on sections of equal thickness. The results on the CO histochemistry are based on systematic observations of one researcher performed blind to the treatments, on two sections per animal per CNS region, from six animals in each group. CO staining was semi-quantitatively estimated on a four-level scale, as follows: 0 = no reaction, + (1) = weak staining, ++ (2) = moderate staining, +++ (3) = intense staining, separately for the neuronal somata and the neuropil in each region.

6. Statistical methods

BMDP (II, V) and Statistical Package for the Social Sciences (SPSS) for Windows (I, III, IV) statistical software were used to analyse the data. The body weights were analysed with analysis of variance (ANOVA), and post-hoc evaluations were made with 95% confidence intervals (II) Bonferroni-corrected t-tests (III) or Student t-test (I). The weight gain was analysed with repeated measures ANOVA (III). The survival of the rats was estimated by the COX regression method, and differences in autopsy findings with Chi-square test. The differences between the groups were evaluated with 1- or 2-way ANOVA, and Bonferroni corrected t-tests (III, IV) or 95% confidence intervals (II) were used

for further analysis. Pearson's correlation analysis was used to measure the relations between blood ethanol and acetaldehyde concentration, ethanol consumption and body weight, and morphometric parameters.

RESULTS

1. Morbidity and mortality during ethanol exposure

Lifelong ethanol consumption did not reduce or improve the survival of the rats (I, II). In addition, there was no gender difference in the mortality of ethanol-exposed rats. However, the survival rate of the ANA rats was significantly lower compared to the AA rats (Fig 1A and 1B). The ANA line had 3.6 times higher risk of death than the AA line by the age of 24 months (B(1)=1.27, p<0.0001, Exp(B)=3.6). The autopsy findings showed that ANA rats had significantly higher occurrence of kidney pathology ($\chi^2(1)=40.11$, p<0.0001), particularly polycystic kidney diseases. ANA rats also had higher rates of benign tumors (particularly pheochromocytomas) and cardiovascular diseases (particularly cardiomyopathies, myocarditis and heart failure).

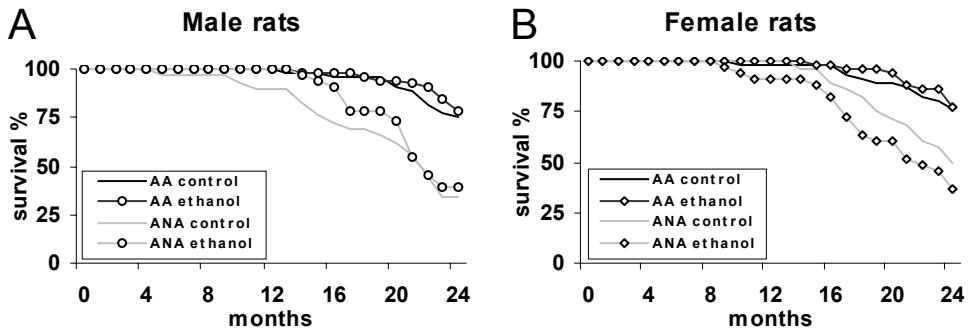

Figure 1. *Survival of AA and ANA rats during the lifelong ethanol exposure showed equal survival between all ethanol-exposed and water consuming groups. In Fig 1A and 1B survival of male and female rats are expressed, respectively. Difference in survival between AA and ANA rats was significant, but the survival curves were similar between the genders.*

Although lifelong ethanol consumption did not affect the survival of AA or ANA rats, the ethanol-exposed rats had a tendency towards a higher rate of malignant tumors than the control rats ($\chi^2(1)=2.92$, p=0.087), and the difference was significant if the rats surviving up to 24 months of age were included in the analysis ($\chi^2(1)=4.50$, p=0.034). The specific malignancies in the ethanol-exposed and the control rats are shown in Table 2. No difference between AA and ANA rats or between the genders in the incidence of malignant neoplasms was found.

5½ months of intermittent or continuous ethanol exposure (III, IV) of male Wistar rats caused no deaths, but one control rat died during the experiment. In gross pathological examination at the end of the experiment no significant pathology was found in any of the rats. When Wistar male rats were exposed to repeated ethanol intoxications and withdrawals for 5 weaks, two *old EtOH* rats died (V). In both cases gross autopsy revealed no specific pathological findings, and the probable cause of death was ethanol intoxication. Gross pathological autopsies were made to all rats used in the histological studies and there were no macroscopic pathologies observed in any of the rats.

Table 2. *The cumulative number of specific malignant neoplasms found in the histopathological autopsies of AA and ANA rats. The malignant neoplasm was the cause of death or the reason to sacrifice in all the cases. Both genders and both lines (AA and ANA) of rats are pooled. The total number of cancers was significantly different (p=0.034) between the groups.*

	Control (n=145)	Ethanol (n=172)
Autopsied	51	59
Neoplastic proliferations of white cells	1	5
Lymphocytic leukaemia	1	1
Monocytic leukaemia	0	1
Malignant lymphoma	0	1
Histiocytic lymphoma	0	2
Carcinomas	3	11
Squamous cell carcinoma of external ear	0	1
Carcinoma of adrenal cortex	0	1
Adenocarcinoma of Zymbal gland	0	3
Endometral adenocarcinoma of uterus	0	1
Adenocarcinoma of anterior pituitary gland	1	1
Invasive papillary carcinoma of female breast	1	0
Invasive apocrine carcinoma of female breast	0	1
Infiltrating duct carcinoma of female breast	0	1
Adenocarcinoma (unknown origin)	1	2
Sarcomas	1	2
Endometrial stromal sarcoma	1	0
Rhabdomyosarcoma of heart	0	1
Angiosarcoma of mesentery	0	1
TOTAL	**5**	**18**

2. Body weight

The body weighs of rats were measured throughout the experiments. Lifelong ethanol consumption induced neither reduction nor gain in body weight

compared to the water-consuming groups. The male rats were significantly heavier than the female rats, and the weights of AA and ANA rats were equal at the end of the experiment. However when the body weights were measured at the age of 12 months, both male and female ANA rats were significantly heavier than respective AA rats (I, II). Wistar rats exposed to ethanol intermittently for 5½ months had poorer weight gain compared to the continuously ethanol-exposed rats and to the water-consuming rats. Also body weight at the end of the intermittent ethanol exposure was significantly lower than body weight in the continuously exposed or control groups (III, IV). Repeated ethanol intoxications and withdrawals for 5 weeks caused impaired progression in weight gain in the *young EtOH* (Wistar) group but not in the *old EtOH* group compared to the pair-fed controls (*sucrose*) (V). Summary of the body weights of ethanol-exposed groups are shown in Table 3.

Table 3. *Body weights of the ethanol-exposed rats measured at the end of the experiment. Age expressed in months. Weights are expressed in mean ± SD. Difference means the comparison to respective controls (I: 24-months-old control; III: control; V: young sucrose, old sucrose). Significant difference between the ethanol-exposed and the respective groups (p<0.05 *, t-test).*

Study	Group	Age (months)	Weight (g)	Difference (g)
I	AA ethanol male	24	565 ± 83	-17
	ANA ethanol male	24	555 ± 77	-48
	AA ethanol female	24	322 ± 54	+18
	ANA ethanol female	24	304 ± 55	+2
III	Wistar *continuous* male	7.5	585 ± 28	-17
	Wistar *intermittent* male	7.5	547 ± 32	-55 *
V	Wistar *young EtOH* male	4	317 ± 17	-28 *
	Wistar *old EtOH* male	30	446 ± 32	-27

3. Ethanol consumption

The average ethanol consumption during the experiments is shown in Table 4. The AA and ANA rats (studies I and II) consumed ethanol for 21 months, and therefore their total ethanol consumption was the highest. Ethanol consumption of the female rats was higher than that of the male rats. No line difference was observed. Daily ethanol consumption in intermittently and continuously exposed male Wistar rats (studies III and IV) was equal, but the *intermittent* rats had ethanol only on 4 days a week. Therefore, the weekly ethanol consumption was higher in continuously exposed rats. However, the daily ethanol consumption in the *intermittent* and the *continuous* male Wistar rats was higher compared to the lifelong ethanol-exposed male AA and ANA rats. In study V ethanol was force-fed to the rats according to the level of intoxication, which produced the highest

daily ethanol exposures in this series of studies. There was a significant difference between the *young* and the *old EtOH* groups in ethanol doses.

Table 4. *The average ethanol consumption of the rats in each experiment. The values are expressed in grams of ethanol/ kg of body weight. The concentrations of ethanol (v/v) were 12 % (II), 10 % (III, IV) and 25 % (V).*

Study	Group	Daily	Weekly	Total
II	AA ethanol male	3.5	24.5	2058
	ANA ethanol male	3.6	25.2	2142
	AA ethanol female	6.7	46.9	3940
	ANA ethanol female	5.5	38.5	3234
III, IV	Wistar *continuous* male	5.7	39.6	911
	Wistar *intermittent* male	5.8	23.0	529
V	Wistar *young EtOH* male	9.5	38.0	190
	Wistar *old EtOH* male	8.2	32.8	164

4. Superior cervical ganglion (II, III)

The volumes of the superior cervical ganglia (SCG) were measured after lifelong ethanol consumption in AA and ANA rats, and after a 5½-month intermittent or continuous ethanol exposure in male Wistar rats. Although the volume of SCG increased with aging, there was no volumetric change in the SCG after lifelong ethanol consumption compared to the old controls (AA and ANA rats). The increased volume of SCG with ageing was due to an increased volume of non-neuronal tissue (neuropil, interstitial elements), but the volume of neurons did not change with ageing. The SCG volumes were equal after 5½ months of intermittent or continuous ethanol exposure and in the control (Wistar) rats.

After lifelong ethanol consumption no loss of SCG neurons was found in either AA or ANA rats. As there was no difference between males and females either, in Fig 2A both genders and both lines (AA and ANA) are pooled together. Although no statistically significant decrease in the number of SCG neurons was found, ethanol-exposed females showed a significant ethanol consumption dependent decrease in SCG neuron number ($r=-0.70$, $p<0.01$). Similar correlation was not found in males ($r=-0.12$, p=n.s.). In study III the male Wistar rats were exposed to ethanol for 5½ months. The duration of the experiment was shorter but daily ethanol consumption was higher compared to study II. After 5½ months of intermittent ethanol consumption the total number of SCG neurons decreased by 28% ($p<0.001$) and by 22% ($p<0.01$) compared to the water exposed control rats and continuously ethanol exposed rats, respectively (Fig 2B). However, correlation between individual ethanol consumption and the number of neurons was found neither in the intermittently ($r=-0.43$, $p=0.25$) nor in the continuously ($r=0.47$, $p=0.20$) ethanol-exposed rats.

A significant increase in the amount of intraneuronal lipopigments after lifelong ethanol exposure was found in the ethanol exposed AA and ANA male rats, but not in the female rats. However, the volume of lipopigment increased in both sexes during aging. After lifelong ethanol exposure the overall level of TH-IR in the SCG was increased in the male rats compared to the ethanol-exposed female rats and the water-consuming male control rats. The rats exposed to ethanol intermittently for 5½-months and those exposed continuously showed no differences in overall FIF or TH-IR intensities compared to water consuming controls, but the continuously ethanol-exposed rats had plenty of neurons showing decreased intensity of FIF and TH-IR.

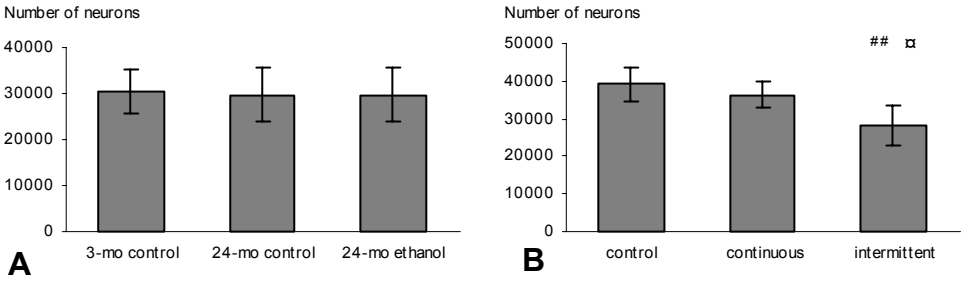

Figure 2. *Number of neurons in the superior cervical ganglion of AA and ANA rats after lifelong ethanol consumption in AA and ANA rats (2A) and in the SCG of male Wistar rats after a 5½-month continuous or intermittent ethanol exposure (2B). In 2A both genders of AA and ANA rats are pooled together since there were no differences between the male and female rats. The number of neurons in 3-mo control, 24-mo control and 24-mo ethanol groups are equal. In 2B the intermittent group has a significantly decreased number of neurons compared to the control group ($p<0.001$, ##) and the continuously ethanol exposed group ($p<0.01$, ¤).*

5. Cerebellar microglia (IV)

In male Wistar rats (n=18) microglial cells were seen throughout the cerebellar vermis, and plenty of them were located in the vicinity of blood vessels. Most of the microglia were ramified (i.e. resting), but a few ameboid (i.e. activated) microglia were found in all the groups. The number of microglia was measured from each layer (molecular, granular and white matter) of cerebellar vermis folia II and X after 5½ months of intermittent or continuous ethanol consumption and in the water-consuming control animals. The volume of cerebellar folia II and X was equal in all the groups. There was a significant increase in the total number of microglia in the molecular layer of folium II in the *intermittent* group compared to the *continuous* ($p<0.05$) and *control* ($p<0.05$) groups. There was no

difference in the number of microglia between the groups in the other layers. In folium X no difference between the groups was found in any of the layers.

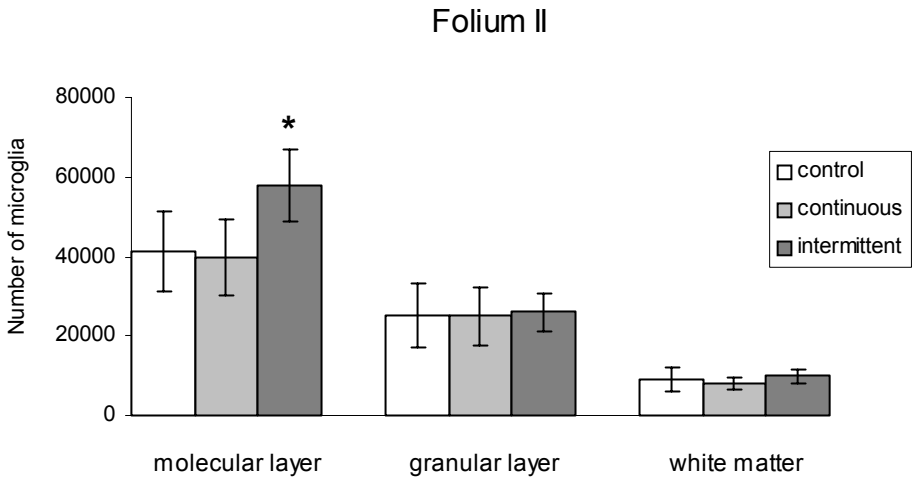

Figure 3. *The number of folium II microglia in molecular layer, granular layer and white matter of controls, continuously and intermittently ethanol-exposed rats. Mean ± SD. * Significant difference ($p<0.05$, t-test) from the controls and continuously ethanol-exposed group.*

6. Cytochrome oxidase activity (V)

Cytochrome oxidase (CO) staining intensity decreased in medial prefrontal cortex (MPF), anterior superior cerebellar vermis, and LC in male Wistar rats (n=48) exposed to heavy ethanol feeding and ethanol withdrawals for 5 weeks. There was no obvious difference in staining between water consuming and sucrose-fed rats. In the MPF the most intense staining was found in layers III and V, and the difference between the cortical layers was most apparent in the *young control* and *old control* groups, and the least obvious in the *young EtOH* and *old EtOH* rats. There was no marked difference between young and old groups. In the cerebellar vermis the granular layer had most intense CO staining, and white matter lowest staining intensity. CO activity decreased in granular layer folia I-II (anterior superior part of vermis) in both the *young* and *old EtOH* rats, but in the Purkinje cells a decreased activity was obvious only in the *old EtOH* animals, and not in the *young EtOH* animals, compared to the respective *sucrose* and *control* groups. In the LC increased CO staining intensity was found in the old rats compared to the young rats, and ethanol-induced decrease in LC CO activity was less marked in the *old EtOH* group than the *young EtOH* group compared to the *sucrose* and *control* rats, respectively. Summary of the results is represented in Figure 4.

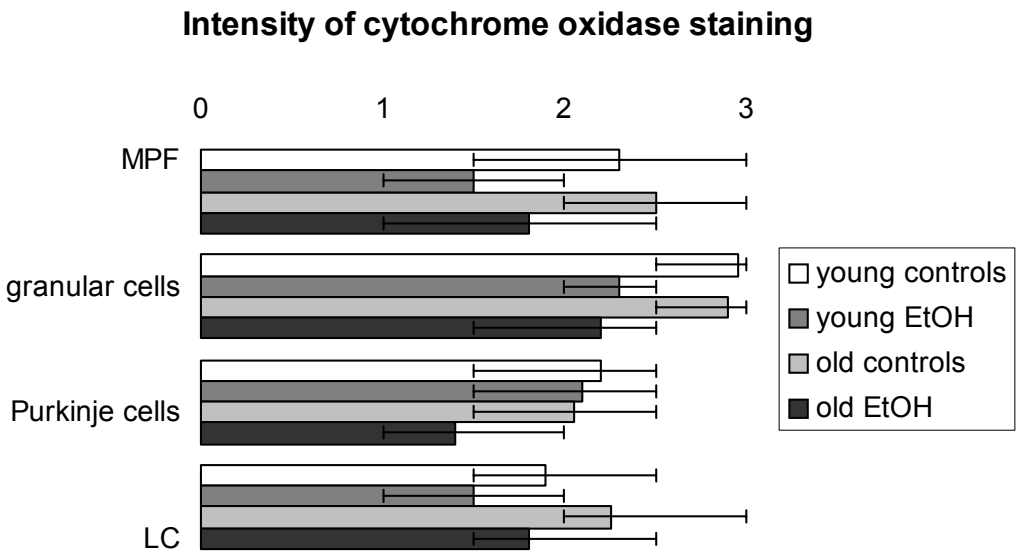

Figure 4. *Cytochrome oxidase (CO) staining intensity in neurons of medial prefrontal cortex (MPF), granular cells of cerebellar vermis, Purkinje cells of cerebellar vermis and neurons of locus coeruleus (LC) of young and old controls and ethanol-exposed (EtOH) rats. Water consuming and sucrose-fed contol groups are pooled. The CO staining was estimated from 0 to 3. The figure represents means (bars) and range (error bars) of values.*

DISCUSSION

1. Methodological considerations

1.1. Animals

In the present series of experiments three different ethanol exposures were used. In studies I-II both genders of alcohol-preferring (AA) and alcohol-avoiding (ANA) rats were used, whereas in the rest of the studies the experimental animals were male Wistar albino rats (III, IV, V). AA and ANA rats differ from each other and from Wistar rats by their different voluntary ethanol consumption (Eriksson 1971, Aalto 1986), ethanol metabolism (Eriksson 1973, Hilakivi et al. 1984, Koivisto et al. 1993) and behaviour (Eriksson 1981, Sinclair et al. 1989). Although AA and ANA rats are originally bred from Wistar rats (Eriksson 1968), ethanol exposure may cause different effects on each line of rats (AA *vs.* ANA *vs.* Wistar).

In the present study the survival of ANA rats was significantly reduced compared to the survival of AA rats. The increased morbidity of ANA rats was mostly due to kidney diseases, but they also had more cardiovascular diseases and benign tumors (particularly pheochromocytomas) compared to the AA rats. Microscopic studies revealed that ANA rats suffered from polycystic kidney disease, suggesting that during the breeding process of ANA rats, genes predisposing to cystic kidney disease have been unintentionally co-selected. Polycystic kidney disease in humans is often associated with elevated blood pressure, contributing to the development of cardiovascular diseases. Previously, a rat model for autosomal dominant polycystic kidney disease, called Han:SPRD cy/+ rats, has been developed (Gretz et al. 1996). Homozygous Han:SPRD rats die by the age of 3 months, but heterozygous rats develop a slowly progressive polycystic kidney disease and die at about the age of 17 months (Schäfer et al. 1994). The abnormal laboratory findings in Han:SPRD cy/+ rats include increased serum creatinine and urea due to decreased glomerular filtration rate (Braun et al. 1996). These parameters have not been studied in ANA rats yet. However, there have been no differences in postnatal or early lifetime mortality between AA and ANA rats (Sarviharju Maija, oral communication), therefore the genotype of polycystic kidney disease may be different from that of

Han:SPRD rats. Also the median life time of ANA rats was 22 months compared to the 17 months of Han:SPRD cy/+ rats.

The ANA rats had higher rates of kidney diseases, benign tumors and cardiovascular diseases than the AA rats. In the present study lifelong ethanol exposure and aging induced similar changes in the superior cervical ganglia (SCG) of AA and ANA rats. Previously when the same rats as used in the present experiment were studied the number of locus coeruleus (LC) neurons decreased due to the lifelong ethanol consumption but no line difference (AA *vs*. ANA) was found (Lu et al. 1997, Rintala et al. 1998). However, there was atrophy in the molecular layer of cerebellar vermis folium II in ANA female rats after lifelong ethanol consumption that was not found in AA rats or ANA male rats (Rintala et al. 1997). Although the survival rate of ANA rats was significantly lower compared to that of AA rats, and their kidney, cardiovascular and benign neoplasm morbidity significantly higher, the ethanol- and age related line differences (AA *vs*. ANA) found in the present study and in the previous studies were rather minor. It might be that the ANA rats surviving till the end of the experiment were selected for a better renal and cardiovascular health than the average ANA rats. However, further studies are needed to evaluate the impact of the kidney disease found in the ANA rats. Because the study did not include a non-selected line of rat, it cannot be determined whether the AA rats lived particularly long or the ANA rats died particularly early. However, the results showed that genetically alcohol-preferring AA rats had decreased mortality compared to the genetically alcohol-avoiding ANA rats. Interestingly, an analogous situation is seen in humans, since abstainers have higher mortality compared to moderate drinkers (Single et al. 1999), mainly due to differences in the occurrence of cardiovascular disease.

1.2. Ethanol exposures

At the end of the present study (II) the AA and ANA male rats consumed ethanol 3.5 and 3.6 g/kg/day, and female rats 6.7 and 5.5 g/kg/day, respectively. The AA and ANA females and the ANA males increased their consumption with age, while in the AA males ethanol consumption was constant (Sarviharju et al. 2001). As the ethanol elimination rate after chronic ethanol consumption has been shown to increase from 11.3 up to 12.3 g/kg/day in AA rats and non-significantly decrease from 10.5 to 9.8 g/kg/day ($p=0.06$) in ANA rats (Forsander and Sinclair 1992), the rats consumed much less alcohol than their metabolic capacity would allow. The ethanol elimination rate is about the same in common laboratory rat than in AA and ANA rats (Lundqvist et al. 1995). In study III and IV intermittently and continuously ethanol-exposed rats consumed ethanol 5.8 g/kg/day and 5.7 g/kg/day, respectively. Their blood ethanol concentrations (BEC) measured were not very high (*intermittent:* 3.1 mmol/l

(range 0-10.2 mmol/l), *continuous:* 3.6 (range 0-10.2 mmol/l)), but most likely the peak BECs were higher. Previously daily intraperitoneal ethanol injections (3.0 g/kg) have been shown to lead to a BEC of 43 mmol/l and significant loss of hippocampal cells, whereas continuous *per oral* ethanol consumption (7.8 g/kg/day) caused BEC levels 1-20 mmol/l (Lundqvist et al. 1995). When the rats are offered ethanol as the only source of fluid the ethanol consumption is not very heavy, but the duration of experiment can be long and the experiment is more comfortable to the animals.

As BECs rarely reach the levels of intoxication when the rats drink ethanol solution *ad libitum*, ethanol was force-fed to the rats in study V. The advantage of intragastric ethanol intubation is that the researcher can decide the amount of ethanol consumption. In study V constant intoxication level was maintained (Riihioja et al. 1999a, 1999b). The disadvantages of force-feeding method are the stress caused by ethanol feeding and possible nutritional deficiencies. The sucrose fed controls were correspondingly force-fed with isocaloric sucrose solution, and they were pair-fed with food. Therefore, the sucrose-fed rats had a similar feeding stress and intake of nutrients than the ethanol-fed rats. However, heavy ethanol feeding caused diarrhoea to some of the rats, and therefore the absorption of nutrients may not be identical between the groups. When ethanol was force-fed to the rats, BEC values measured before the morning ethanol doses were 17.7 mmol in young ethanol-exposed rats and 25.2 mmol/l in old ethanol exposed rats. 1h after ethanol feeding the BEC values in young and old rats were 36.6 mmol/l and 46.8 mmol/l, respectively. The ethanol consumption in study V can therefore be classified as fairly heavy, and objectively estimated the rats were intoxicated during the days they where ethanol-fed.

Voluntary ethanol consumption of AA and ANA rats was measured in both the ethanol-exposed and the water-consuming groups. 3-month-old control male and female AA rats consumed ethanol 6.0 and 6.6 g/kg/day, respectively, for 3 weeks, and at the age of 23 months 4.1 and 5.5 g/kg/day, respectively. In male and female control ANA rats the ethanol consumptions, respectively, were 0.1 and 0.3 g/kg/day (3-month-old) and 0.3 and 0.4 g/kg/day (23-month-old) (Sarviharju et al. 2001). The voluntary ethanol consumption of the control rats may diminish the differences found between the ethanol-exposed and the water-consuming groups.

1.3. Morphometric methods

The number of SCG neurons and cerebellar microglia were measured by using the disector method, which is an unbiased stereological procedure for the estimation of arbitrary particles (Sterio 1984, Gundersen et al. 1986). Instead of particle density achieved by conventional 2D method, the total number of

particles can be measured with the stereological method. This method has been used in particle counting in the neuronal tissue (West 1993) and in other tissues as well (Mayhew and Gundersen 1996). In studies II and III physical disector pairs were utilized and in study IV optical disector was used. Similar results are achieved by both methods but the optical disector is time saving and more reliable if cell density is high (Gundersen et al. 1988). In the present study both methods were estimated equally reliable, but the efficiency of the optical disector was noticeable. Cavalieri's principle was used to measure the volume of SCG and cerebellar folia. The method demands systematic sampling of the studied tissue with a random starting point (Gundersen and Jensen 1987). The most important reason for using the Cavalieri's principle was its efficiency: it is easy, fast and reliable (Gundersen and Jensen 1987). Stereological methods were also used to measure the density of neurons (II, III), the amount of intraneural lipopigments (II), and the total volumes of SCG neurons and non-neuronal elements (II) (Gundersen et al. 1988).

The intensities of formaldehyde-induced histofluorescence (FIF), tyrosine hydroxylase immunoreactivity (TH-IR) and cytochrome oxidase (CO) histochemistry were evaluated by a systematic light or fluorescence microscopic observation. FIF intensities were also estimated with pseudocolor video prints. All the measurements were made blind to treatment (rats were coded and codes were broken after completion of measurements) by a single researcher. Previously computer-assisted methods have also been used for analysing the intensity of (immuno)-histochemical stainings leading to semi-quantitative data (Shetty and Phillips 1992, Huang et al. 1996, Knapp and Crew 1999, Rintala et al. 2001). Although there are advantages in the computer-assisted methods, the variation of tissue fixation, background staining and the distribution of staining may easily biase the results. Therefore the visual observation and grading of reaction intensities were considered the best methods for the present study.

2. Mortality and morbidity during lifelong ethanol consumption

Although alcohol consumption causes 3 to 9% of all deaths in the Western countries (Shultz et al. 1990, Pignon and Hill 1991, Yanez et al. 1993, Holman and English 1995, Cipriani et al 1998, Mäkelä 1998, Single et al. 1999), moderate alcohol consumption decreases mortality by decreasing the incidence of coronary heart disease, myocardial infarction and stroke (Mäkelä et al. 1997, Hart et al. 1999, Dawson 2000, Sjögren et al. 2000). The correlation between alcohol consumption and mortality is therefore a J-shaped curve. It is also suggested that other factors, rather than alcohol consumption *per se*, cause the J-

shaped association between alcohol consumption and mortality (Andreasson 1998). Alcohol consumption decreases the diseases that cause deaths at an advanced age (coronary heart disease, myocardial infarction, stroke) leading to the prevention of mortality among the aged. On the contrary, alcohol consumption increases liver diseases, cardiomyopathy, alcohol dependence, pancreatitis, intoxications, accidents, suicides and homicides leading to increased alcohol-induced mortality in young age groups. Therefore, alcohol consumption contributes to ca. 25% of deaths in people under the age of 50, and decreases the average life expectancy by 10% (Sjögren et al. 2000).

In a previous study, a 21-month voluntary ethanol consumption did not change the survival of AA rats compared to water-consuming AA controls (Hervonen et al. 1992). In Sprague-Dawley rats a 24-month ethanol exposure increased mortality compared to pair-fed controls (Mendenhall et al. 1993). In spontaneously hypertensive rats, however, chronic ethanol consumption decreased coronary heart disease and renal arteriopathy (primary causes of deaths in spontaneously hypertensive rats), which resulted in decreased mortality and prolonged life-span (Schlicht et al. 1992). In the present study lifelong ethanol consumption did not cause premature deaths in either gender of AA or ANA rats. Neither was ethanol-induced prolongation of the life-span found. If AA and ANA rats consume similar amounts of ethanol, as the male rats did in the present study, the acetaldehyde levels of ANA rats are higher compared to AA rats (Eriksson 1973, Koivula et al. 1975, Hilakivi et al. 1984, Koivisto et al. 1993). Acetaldehyde is responsible for several unwanted effects of ethanol (flushing, nausea, headache, increased heart rate etc.) (Eriksson 2001), and has been related to many alcohol-induced diseases, such as alcoholic liver disease (Matysiak-Budnik et al. 1996), fetal alcohol syndrome (Kaufman 1997), cardiomyopathy (Liang et al. 1999), pancreatitis (Nordback et al. 1991) and neuronal damage (Phillips and Cragg 1983, Holownia et al. 1999). Although the ethanol-exposed rats in the present studies did not show increased mortality compared to the control rats, lifelong ethanol consumption increased the incidence of malignant tumors.

Chronic ethanol consumption in humans is related to an increased incidence of several cancers (IARC 1998, Bagnardi et al. 2001). It has been suggested that ethanol treatment increases the malignancy of cancer cells *in vitro* (Hsu et al. 1991, Kornfehl et al. 1999, Izevbigie et al. 2002) and the incidence of colorectum cancer *in vivo* (Seitz et al. 1985, Niwa et al. 1991), but not the incidence of breast cancer in female rats (McDermott et al. 1992). However, ethanol itself is not considered carcinogenic, but may be cocarcinogenic or act as a tumor promoter (Takada et al. 1986, Seitz et al. 1998, IARC 1998). Acetaldehyde, on the contrary, seems to have direct mutagenic effects (Homann 2001, Salaspuro 2003). The risk of digestive tract and pulmonary cancers is increased 3- to 12-fold, if acetaldehyde metabolism is disturbed (ALDH-2 defect) leading to accumulation of acetaldehyde after ethanol consumption

(Yokoyama et al. 1998). In addition, microbes of gastrointestinal tract (mouth, stomach and large bowel) have ADH activity and are capable of metabolising ethanol to acetaldehyde (Salaspuro 1996, 1997). Therefore, local accumulation of acetaldehyde is found, if microbes proliferate in the saliva (poor dental hygiene and tobacco smoking) (Homann et al. 2000, 2001), in the gastric juice (atrophic gastritis and medication to increase pH) (Väkeväinen et al. 2000, 2002) or in the large bowel (antibiotics) (Tillonen et al. 1999, 2000), which may lead to an increased occurrence of oropharyngeal, stomach and colorectal cancers, respectively (Salaspuro 2003). The International Agency for Research on Cancer summarises that alcoholic beverages are carcinogenic in humans, although evidence from experimental studies is insufficient (IARC 1998). As for acetaldehyde, the carcinogenecity is evident in experimental animals, but poorly documented in humans (IARC 1999).

In the present study the main aim was not to analyze the relation between the chronic ethanol exposure and cancer, but the autopsy findings revealed that long-term alcohol consumption tended to increase the rate of cancers. The effect reached significance when the rats over 24 months of age were included in the analysis. Previously, systematic autopsies during lifelong ethanol consumption of rats have not been performed, but the results of the present study are in line with previous epidemiological human studies. In the present study, the number of cancers was too low for further analysis of specific malignancies. Examination of the breasts of female rats was not included in the study protocol, and therefore there is no data available on the incidence of breast cancer in the present experiment.

3. Ethanol-induced changes in adrenergic neurons

In the present study peripheral sympathetic neurons (SCG) were examined. Also CNS adrenergic neurons (LC) were studied. The SCG is a well defined and homogenous neuron population (Eränkö 1971, Burnstock and Costa 1975, Gabella 1976), and a simple model for studying neurons *in vivo* (Hervonen et al. 1986). Previously it has been shown that long-term (25 months) voluntary ethanol consumption decreases the neuronal packing density, increases the amount of lipopigment and decreases the intensity of FIF and TH-IR in the SCG of male AA rats (Jaatinen et al. 1992). A 4-week heavy ethanol feeding has been found to induce vacuolation of SCG neurons, decrease neuronal packing density, and increase FIF and TH-IR intensities in the SCG (Jaatinen et al. 1993, Jaatinen and Hervonen 1994). However, in young rats (4 months of age) neuronal vacuolation, the increase of FIF intensities and the proportion of TH negative neurons were more prominent compared to rats aged 12 and 24 months (Jaatinen and Hervonen 1994). Decrease in neuronal packing density was most severe in

the oldest rats and the ethanol-induced changes were least significant in the 12-month-old rats (Jaatinen and Hervonen 1994).

In the present study the total number of SCG neurons was not changed after lifelong ethanol consumption in either genders of AA and ANA rats or after a 5½-month continuous ethanol consumption in Wistar rats. However, 5½ months of intermittent ethanol consumption decreased the number of SCG neurons, suggesting that binge type of drinking is more harmful to the sympathetic neurons than continuous drinking. Lipopigmentation and TH-IR intensity increased during lifelong ethanol consumption in male rats but not in female rats. Interestingly FIF or TH-IR intensities were not increased in the rats exposed intermittently or continuously to ethanol for 5½ months. This may be due to the resistance of "middle-aged" rat to ethanol-induced changes in catecholamine synthesis (TH-IR) and content (FIF) (Jaatinen and Hervonen 1994). Even though there were plenty of similarities between studies II and III (standard conditions, food *ad lib*, group cages, liquid N_2 fixation, FIF staining, physical disector morphometry) there were also differences, such as the duration of experiments, the strains of rats, the food, the ethanol concentration, age at the beginning of the experiment, and the ethanol consumption level in male rats. However the results indicated that intermittent ethanol exposure, unlike chronic continuous ethanol exposure, did cause loss of SCG neurons. In the present study we could not replicate our earlier finding of a decrease in neuronal packing density after lifelong ethanol consumption (Jaatinen et al. 1993, Jaatinen and Hervonen 1994). One of the reasons for the discrepancy may be the different morphometric method used. Also previously the duration of ethanol exposure was longer (25 *vs.* 21 mo) or ethanol was force-fed leading to higher daily doses (Jaatinen et al. 1993, Jaatinen and Hervonen 1994). In the present study the effects of heavy ethanol feeding on SCG were not studied, but the increased intensity of TH-IR and the accumulation of lipopigment after long-term ethanol consumption in male rats are in line with the previous studies (Jaatinen et al. 1993, Jaatinen and Hervonen 1994).

The effect of lifelong ethanol exposure on LC of the same rats as studied in the present study II has been reported previously. Lifelong ethanol exposure decreased the number of LC neurons in AA and ANA female rats, and in AA male rats, but not in ANA male rats (Lu et al. 1997, Rintala et al. 1998). As the ANA line of rats produces higher acetaldehyde levels during ethanol oxidation than the AA rats (Eriksson 1973, Hilakivi et al. 1984, Koivisto et al. 1993), acetaldehyde did not seem to be responsible for the ethanol-induced damage in LC (Rintala et al. 1998). 17 weeks of heavy ethanol consumption has been reported to decrease the synapse-to-neuron ratio in the LC (Kjellström et al. 1993). In the present study, CO activity in LC neurons was considerably decreased in the *young EtOH* rats, and slightly decreased in the *old EtOH* rats. In the same rats the loss of LC neurons has been reported in the *old EtOH* rats, but in the *young EtOH* rats loss was not significant (Riihioja et al. 1999b). Decreased

CO activity reflects impaired neuronal energy metabolism (Wong-Riley 1989). Only slightly decreased CO activity of the *old EtOH* rats may mean that the fewer LC neurons in a compensatory manner increase their oxidative energy metabolism, or the neurons with low energy metabolism are lost and the neurons having high CO activity survive.

In summary, previous studies have suggested that the number of adrenergic neurons in LC decreases after long-term chronic ethanol consumption (Lu et al. 1997, Rintala et al. 1998), and also after repeated ethanol intoxications and withdrawals in aged rats (Riihioja et al. 1999b). However, in the present study lifelong or 5½-month continuous ethanol consumption did not induce any loss of adrenergic sympathetic neurons in SCG. Therefore, the central adrenergic neurons seem to be more vulnerable to ethanol-induced degeneration than the peripheral ones. However, a negative correlation between individual ethanol consumption and the number of SCG neurons was found in female rats after lifelong ethanol consumption. Since female rats consumed more ethanol throughout the experiment (Sarviharju et al. 2001), higher blood ethanol concentrations may have led to degeneration of rat SCG neurons as has been suggested previously in CNS neurons (Bonthius and West 1990, Lundqvist et al. 1994). Because the SCG degeneration was most severe in the intermittently ethanol-exposed group although the weekly ethanol consumption the lower compared to other groups, repeated ethanol withdrawals might be more vulnerable to SCG neurons than chronic ethanol intoxication *per se*. The BECs were measured once during the 5½-month ethanol exposure. Although the values were similar in intermittently and continuously exposed rats, higher BEC peaks in the intermittently exposed rats could not be excluded since their daily drinking rhythm was not evaluated. The rats consume most of their daily ethanol dose during the dark phase (Boyle et al. 1997), and AA and ANA rats have three peaks of daily fluid consumption: 6-7 p.m., 11 p.m. and 3-4 a.m. (lights on from 6 a.m. to 6 p.m.) (Eriksson 1972). On the other hand, it has been shown previously that rats consume relatively large amounts of ethanol immediately when ethanol is available after one hour of ethanol deprivation (Boyle et al. 1997). Therefore it is possible that intermittently exposed rats had a high BEC peak immediately after having access to ethanol.

4. Ethanol-induced neuronal damage – possible mechanisms

4.1. Acetaldehyde

Acetaldehyde is a reactive metabolite of ethanol (Lindros 1978) having also neurotoxic effects (Hunt 1996). It is capable of causing gene mutations in human lymphocytes *in vitro* (He and Lambert 1990). Acetaldehyde may also produce adducts with various proteins, such as haemoglobin (Sillanaukee and Koivula 1990), lipoproteins (Wehr et al. 1993) and cytochrome enzymes (Behrens et al. 1988). Acetaldehyde protein adducts have also been found in the liver (Niemelä et al. 1991, 1994, 1998) and in the CNS (Rintala et al. 2000). Acetaldehyde and acetaldehyde protein adducts are suggested to cause degeneration of neurons (Hunt 1996), but also the formation of ROS during ethanol metabolism may be crucial in acetaldehyde-related neuronal damage (see page 48, Oxidative stress). The ANA rats produce higher acetaldehyde levels during ethanol metabolism than AA rats if they are exposed to equal amounts of ethanol (Eriksson 1973, Koivula et al. 1975, Hilakivi et al. 1984, Koivisto et al. 1993). Therefore, if acetaldehyde plays a central role in the degeneration of neurons, ANA rats should be more vulnerable to ethanol-induced neuronal damage. However, no difference in neuronal vulnerability was found between the AA and ANA line of rats in peripheral sympathetic neurons in the present study or in CNS adrenergic neurons in a previous study (Rintala et al. 1998). Acetaldehyde levels were equal in the rats that had been exposed to ethanol intermittently for 5½ months and those exposed to ethanol continuously, and therefore total acetaldehyde exposure was lower in the intermittently exposed rats. Although in the present study no evidence of acetaldehyde-induced neuronal toxicity was found, the local accumulation of acetaldehyde and by-products of ethanol metabolism (e.g. ROS) might be more crucial to the degeneration of neurons than blood acetaldehyde levels.

4.2. Microglia

The physiological function of microglia is to phagosytosize degenerated tissue and to secrete cytotoxins as a part of the CNS immune defense (Nakajima and Kohsaka 1993). This normal action of microglia may harm the normal neuronal tissue as well. As shown in the present study, intermittent ethanol exposure increased the number of microglia in the molecular layer of cerebellar vermis folium II, whereas continuous ethanol consumption did not induce any

microgliosis. Previously, 40 weeks of continuous ethanol consumption did not alter the density of microglia in rat cerebellar cortex (Dlugos and Pentney 2001). Although in the study of Dlugos and Pentney (2001) the number of microglia was estimated by using areal density, the results are in line with the present study showing no microgliosis after continuous ethanol exposure. *In vitro* microglia treated with ethanol increase their ROS production (Colton et al. 1998). Oxidative stress has been suggested to lead to degeneration of neurons (Cohen and Werner 1993, West et al. 1994, Mantle and Preedy 1999), which in turn may increase the number and activity of microglia. This ethanol- and withdrawal-induced vicious circle may be one of the central mechanisms contributing to ethanol-induced neuronal damage.

The anterior superior part (i.e. folia I-II) of vermis is the most vulnerable cerebellar area to ethanol-induced degeneration both in human alcoholics and in experimental animals (Viktor et al. 1959, Torvik and Torp 1986, Phillips et al. 1987, Karhunen et al. 1994, Rintala et al. 1997). In the same area microgliosis was found in the present study. In AIDS patients the cerebral atrophy has been found to strongly correlate to cortical microgliosis suggesting that microglia phagocytosize degenerated neuronal tissue or that microglia cause neuronal degeneration (Gelman 1993). Therefore, microglia may have a crucial role in ethanol-induced CNS degeneration, and may be used as an early marker of ethanol-induced nervous system damage.

4.3. Cytochrome oxidase

CO is the complex IV of oxidative phosphorylation and the terminal enzyme in the respiratory chain located on the inner membrane of mitochondria (Capaldi 1990). Its activity can be used as a marker for neuronal activity (Wong-Riley 1989). Energy formed via oxidative phosphorylation generates small amounts of ROS, but if CO activity is reduced, aberrant oxidative metabolic processes form larger amounts of ROS (Abe et al. 1993, Chandrasekaran et al. 1994). Therefore CO is a marker of neuronal activity but also decreased CO activity may be related to an increased production of ROS.

In the present study a decreased activity of CO was found in the anterior superior vermis, MPF and LC of rats exposed to intermittent ethanol feeding for 5 weeks. Previously, the expression of CO mRNA in rat hippocampus has been shown to be decreased after a 10-day intraperitoneal ethanol treatment (Kim et al. 2001). In mammillary bodies, no significant change in CO activity after ethanol consumption was found (Rubio et al. 1996). In whole brain homogenates there was a reduced CO activity in 3-month-old rats fed with ethanol liquid diet for one month (Marin-Garcia et al. 1995). However, in 18-month-old rats no change in whole brain CO activity was observed after similar ethanol-exposure

(Marin-Garcia et al. 1995). In contrast, in the present study the *old EtOH* rats had decreased CO activity in the Purkinje cells of anterior superior vermis, whereas in the *young EtOH* rats the Purkinje cell CO activity was unchanged. On the other hand, the lack of change in whole brain CO levels (Marin-Garcia et al. 1995) does not rule out the possibility of there having been regional changes in CO activities, reflecting regional differences in ethanol-induced vulnerability.

Local cerebral glucose utilization has been widely used to estimate neuronal activity after ethanol consumption. It reflects acute neuronal activity compared to the CO measuring a longer period of time (Hevner et al. 1995). In line with the present study, previously local cerebral glucose utilization has been shown to decrease during ethanol withdrawal in cortical and limbic regions (Clemmesen et al. 1988). Although acute low ethanol doses have been reported to increase local cerebral glucose utilization in motor and limbic areas, higher doses decrease glucose utilization in several brain structures (Eckardt et al. 1988, Grünwald et al. 1993, Williams-Hemby and Porrino 1994, 1997). Also limited (4 hours/day) ethanol exposure for 8 weeks has been found to depress local cerebral glucose utilization throughout the CNS in alcohol-preferring rats (Smith et al. 2001, 2002).

The present study suggests that repeated heavy ethanol intoxications and withdrawals decrease metabolic activity in several CNS areas. In neurons, energy is mostly used for active ion pumping to maintain the resting membrane potential, for fast axoplasmic transport, and for the synthesis of macromolecules and neurotransmitters. Impaired energy metabolism may enhance excitotoxic neuronal degeneration by deteriorating the membrane potential, and by impairing processes involved in buffering intracellular calcium (Beal 1992, Bowling et al. 1993). Impaired CO function also increases the accumulation of ROS (Abe et al. 1993).

4.4. Oxidative stress

Reactive oxygen species (ROS) are single-electron reduction products of oxygen (Cheeseman and Slater 1993). The most important ROS are hydroxyl radical ($OH\cdot$), superoxide anion ($O_2^{-}\cdot$), and hydrogen peroxide (H_2O_2). All cells generate ROS as by-products of normal oxidative metabolism (Cheeseman and Slater 1993). The mechanisms of ROS production include e.g. mitochondrial electron transport chain, lipid peroxidation, metabolism of purines and prostanoids, autooxidation of catecholamines and metabolism of ethanol via CYP2E1 (Ramasarma 1982, Halliwell and Gutteridge 1989, Persson et al. 1990, Cohen and Werner 1993, Roy et al. 1994, Mantle and Preedy 1999). Also activated microglia produce ROS, for example superoxide anions (Colton et al. 1998). All cells are susceptible to the oxidative damage but neurons are particularly

vulnerable since their oxygen consumption is high (Cohen and Werner 1993, West et al. 1994, Mantle and Preedy 1999). The cells have capability to resist oxidative stress by antioxidants (Halliwell and Gutteridge 1989).

Acute and chronic ethanol administration has been found to decrease the amount or activity of both anti-oxidative enzymes (superoxide dismutase and glutathione peroxidase) and low molecular weight antioxidants (ascorbic acid, α-tocopherol) in CNS (Ledig et al. 1981, Uysal et al. 1989, Nordmann et al. 1992, Montoliu et al. 1994, Rouach et al. 1997). Ethanol exposure also increases the production of prooxidants. Ethanol metabolism via CYP2E1 has been shown to produce significant amounts of ROS (Ekström et al. 1986, Persson et al. 1990). Ethanol molecule itself can be reduced to a free radical molecule, the hydroxyethyl radical ($CH_3 \cdot CHOH$) (Hunt 1993). In cultured cerebellar granule cells ethanol exposure increases the level of ROS and decreases the viability of neurons (Huentelman et al. 1999). It has been shown that the number of Purkinje cells decreases in ethanol-treated neonatal rats, but the loss is prevented by the inclusion of α-tocopherol in the diet (Heaton et al. 2000).

Lipopigments accumulate to postmitotic cells with aging (Sohal 1981). The rate of lipopigment accumulation has been suggested to relate to the level of oxidative stress, increased functional activity or disturbance of lysosomal function (Sohal and Brunk 1990). *In vitro* the formation of lipopigments in human glial cells increases when oxygen concentration in their atmosphere increases, showing the relation between oxygen metabolites and lipopigment accumulation (Thaw et al. 1984). Chronic ethanol exposure increases lipopigmentation in rat prefrontal cortex, hippocampus and cerebellum (Tavares and Paula-Barbosa 1982, Tavares et al. 1985, Borges et al. 1986, Cadete-Leite et al. 1988c) Also long-term (25 months) voluntary ethanol consumption has been shown to increase the amount of lipopigment in the SCG of male AA rats (Jaatinen et al.1992). In the present study lifelong ethanol exposure increased the accumulation of lipopigments in the SCG of male rats. This was most likely due to the increased functional activity of SCG neurons (increased TH-IR in male rats), but other mechanisms may also have increased the production of ROS during chronic ethanol exposure.

As brain macrophages, microglia are capable of producing ROS for functional responses during injury or infection of the CNS (Banati et al. 1993). Ethanol-exposed cultured microglia increase their ROS formation (Colton et al. 1998). This may be due to the disruption of mitochondrial electron transport chain in ethanol-treated microglia (Nordmann et al. 1992, Hunt 1993, Devi et al. 1994). Although *in vitro* 24 hours 20 mM ethanol treatment increased microglial phagocytic activity (Colton et al. 1998), 6 days 100 mM ethanol treatment decreased it (Aroor and Baker 1998). In the present study the number of cerebellar microglia was increased in the granular layer of folium II in the

intermittent rats. Microgliosis may lead to an increased production of ROS if the microglia are activated.

It has been suggested that the leakage of electrons from oxidative phosphorylation is the principal source of ROS (Chance et al. 1979). However, aberrant oxidative metabolic processes form more ROS if similar amount of energy is produced (Abe et al. 1993, Chandrasekaran et al. 1994). Since most of the oxygen in the CNS is used in oxidative phosphorylation, the proper function of CO is critical for regulating ROS production in brain. After a 10-day ethanol exposure CO expression decreased in the hippocampus, suggesting elevation in hippocampal oxidative stress (Kim et al. 2001). In the present study, CO activity decreased in prefrontal cortex, LC and cerebellum after 5 weeks of repeated ethanol intoxications and ethanol withdrawals, which may increase oxidative stress in those CNS areas.

4.5. Withdrawal-induced excitotoxicity

It has been found that chronic ethanol consumption causes degeneration of neurons in cerebellum (Tavares et al. 1987), prefrontal cortex (Cadete-Leite et al. 1990), hippocampus (Cadete-Leite 1988b, Bengoechea and Gonzalo 1991), SCG (Jaatinen et al. 1992, 1993, Jaatinen and Hervonen 1994) and LC (Lu et al. 1997, Rintala et al. 1998, Riihioja et al. 1999b) of adult rats. Although ethanol exposure without a withdrawal period causes degeneration of neurons, according to several studies recovery from chronic ethanol exposure and/or repeated ethanol withdrawals are probably more harmful to nervous system than chronic continuous ethanol exposure (Walker et al. 1980, Cadete-Leite et al. 1988a, 1989, 1990, Paula-Barbosa et al. 1993, Lundqvist et al. 1994, 1995). In the present study intermittent ethanol exposure caused loss of SCG neurons and induced microgliosis in cerebellar vermis. After lifelong continuous ethanol consumption followed by a gradual withdrawal from ethanol, no significant degeneration of neurons was seen. The results are in line with the previous findings suggesting that sudden ethanol withdrawal may be more harmful to the neurons than chronic ethanol consumption *per se*.

It has been suggested that high ethanol concentrations may cause the degeneration of neurons. Binge-type drinking may produce higher BEC values than continuous ethanol exposure, and thereby induce neuronal damage (West and Goodlett 1990, West et al. 1990, Lundqvist et al. 1995). Indeed, this may be the case in the developing nervous system: It has been shown that smaller cumulative ethanol doses are more vulnerable to the developing CNS of rat pups than larger doses, if smaller doses produce higher BEC peaks (Bonthius and West 1990). The rats exposed to ethanol intermittently for 5½ months and those exposed continuously consumed similar amounts of ethanol during the ethanol

exposure days, and there were no differences in the BEC values between the groups. During continuous access to ethanol, ethanol consumption is highest during the night time (Aalto 1986). Circadian drinking rhythm is not known in intermittently ethanol-exposed rats, and was not studied in the present study, either. However, it has been previously shown that ethanol consumption reaches the peak immediately when ethanol is available after ethanol deprivation (Boyle et al. 1997). Therefore, more blood samples would be needed to estimate BEC values in intermittently and continuously ethanol-exposed rats throughout the day. However, the inverse correlation between individual ethanol consumption and the number of SCG neurons in female rats after lifelong ethanol consumption may be an evidence of SCG vulnerability to higher doses of ethanol.

Ethanol withdrawal causes hyperactivity in the nervous system, as the excitatory NMDA-receptors and the sympathetic nervous system are activated, while the inhibitory $GABA_A$-receptors are inhibited (see pages 14-15). The activation of NMDA-receptors increases intracellular Ca^{2+} concentration, possibly leading to neurotoxic effects (Ahern et al. 1994). NMDA antagonists have been found to prevent ethanol-induced Ca^{2+} influx and the degeneration of neurons in rat cerebellum (Hoffman et al. 1995). The deterioration of $GABA_A$-receptor function could in part cause withdrawal symptoms and therefore benzodiazepines ($GABA_A$-receptor agonists) are widely used against ethanol withdrawal symptoms (Litten and Allen 1991). Sympathetic overactivity can be reduced by β-adrenergic blockers (Litten and Allen 1991) or by α_2-adrenoceptor agonists (e.g. clonidine and dexmedetomidine). Dexmedetomidine alleviates withdrawal symptoms (Riihioja et al. 1997a, 1997b, 1999a), and is protective against ethanol- and/or withdrawal-induced degeneration in peripheral (Jaatinen et al. 1995) and central (Riihioja et al. 1999a) adrenergic neurons. In the present study sympathetic overactivity in SCG was found after lifelong ethanol consumption in AA and ANA male rats. The overactivity of adrenergic neurons may increase the oxidation of catecholamines, leading to an enhanced production of ROS. The possible mechanisms leading to the increased sympathetic activity in the male rats compared to the female rats are discussed in the next chapter.

5. Effect of gender on ethanol-induced nervous system changes

Previously, it has been suggested that the nervous system of females may be more vulnerable to ethanol-induced damage than the nervous system of males (Harper et al. 1990, Lancaster 1994). This view has been supported by computer tomography and histopathological studies showing comparable brain atrophy in

female alcoholics with a shorter drinking history than males (Jacobson 1986, Harper and Krill 1990, Harper et al. 1990, Mann et al. 1992). However, recently a significant decrease in cortical gray and white matter volumes, and an increase in CNS ventricle volumes and size of sulci were found in alcoholic males, whereas in female alcoholics no deficits were detectable (Pfefferbaum et al. 2001). The result was the same even when adjusted for lifetime ethanol consumption. Also positron emission tomography showed deficient cerebral glucose utilization in male alcoholics but not in the females (Wang et al. 1998).

In experimental studies, no significant differences between the genders have been found in the nervous system after ethanol exposure (Lu et al. 1997, Rintala et al. 1998, Savage et al. 2000), excluding the reduction in the molecular layer of cerebellum vermis folium II volume found in ANA female rats, but not in ANA male rats (Rintala et al. 1997). In the present study both sexes had an equal number of SCG neurons after lifelong ethanol exposure. However, in females there was a significant negative correlation between ethanol consumption and the number of neurons. This may be caused by vulnerability of SCG neurons to high ethanol doses rather than differences between genders since female rats in the present study consumed more ethanol than males, in line with previous findings (Eriksson and Malmström 1967, Krishnan and Maickel 1991, Lancaster and Spiegel 1992). However, when the individual ethanol consumption was used as a covariate, there was no difference between the genders in the number of SCG neurons. TH-IR was more heterogeneous and on an average higher, and the amount of intraneuronal lipopigmentation significantly higher in the ethanol-exposed male rats than in the ethanol-exposed female rats or in the male control rats, which indicates a higher oxidative stress in male rats due to chronic ethanol exposure (Hervonen et al. 1986, Sohal and Brunk 1990). An ethanol-related increase in lipopigmentation has been reported in the SCG (Jaatinen et al. 1992), cerebellum (Tavares et al. 1985), and hippocampus (Borges et al. 1986) of ethanol-exposed male rats, but females have not been studied previously. In survival and morbidity no sex differences were seen. In the present study gender differences were found in the amount of drinking, SCG lipopigmentation and TH-IR after lifelong ethanol consumption, but the results rather suggested that males might be more vulnerable to ethanol-induced changes in peripheral sympathetic neurons than females. It could be speculated that in male rats ethanol exposure increases sympathetic activity more than in female rats, at least when the rats are housed in group cages. At least, during ethanol withdrawal male rats are more anxious than female rats (Varlinskaya and Spear 2004).

6. Clinical implications

The results of the experimental studies should be interpreted with caution as far as human alcoholics are concerned (Ponnappa and Rubin 2000). Previously,

discrepancies in the effects of chronic ethanol exposure on CNS between experimental and human studies have been found. For example, it has been reported that ethanol exposure degenerates neurons in rat hippocampus CA1 (Bonthius and West 1990), but not in human alcoholics (Harding et al. 1997). The number of LC neurons has decreased during chronic ethanol consumption in rat (Kjellström et al. 1993, Lu et al. 1997, Rintala et al. 1998) but not in human alcoholics (Halliday et al. 1992, Baker et al. 1994). However, one study showed a loss of LC neurons in human alcoholics compared to controls (Arango et al. 1994). In humans cerebellar Purkinje cells have been vulnerable to long-term ethanol exposure (Karhunen et al. 1994) and granular cells more resistant (Baker et al. 1999), but the situation has been rather the opposite in rats (Tavares and Paula-Barbosa. 1982, Pentney 1993). However, several different morphometric methods were used in previous studies and the experimental settings were not comparable.

Moderate lifelong ethanol consumption did not significantly affect the SCG neurons but shorter intermittent ethanol exposure decreased the number of SCG neurons by 28% and increased microgliosis in cerebellum. According to the present study binge drinking, at least, is harmful to the neurons but small continuous doses may be safer. Although the ethanol consumption was moderate and there were no withdrawals, the risk of cancer was increased. In the present study females were not more vulnerable to the effects of ethanol. However, similar ethanol exposure leads to higher BECs in women, and 10 g of alcohol per day increases the risk of breast cancers by 7% (Aronson 2003). In the present study aging and ethanol consumption did not cause loss of SCG neurons, but after repeated ethanol intoxications and withdrawals 30-month-old rats had impaired energy metabolism in Purkinje cells compared to the *young EtOH* rats. It may be that binge drinking causes more harmful consequences in the aging brain than in the young brain.

SUMMARY AND CONCLUSIONS

The main findings of the study are:

1. Lifelong ethanol consumption did not change the survival of AA or ANA rats of either gender. However, the survival of the ANA rats in general was significantly lower compared to the AA rats. Autopsy findings showed that the ANA rats had significantly more kidney diseases, particularly polycystic kidney disease. The ANA rats also had more benign tumors and cardiovascular diseases than the AA rats. Although ethanol consumption did not increase mortality, the ethanol-exposed rats tended to have a higher rate of malignant neoplasms than the control rats. Although polycystic kidney disease was the most probable cause of premature death in the ANA line, the effects of the kidney disease in the ANA rats on their homeostasis and renal function need to be studied further.

2. Lifelong ethanol consumption did not change the SCG neuronal density, volume, or total number of neurons in either sex of AA or ANA rats. However, there was a significant negative correlation between individual ethanol consumption and the number of SCG neurons in female rats. No AA *vs.* ANA line difference was found in ethanol-induced neuropathology of the SCG. Although the amount of intraneuronal lipopigments increased due to lifelong ethanol consumption only in male rats, no other gender differences in SCG neuropathology were found.

3. 5½ months of intermittent ethanol exposure decreased the total number of SCG neurons in unselected Wistar rats. Continuous ethanol exposure did not change the number of SCG neurons, even though the continuously ethanol-exposed rats consumed 1.7 times more ethanol than the intermittently exposed ones. Therefore, intermittent ethanol consumption seemed to be more harmful to the sympathetic neurons than continuous ethanol consumption.

4. Intermittent consumption of ethanol for 5½ months increased the number of microglia in the molecular layer of rat cerebellar vermis folium II compared to control animals. However, 5½ months of continuous ethanol consumption did not induce any change in the number of cerebellar microglia. In the present study the morphology of microglia was similar in all the groups, and most of the microglia were ramified. The slow nature of ethanol-induced

CNS damage may be the reason for the equal number of ameboid microglia in all the groups.

5. Histochemical demonstration showed that repeated ethanol intoxications and ethanol withdrawals decreased CO activity in the LC of both 4- and 30-month-old rats, but the decrease was more pronounced in 4-month-old rats. Ethanol exposure decreased CO activity in prefrontal cortex and cerebellar granule cells in both 4- and 30-month-old rats. 30-month-old ethanol-exposed rats had decreased CO activity in cerebellar Purkinje neurons compared to the controls of the same age and 4-month-old ethanol-exposed rats. Decreased CO activity may indicate impaired regional energy metabolism and may contribute to the selective neuronal degeneration caused by chronic ethanol exposure.

In conclusion, the pattern of drinking is an important factor when evaluating the effects of ethanol exposure on the nervous system. In other words, ethanol withdrawal may be more harmful to the neuronal tissue than ethanol consumption *per se*. The present study did not support the hypothesis of the female nervous system being more vulnerable to ethanol than the male one. Finally, the decreased survival and increased occurrence of kidney diseases in the ANA line must be taken into account when estimating previous, as well as future studies on AA and ANA rats.

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REFERENCES

Aalto J (1986): Circadian drinking rhythms and blood alcohol levels in two rat lines developed for their alcohol consumption. Alcohol 3:73-75.

Aalto J and Kiianmaa K (1987): Role of brain monoaminic systems in the increased ethanol drinking caused by REM-sleep deprivation. Alcohol Alcohol Suppl 1:313-317.

Abdel-Rahman AA and Wooles WR (1987): Ethanol-induced hypertension involves impairment of baroreceptors. Hypertension 10:67-73.

Abe K, Kawagoe J, Lee TH, Aoki M and Kogure K (1993): Disturbance of mitochondrial DNA expression in gerbil hippocampus after transient forebrain ischemia. Brain Res Mol Brain Res 19:69-75.

Acarin L, Vela JM, Gonzalez B and Castellano B (1994): Demonstration of poly-N-acetyl lactosamine residues in ameboid and ramified microglial cells in rat brain by tomato lectin binding. J Histochem Cytochem 42:1033-1041.

Adams KM, Gilman S, Koeppe RA, Kluin KJ, Brunberg JA, Dede D, Berent S and Kroll PD (1993): Neuropsychological deficits are correlated with frontal hypometabolism in positron emission tomography studies of older alcoholic patients. Alcohol Clin Exp Res 17:205-210.

Ahern KB, Lustig HS and Greenberg DA (1994): Enhancement of NMDA toxicity and calcium responses by chronic exposure of cultured cortical neurons to ethanol. Neurosci Lett 165:211-214.

Ahtee L and Svartström-Frazer M (1975): Effect of ethanol dependence and withdrawal on the catecholamines in rat brain and heart. Acta Pharmacol Toxicol 36:289-298.

Airaksinen MM and Peura P (1987): Mechanism of alcohol withdrawal syndrome. Med Biol 65:105-112.

Alho H, Partanen M and Hervonen A (1983): Microfluorimetric quantification of catecholamine fluorescence in rat sympathetic ganglia. Histochem J 15:1203-1215.

Allan AM and Harris RA (1987): Acute and chronic ethanol treatments alter gamma-aminobutyric acid-stimulated chloride flux across brain membranes. Pharmacol Biochem Behav 24:665-670.

Alroy J, Ucci AA and Periera ME (1988): Lectin histochemistry: an update. In: Advances in Immunohistochemistry, pp. 93-131. Ed. RA DeLellis, Raven Press, New York.

Anandatheerthavrada HK, Shankar SH, Bhamre S, Boyd MR, Song BJ and Ravindranath V (1993): Induction of brain cytochrome P450IIE1 by chronic ethanol treatment. Brain Res 601:279-285.

Andreasson S (1998): Alcohol and J-shaped curves. Alcohol Clin Exp Res Suppl 22:359-364.

Aragon CM, Rogan F and Amit Z (1992): Ethanol metabolism in rat brain homogenates by a catalase-H_2O_2 system. Biochem Pharmacol 44:93-98.

Arai M, Gordon ER and Lieber CS (1984): Decreased cytochrome oxidase activity in hepatic mitochondria after chronic ethanol consumption and the possible role of decreased cytochrome aa3 content and changes in phospholipids. Biochim Biophys Acta 797:320-327.

Arango V, Underwood M and Mann JJ (1994): Fewer pigmented neurons in the locus coeruleus of uncomplicated alcoholics. Brain Res 650:1-8.

Aronson K (2003): Alcohol: a recently identified risk factor for breast cancer. CMAJ 168:1147-1148.

Aroor AR and Baker RC (1998): Ethanol inhibition of phagocytosis and superoxide anion production by microglia. Alcohol 15:277-280.

Badger TM, Huang J, Ronis M and Lumpkin CK (1993): Induction of cytochrome P450 2E1 during chronic ethanol exposure occurs via transcription of the CYP 2E1 gene when blood alcohol concentrations are high. Biochem Biophys Res Commun 190:780-785.

Bagnardi V, Blangiardo M, La Vecchia C and Corrao G (2001): Alcohol consumption and the risk of cancer. Alcohol Res Health 25:263-270.

Baker KG, Halliday GM and Harper CG (1994): Effect of chronic alcohol consumption on the human locus coeruleus. Alcohol Clin Exp Res 18:1491-1496.

Baker KG, Harding AJ, Halliday GM, Kril JJ and Harper CG (1999): Neuronal loss in functional zones of the cerebellum of chronic alcoholics with and without Wernicke's enkephalopathy. Neuroscience 91:429-438.

Banati RB and Graeber MB (1994): Surveillance, intervention and cytotoxicity: is there a protective role of microglia? Dev Neurosci 16:114-127.

Banati RB, Gehrmann J, Schubert P and Kreutzberg GW (1993): Cytotoxicity of microglia. Glia 7:111-118.

Barres DA, Hart IK, Coles HSR, Burne JF, Voyvodic JT, Richardson WD and Raff MC (1992): Cell death and control of cell survival in the oligodendrocyte lineage. Cell 70:31-46.

Barry JA and Gawrish K (1994): Direct NMR evidence for ethanol binding to the lipid-water interface of phospholipid bilayers. Biochemistry 33:8082-8088.

Barter F and Tanner AR (1987): Autonomic neuropathy in an alcoholic population. Postgrad Med J 63:1033-1036.

Bautista AP and Spitzer JJ (1992): Acute ethanol intoxication stimulates superoxide anion production by in situ perfused rat liver. Hepatology 15:892-898.

Bautista AP and Spitzer JJ (1999): Role of Kupffer cells in the ethanol-induced oxidative stress in the liver. Frontiers Biosci 4:589-595.

Beal MF (1992): Does impairment of energy metabolism result in excitotoxic neuronal death in neurodegenerative illnesses? Ann Neurol 31:119-130.

Beghi E and Monticelli ML (1998): Cronic symmetric symptomatic polyneuropathy in the elderly: a field screening investigation of risk factors for polyneuropathy in two Italian communities. Italian General Practitioner Study Group (IGPST). J Clin Epidemiol 51:697-702.

Behrens UJ, Hoerner M, Lasker JM and Lieber CS (1988): Formation of acetaldehyde adducts with ethanol-inducible P450IIE1 *in vivo*. Biochem Biophys Res Commun 154:548-590.

Bengoechea O and Gonzalo LM (1990): Effect of chronic alcoholism on the human hippocampus. Histol Histopathol 5:349-357.

Bengoechea O and Gonzalo LM (1991): Effects of alcoholization on the rat hippocampus. Neurosci Lett 123:112-114.

Beracochea D, Durkin TP and Jaffard R (1986): On the involvement of the central cholinergic system in memory deficits induced by long-term ethanol consumption in mice. Pharmacol Biochem Behav 24:519-524.

Bode C, Kugler V and Bode JC (1987): Endotoxemia in patients with alcoholic and non-alcoholic cirrhosis and in subjects with no evidence of chronic liver disease following acute alcohol excess. J Hepatol 4:8-14.

Bonthius DJ and West JR (1990): Alcohol-induced neuronal loss in developing rats: increased brain damage with binge exposure. Alcohol Clin Exp Res 14:107-118.

Borges MM, Paula-Barbosa MM and Volk B (1986): Chronic alcohol consumption induces lipofuscin deposition in the rat hippocampus. Neurobiol Aging 7:347-355.

Bowling AC, Mutisya EM, Walker LC, Price DL, Cork LC and Beal MF (1993): Age-dependent impairment of mitochondrial function in primate brain. J Neurochem 60:1964-1967.

Boyle AEL, Smith BR and Amit Z (1997): A descriptive analysis of the structure and temporal pattern of voluntary ethanol intake within an acquisition paradigm. J Stud Alcohol 58:382-391.

Braun C, Ludicke C, Rebsch W, Gretz N, van der Woude FJ and Rohmeiss P (1996): Autoregulation of renal blood flow and pressure-dependent renin release in autosomal dominant polycystic kidney disease of rats. Nephrol Dial Transplant 11:52-57.

Brewer C and Perrett L (1971): Brain damage due to alcohol consumption: An air-encephalographic, psychometric and electroencephalographic study. Br J Addict 66:170-182.

Bühler R, Pestalozzi D, Hess M and von Wartburg JP (1983): Immunohistochemical localization of alcohol dehydrogenase in human kidney, endocrine organs and brain. Pharmacol Biochem Behav Suppl 1:55-59.

Burnstock G and Costa M (1975): Adrenergic Neurons. Chapman and Hall, London.

Cadete-Leite A, Tavares MA and Paula-Barbosa MM (1988a): Alcohol withdrawal does not impede hippocampal granule cell progressive loss in chronic alcohol-fed rats. Neurosci Lett 86:45-50.

Cadete-Leite A, Tavares MA, Uylings HBM and Paula-Barbosa M (1988b): Granule cell loss and dendritic regrowth in hippocampal dentate gyrus of rat after chronic alcohol consumption. Brain Res 473:1-14.

Cadete-Leite A, Alves Mc and Tavares MA (1988c): Lysosomal abnormalities in the pyramidal cells of the rat medial prefrontal cortex after chronic alcohol and withdrawal. J Submicrosc Cytol Pathol 20:115-122.

Cadete-Leite A, Tavares MA, Pacheco MM, Volk B and Paula-Barbosa MM (1989): Hippocampal mossy fiber-CA3 synapses after chronic alcohol consumption and withdrawal. Alcohol 6:303-310.

Cadete-Leite A, Alves MC, Tavares MA and Paula-Barbosa MM (1990): Effects of chronic alcohol intake and withdrawal on the prefrontal neurons and synapses. Alcohol 7:145-152.

Cala LA, Jones B, Mastaglia FL and Wiley B (1978): Brain atrophy and intellectual impairment in heavy drinkers: a clinical, psychometric computerized tomography study. Aust N Z J Med 8:147-153.

Capaldi RA (1990): Structure and function of cytochrome c oxidase. Annu Rev Biochem 59:569-596.

Carlen PL, Wortzman G, Holgate RC, Wilkinson DA and Rankin JG (1978): Reversible cerebral atrophy in recently abstinent chronic alcoholics measured by computer tomographic scans. Science 200:1076-1078.

Chan TC, Wall RA and Sutter MC (1985): Chronic ethanol consumption, stress and hypertension. Hypertension 7:519-524.

Chance B, Sies H and Boveris A (1979): Hydroperoxide metabolism in mammalian organs. Physiol Rev 59:527-605.

Chandrasekaran K, Giordano T, Brady DR, Stoll J, Martin LJ and Rapaport SI (1994): Impairment in mitochondrial cytochrome oxidase gene expression in Alzheimer disease. Mol Brain Res 24:336-340.

Charness ME (1993): Clinical and pathological overview of the brain disorders in alcoholics. In: Alcohol-induced Brain Damage, pp. 15-36. Eds. WA Hunt and SJ Nixon, National Institute of Health, Bethesda, MD.

Cheeseman KH and Slater TF (1993): An introduction to free radical biochemistry. Br Med Bull 49:481-493.

Chen WA, Parnell SE and West JR (1999): Early postnatal alcohol exposure produced long-term deficits in brain weight, but not the number of neurons in the locus coeruleus. Dev Brain Res 118:33-38.

Chen X, Michaelis MR and Michaelis EK (1997): Effects of chronic ethanol treatment on the expression of calcium transport carriers and NMDA/glutamate receptor proteins in brain synaptic membranes. J Neurochem 69:1559-1569.

Cipriani F, Balzi D, Sorso B and Buiatti E (1998): Alcohol-related mortality in Italy. Public Health 112:183-188.

Clemmesen L, Ingvar M, Hemmingsen R and Bolwig TG (1988): Local cerebral glucose consumption during ethanol withdrawal in the rat: effects of single and multiple episodes and previous convulsive seizures. Brain Res 453:204-214.

Cohen G and Werner P (1993): Free radicals, oxidative stress and neurodegeneration. In: Neurogenerative Disease, pp. 139-161. Ed. D Calne, Saunders, Philadelphia.

Cohen G, Sinet PM and Heikkilä R (1980): Ethanol oxidation by rat brain in vivo. Alcohol Clin Exp Res 4:366-370.

Colton CA and Gilbert DL (1987): Production of superoxide anions by a CNS macrophage, microglia. FEBS Lett 223:284-288.

Colton CA, Snell-Callanan J and Chernyshev ON (1998): Ethanol induced changes in superoxide anion and nitric oxide in cultured microglia. Alcohol Clin Exp Res 22:710-716.

Cottrell DA, Blakely EL, Johnson MA, Ince PG, Borthwick GM and Turnbull DM (2001): Cytochrome c oxidase deficient cells accumulate in the hippocampus and choroid plexus with age. Neurobiol Aging 22:265-272.

Cunningham TJ (1982): Naturally occurring death and its regulation by developing neural pathways. Int Rev Cytol 74:163-186.

Curti D, Giangare MC, Redolfi ME, Fugaccia I and Benzi G (1990): Age-related modifications of cytochrome c oxidase activity in discrete brain regions. Mech Ageing Dev 55:171-180.

Danysz W, Dyr W, Jankowska E, Glazewski S and Kostowski W (1992): The involvement of NMDA receptors in acute and chronic effects of ethanol. Alcohol Clin Exp Res 16:499-504.

Daost M, Lhuintre JP, Saligant C, Morre N, Flipo JL and Boismare F (1987): Noradrenaline and GABA brain receptors are co-involved in the voluntary intake of ethanol by rats. Alcohol Alcohol Suppl 1:319-322.

Darriet D, Derr T and Collins RC (1986): Distribution of cytochrome oxidase in rat brain: studies with diaminobenzidine histochemistry in vitro and ^{14}C cyanide tissue labeling in vivo. J Cereb Blood Flow Metab 6:8-14.

Davidson M, Shanley B and Wilce P (1995): Increased NMDA-induced excitability during ethanol withdrawal: a behavioral and histological study. Brain Res 674:91-96.

Davis RE, Miller S, Herrnstadt C, Ghosh SS, Fahy E, Shinobu LA, Galasko D, Thal LJ, Beal MF, Howell N and Parker WD Jr (1997): Mutations in mitochondrial cytochrome c oxidase genes segregate with late-onset Alzheimer disease. Proc Natl Acad Sci USA 94:4526-4531.

Dawson DA (2000): Alcohol consumption, alcohol dependence, and all-cause mortality. Alcohol Clin Exp Res 24:72-81.

De la Monte SM (1988): Disproportionate atrophy of cerebral white matter in chronic alcoholics. Arch Neurol 45:990-992.

De Vegt, Dekker JM, Groeneveld W-JA, Nijpels G, Stehouwer CDA, Bouter LM and Heine RJ (2002): Moderate alcohol consumption is associated with lower risk for incident diabetes and mortality: the Hoorn Study. Diabetes Res Clin Pract 57:53-60.

Deitrich RA, Dunwiddie TV, Harris RA, Erwin VG (1989): Mechanism of action of ethanol: initial central nervous system actions. Pharmacol Rev 41:489-537.

Del Rio-Hortega P (1919): A microglia y su transformation en celulas en basoncito y cuerpous granulo-adiposis. Trab Lab Invest Biol Madrid 18:37-82.

Del Rio-Hortega P (1932): Microglia. In: Cytology and Pathology of the Nervous System, pp. 483-534. Ed. W Penfield, Hoeber, New York.

Devi B, Henderson G, Frosto T and Schenker S (1994): Effect of acute ethanol exposure on cultured fetal rat hepatocytes: relation to mitochondria function. Alcohol Clin Exp Res 18:1436-1442.

Dlugos CA and Pentney RJ (2001): Quantitative immunocytochemistry of glia in the cerebellar cortex of old ethanol-fed rats. Alcohol 23:63-69.

Dong Y-J, Peng T-K and Yin S-J (1996): Expression and activities of class IV alcohol dehydrogenase and class III aldehyde dehydrogenase in human mouth. Alcohol 13:257-262.

Dufour MC (1993): The epidemiology of alcohol-induced brain damage. In: Alcohol-induced Brain Damage, pp. 3-14. Eds. WA Hunt and SJ Nixon, National Institute of Health, Bethesda, MD.

Durand D and Carlen PL (1984): Decreased neuronal inhibition *in vitro* after long-term administration of ethanol. Science 224:1359-1361.

Eckardt MJ, Campbell GA, Marietta CA, Majchrowicz E and Weight FF (1988): Acute ethanol administration selectively alters localized cerebral glucose metabolism. Brain Res 444:53-58.

Eisenhofer G, Lambie DG and Johnson RH (1983): Effects of ethanol on plasma catecholamines and norepinephrine clearance. Clin Pharmacol Ther 34:143-147.

Ekström G, Grönholm T and Ingelman-Sundberg M (1986): Hydroxyl-radical production and ethanol oxidation by liver microsomes isolated from ethanol-treated rats. Biochem J 233:755-761.

Engberg G and Hajos M (1992): Alcohol withdrawal reaction as a result of adaptive changes of excitatory amino acid receptors. Naunyn Schmiedebergs Arch Pharmacol 346:437-441.

Eränkö O (1967): The practical histochemical demonstration of catecholamines by formaldehyde-induced fluorescence. J R Microsc Soc 87:259-276.

Eränkö O (1971): Histochemistry of Nervous Transmission. Progress in Brain Research, vol 34. Elsevier, Amsterdam.

Eriksson CJP (1973): Ethanol and acetaldehyde metabolism in rat strains genetically selected for their ethanol preference. Biochem Pharmacol 22:2283-2292.

Eriksson CJP (1981): Finnish selection studies on alcohol-related behaviors: factors regulating voluntary alcohol consumption. In: Development of Animal Models as Pharmacogenetic Tools, pp. 119-145. Eds. GE McClearn, RA Deitrich and G Erwin, U.S. Government Office, Rockville.

Eriksson CJP (2001): The role of acetaldehyde in the actions of alcohol (update 2000). Alcohol Clin Exp Res 25:15-32.

Eriksson K (1968): Genetic selection for voluntary alcohol consumption in the albino rats. Science 159:739-741.

Eriksson K (1971): Rat strains specially selected for their voluntary alcohol consumption. Ann Med Exp Biol Fenn 49:67-72.

Eriksson K (1972): Alcohol consumption ad blood alcohol in rat strains selected for their behavior towards alcohol. The Finnish Foundation for Alcohol Studies 20:121-125.

Eriksson K and Malmström KK (1967): Sex differences in consumption and elimination of alcohol in albino rats. Ann Med Exp Biol Fenn 45:389-392.

Fadda F and Rossetti ZL (1998): Chronic ethanol consumption: from neuroadaptation to neurodegeneration. Prog Neurobiol 56:385-431.

Faingold CL, Gouemo PN and Riaz A (1998): Ethanol and neurotransmitter interactions– from molecular to integrative effects. Prog Neurobiol 55:509-535.

Farchi G, Fidanza F, Giampaoli S, Mariotti S and Menotti A (2000): Alcohol and survival in the Italian rural cohorts of the Seven Counties Study. Int J Epidem 29:667-671.

Foote SL, Bloom FE and Aston-Jones G (1983): Nucleus locus coeruleus: new evidence of anatomical and physiological specifity. Physiol Rev 63:844-914.

Forsander OA and Sinclair JD (1992): Alcohol oxidation and the regulation of consumption in AA and ANA rats. Alcohol Alcohol 27:411-416.

Franke H (1995): Influence of chronic alcohol treatment on the GFAP-immunoreactivity in astrocytes of the hippocampus in rats. Acta Histochem 97:263-271.

Gabella G (1976): Structure of the Autonomic Nervous System. Chapman and Hall, London.

Gehrmann J, Bonnekoh P, Miyazawa T, Oschlies U, Dux E, Hossmann KA and Kreutzberg GW (1992): The microglial reaction in the rat hippocampus following global ischemia: immuno-electron microscopy. Acta Neuropathol (Berl) 84:588-595.

Gelman BB (1993): Diffuse microgliosis associated with cerebral atrophy in the acquired immunodeficiency syndrome. Ann Neurol 34:65-70.

Gill M, Menez JF, Lucas D and Deitrich RA (1992): Enzymatic production of acetaldehyde from ethanol in rat brain tissue. Alcohol Clin Exp Res 16:910-915.

Giulian D and Baker TJ (1986): Characterization of ameboid microglia isolated from developing mammalian brain. J Neurosci 6:2163-2178.

Giulian D, Baker TJ, Shin LC and Lachman LB (1986): Interleukin 1 of the central nervous system is produced by ameboid microglia. J Exp Med 164:594-604.

Goldstein IJ and Hayes CE (1978): The lectins: carbohydrate-binding proteins of plants and animals. Adv Carbohydr Chem Biochem 35:127-340.

Graham DG (1978): Oxidative pathways for catecholamines in the genesis of neuromelanin and cytotoxic quinines. Mol Pharmacol 14:633-643.

Grant KA, Valverius P, Hudspith M and Tabakoff B (1990): Ethanol withdrawal seizures and NMDA receptor complex. Eur J Pharmacol 176:289-296.

Gretz N, Kranzlin B, Pey R, Schieren G, Bach J, Obermuller N, Ceccherini I, Kloting I, Rohmeiss P, Bachmann S and Hafner M (1996): Rat model of autosomal dominant polycystic kidney disease. Nephrol Dial Transplant 11:46-51.

Gronbaek M, Deis A, Sorensen TI, Becker U, Schnohr P and Jensen G (1995): Mortality associated with moderate intakes of wine, beer, or spirits. BMJ 310:1165-1169.

Gronbaek M, Becker U, Johansen D, Gottschau A, Schnohr P, Hein HO, Jensen G and Sorensen TI (2000): Type of alcohol consumed and mortality from all causes, coronary heart disease, and cancer. Ann Int Med 133:411-419.

Grünwald F, Schrock H, Biersack HJ and Kuschinsky W (1993): Changes in local cerebral glucose utilization in the awake rat during acute and chronic administration of ethanol. J Nucl Med 34:793-798.

Guerri C and Reneu-Piqueras J (1997): Alcohol, astroglia and brain development. Mol Neurobiol 15:165-170.

Gundersen HJG (1977): Notes on the estimation of the numerical density of arbitrary profiles: the edge effect. J Microsc. 111:219-223.

Gundersen HJG (1986): Stereology of arbitrary particles. A review of unbiased number and size estimators and the presentation of some new ones, in memory of William R. Thompson. J Microsc 143:3-45.

Gundersen HJG and Jensen EB (1987): The efficiency of systematic sampling in stereology and its prediction. J Microsc 147:229-263.

Gundersen HJG, Bendtsen TF, Korbo L, Marcussen N, Moller A, Nielsen K, Nyengaard JR, Pakkenberg B, Sorensen FB, Vesterby A and West MJ (1988): Some new, simple and efficient stereological methods and their use in pathological research and diagnosis. APMIS 96:379-394.

Halliday G, Ell J and Harper C (1992): The locus coeruleus and memory: a study of chronic alcoholics with and without the memory impairment of Korsakoff's psychosis. Brain Res 598:33-37.

Halliwell B and Gutteridge JMC (1989): Free radical in biology and medicine. Oxford University Press, New York.

Hansson T, Tindberg N, Ingelman-Sundberg M and Köhler C (1990): Regional distribution of cytochrome P-450 2E1 in the rat nervous system. Neuroscience 34:451-463.

Harding AJ, Wong A, Svoboda M, Kril JJ and Halliday GM (1997): Chronic alcohol consumption does not cause hippocampal neuron loss in humans. Hippocampus 7:78-87.

Harper C and Kril J (1985): Brain atrophy in chronic alcoholic patients: a qualitative pathological study. J Neurol Neurosurg Psychiatry 48:211-217.

Harper CG and Kril JJ (1990): Neuropathology of alcoholism. Alcohol Alcohol 25:207-216.

Harper CG, Kril JJ and Holloway RL (1985): Brain shrinkage in chronic alcoholics – a pathological study. BMJ 290:501-504.

Harper CG, Smith NA and Kril JJ (1990): The effects of alcohol on the female brain: a neuropathological study. Alcohol Alcohol 25:445-448.

Hart CL, Smith GD, Hole DJ and Hawthorne VM (1999): Alcohol consumption and mortality from all causes, coronary heart disease, and stroke: results from a prospective cohort study of scottish men with 21 years of follow up. BMJ 318:1725-1729.

Hawley RJ, Major LF, Schulman E and Linnoila M (1985): Cerebrospinal fluid 3-methoxy-4-hydroxyphenylglycol and norepinephrine levels in alcohol withdrawal. Arch Gen Psychiatry 42:1056-1062.

Hawley RJ, Nemeroff CB, Bisette G, Guidotti A, Rawlings R and Linnoila M (1994): Neurochemical correlates of sympathetic activation during severe alcohol withdrawal. Alcohol Clin Exp Res 18:1312-1316.

He SM and Lambert B (1990): Acetaldehyde-induced mutation at the *hprt* locus in human lymphocytes in vitro. Environ Mol Mutagen 16:57-63.

Heaton MB, Mitchell JJ and Paiva M (2000): Amelioration of ethanol-induced neurotoxicity in the neonatal rat central nervous system by antioxidant therapy. Alcohol Clin Exp Res 24:512-518.

Hein HO, Suadicani P and Gyntelberg F (1996): Alcohol consumption, serum low density lipoprotein cholesterol concentration, and risk of ischaemic heart disease: six year follow up in the Copenhagen male study. BMJ 312:736-741.

Hemmingsen R, Barry DI, Hertz MM and Klinken L (1979): Cerebral blood flow and oxygen consumption during ethanol withdrawal in the rat. Brain Res 173:259-269.

Hervonen A, Partanen M, Helén P, Koistinaho J, Alho H, Baker DM, Johnson JE and Santer RM (1986): The sympathetic neuron as a model of neuronal aging. In Neurochemistry: Modern Methods and Applications, pp. 569-586. Eds. P Panula, H Päivärinta and S Soinila. Alan R Liss, New York.

Hervonen A, Jaatinen P, Sarviharju M and Kiianmaa K (1992): Interaction of aging and lifelong ethanol ingestion on ethanol-related behaviors and longevity. Exp Gerontol 27:335-345.

Hevner RF, Liu S and Wong-Riley MT (1995): A metabolic map of cytochrome oxidase in the rat brain: histochemical, densitometric and biochemical studies. Neuroscience 65:313-342.

Hickey WF and Kimura H (1988): Perivascular microglial cells of the CNS are bone marrow derived and present antigen in vivo. Science 239:290-292.

Hilakivi L, Eriksson CJP, Sarviharju M and Sinclair JD (1984): Revitalization of the AA and ANA rat lines: effect on some line characteristics. Alcohol 1:71-75.

Hilakivi LA, Hilakivi I and Kiianmaa K (1987): Neonatal antidepressant administration suppresses concurrent active (REM) sleep and increases adult alcohol consumption in rats. Alcohol Alcohol Suppl 1:339-343.

Hoffman PL, Iorio KR, Snell LD and Tabakoff B (1995): Attenuation of glutamate-induced neurotoxicity in chronically ethanol-exposed cerebellar granule cells by NMDA receptor antagonists and ganglioside GM1. Alcohol Clin Exp Res 19:721-726.

Hoffman PL, Rabe CS, Moses F and Tabakoff B (1989) N-methyl-D-asparate receptors and ethanol: inhibition of calcium flux and cyclic GMS production. J Neurochem 52:1937-1940.

Holbrook TL, Barrett-Connors W and Wingard A (1990): A prospective population-based study of alcohol use and non-insulin-dependent diabetes mellitus. Am J Epidemiol 132:902-909.

Holman CD and English DR (1995): An improved aetiologic fraction for alcohol-caused mortality. Austr J Pub Health 19:138-141.

Holownia A, Ledig M, Braszko JJ, Menez JF (1999): Acetaldehyde cytotoxicity in cultured rat astrocytes. Brain Res 833:202-208.

Homann N (2001): Alcohol and upper gastrointestinal tract cancer: the role of local acetaldehyde production. Addict Biol 6:309-323.

Homann N, Tillonen J, Meurman JH, Rintamäki H, Lindqvist C, Rautio M, Jousimies-Somer H, Salaspuro M (2000): Increased salivary acetaldehyde levels in heavy drinkers and smokers: a microbiological approach to oral cavity cancer. Carcinogenesis 21:663-668.

Homann N, Tillonen J, Rintamäki H, Salaspuro M, Lindqvist C, Meurman JH (2001): Poor dental status increases acetaldehyde production from ethanol in saliva: a possible link to increased oral cancer risk among heavy drinkers. Oral Oncol 37:153-158.

Howes LG and Reid JL (1985): Changes in plasma free 3,4-dihydroxyphenylethylene glycol and noradrenaline levels after acute alcohol administration. Clin Sci 69:423-428.

Hsu TC, Furlong C and Spitz MR (1991): Ethyl alcohol as a cocarcinogen with special reference to the aerodigestive tract: a cytogenetic study. Anticancer Res 11:1097-1101.

Huang X, Chen S and Tietz EI (1996): Immunocytochemical detection of regional protein changes in rat brain sections using computer-assisted image analysis. J Histochem Cytochem 44:981-987.

Huentelman MJ, Peters GM, Ervine WE, Polutnik SM and Johnson P (1999): Ethanol has different effects on rat neuron and thymocyte reactive oxygen species and cell viability. Comp Biochem Physiol C Toxicol Pharmacol 124:83-89.

Hunt WA (1993): Role of free radical reactions in ethanol-induced brain damage: an introduction. In: Alcohol-induced Brain Damage, pp. 327-338. Eds. WA Hunt and SJ Nixon. National Institute of Health, Bethesda, MD.

Hunt WA (1996): Role of acetaldehyde in the actions of ethanol on the brain – a review. Alcohol 13:147-151.

Hunt WA and Dalton TK (1981): Neurotransmitter receptor binding in various brain regions in ethanol-dependent rats. Pharmacol Biochem Behav 14:733-739.

ICD-10 (1993): The ICD-10 classification of mental and behavioural disorders: diagnostic criteria for research. World Health Organization, Geneva.

Ikeda K, Kobayashi T, Kumanishi T, Yano R, Sora I and Niki H (2002): Molecular mechanisms of analgesia induced by opioids and ethanol: is the GIRK channel on of the keys? Neurosci Res 44:121-131.

Imamota K and Leblond CP (1978): Radioautographic investigation of gliogenesis in the corpus callosum of young rats. II. Origin of microglial cells. J Comp Neurol 180:139-164.

Ingelman-Sundberg M, Johansson I, Hu Y, Terelius Y, Eliasson E, Clot P and Albano E (1993): Ethanol-inducible cytochrome P450 2E1: genetic polymorphism, regulation and possible role in the etiology of alcohol-induced liver disease. Alcohol 10:447-452.

IARC (1998): Monographs on the Evaluation of Carcinogenic Risk to Humans: Alcohol Drinking. International Agency for Research on Cancer, Lyon.

IARC (1999): Monographs on the Evaluation of Carcinogenic Risk to Humans: Acetaldehyde. International Agency for Research on Cancer, Lyon.

Iorio KR, Tabakoff B and Hoffman PL (1993): Glutamate-induced neurotoxicity is increased in cerebellar granule cells exposed chronically to ethanol. Eur J Pharmac 248:209-212.

Ireland MA, Vandongen R, Davidson L, Beilin LJ and Rouse IL (1984): Acute effects of moderate alcohol consumption on blood pressure and plasma catecholamines. Clin Sci 66:643-648.

Izevbigie EB, Ekunwe SI, Jordan J and Howard CB (2002): Ethanol modulates the growth of human breast cancer cells in vitro. Exp Biol Med. 227:260-265.

Jaakkola M and Nordback I (1993): Pancreatitis in Finland between 1970 and 1989. Gut 34:1255-1260.

Jaakkola M, Sillanaukee P, Löf K, Koivula T and Nordback I (1994): Amount of alcohol is an important determinant of the severity of acute alcoholic pancreatitis. Surgery 115:31-38.

Jaatinen P and Hervonen A (1994): Reactions of rat sympathetic neurons to ethanol exposure are age-dependent. Neurobiol Aging 15:419-428.

Jaatinen P, Kiianmaa K and Hervonen A (1992). Lifelong ethanol consumption enhances the age-related changes in rat sympathetic neurons. Mech Ageing Dev 63:193-205.

Jaatinen P, Kiianmaa K, Lahtivirta S and Hervonen A (1993): Ethanol-induced vacuolation in rat peripheral nervous system. J Auton Nerv Syst 46:107-121.

Jaatinen P, Riihioja P, Haapalinna A, Heinonen E, Kiianmaa K and Hervonen A (1995): Prevention of ethanol-induced sympathetic overactivity and degeneration by dexmedetomidine. Alcohol 12:439-446.

Jacobson R (1986): The contributions of sex and drinking history to the CT brain scan changes in alcoholics. Psychol Med 16:547-559.

Jensen GB and Pakkenberg B (1993): Do alcoholics drink their neurons away? Lancet 342:1201-1204.

Johnson RH and Robinson BJ (1988): Mortality in alcoholics with autonomic neuropathy. J Neurol Neurosurg Psych 51:476-480.

Kajander OA, Kupari M, Laippala P, Savolainen V, Pajarinen J, Penttilä A and Karhunen PJ (2001): Dose dependent but non-linear effects of alcohol on the left and right ventricle. Heart 86:417-423.

Kalant H and Le AD (1983): Effects of ethanol on thermoregulation. Pharmacol Ther 23:313-364.

Karhunen PJ, Erkinjuntti T and Laippala P (1994): Moderate alcohol consumption and loss of cerebellar Purkinje cells. BMJ 308:1663-1667.

Kaufman MH (1997): The teratogenic effects of alcohol following exposure during pregnancy, and its influence on the chromosome constitution of the pre-ovulatory egg. Alcohol Alcohol 32:113-128.

Kerr TJ, Maxwell DS and Crabb DW (1989): Immunohistochemistry of alcohol dehydrogenase in the rat central nervous system. Alcohol Clin Exp Res 13:730-736.

Kiechl S, Willeit J, Poewe W, Egger G, Oberhollenzer F, Muggeo M and Bonora E (1996): Insulin sensitivity and regular alcohol consumption: large, prospective, cross sectional population study (Bruneck study). BMJ 313:1040-1044.

Kiianmaa K, Stenius K and Sinclair JD (1991): Determinants of alcohol preference in the AA and ANA rat lines selected for differential ethanol intake. Alcohol Alcohol Suppl 1:115-120.

Kim JH, Kim JE, Kim HJ, Roh GS, Yoo JM, Kang SS, Cho YY, Cho GJ and Choi WS (2001): Ethanol decreases the expression of mitochondrial cytochrome c oxidase mRNA in the rat. Neurosci Lett 305:107-110.

Kimelberg HK, Cheema M, O'Connor ER, Tong H, Goderie SK and Rossman PA (1993): Ethanol-induced aspartate and taurine release from primary astrocyte cultures. J Neurochem 60:1682-1689.

Kish SJ, Bergeron C, Rajput A, Dozic S, Mastrogiacomo F, Chang LJ, Wilson JM, DiStefano LM and Nobrega JN (1992): Brain cytochrome oxidase in Alzheimer's disease. J Neurochem 59:776-779.

Kjellström C, Almström S and Conradi N (1993): Decreased synapse-to-neuron ratio in rat locus ceruleus following chronic ethanol feeding. Alcohol Clin Exp Res 17:406-410.

Kloppel G (1999): Progression from acute to chronic pancreatitis. A pathologist's view. Surg Clin North Am 79:801-814.

Knapp DJ and Crew FT (1999): Induction of cyclooxygenase-2 in brain during acute and chronic ethanol treatment and ethanol withdrawal. Alcohol Clin Exp Res 23:633-643.

Koivisto T, Carr LG, Li TK and Eriksson CJP (1993): Mitochondrial aldehyde dehydrogenase (ALDH2) polymorphism in AA and ANA rats: lack of genotype and phenotype line differences. Pharmacol Biochem Behav 45:215-220.

Koivula T, Koivusalo M and Lindroos KO (1975): Liver aldehyde and alcohol dehydrogenase activities in rat liver. Biochem Pharmacol 24:1807-1811.

Korbo L (1999): Glial cell loss in the hippocampus of alcoholics. Alcohol Clin Exp Res 23:164-168.

Kornfehl J, Temmel A, Formanek M and Knerer B (1999): Effects of ethanol treatment of proliferation and differentiation in a head and neck squamous cell carcinoma cell line. Alcohol Clin Exp Res 23:1102-1107.

Korpi ER, Päivärinta P, Sjöholm B, Koulu M (1991): Hypothalamic monoamines and food intake in alcohol-preferring AA and alcohol-avoiding ANA rats. Alcohol 8:137-141.

Koskinen P and Kupari M (1991): Alcohol consumption of patients with supraventricular tachyarrhythmias other than atrial fibrillation. Alcohol Alcohol 26:199-206.

Koskinen P, Kupari M, Leinonen H and Luomanmäki K (1987): Alcohol and new onset atrial fibrillation: a case-control study of current series. British Heart Journal 57:468-473.

Krishnan S and Maickel RP (1991): The effect of Hoe-427 (an ACTH4-9 analog) on free-choice ethanol consumption in male and female rats. Life Sci 49:2005-2011.

Laakso MP, Vaurio O, Savolainen L, Repo E, Soininen H, Aronen HJ and Tiihonen J (2000): A volumetric MRI study of the hippocampus in type 1 and 2 alcoholism. Behav Brain Res 109:177-186.

Lancaster EF (1994): Gender differences in the brain: implications for the study of human alcoholism. Alc Clin Exp Res 18:740-746.

Lancaster FE and Spiegel KS (1992): Sex differences in pattern of drinking. Alcohol 9:415-420.

Lazarus R, Sparrow D and Weiss ST (1997): Alcohol intake and insulin levels: The Normative Aging Study. Am J Epidemiol 145:909-916.

Ledig M, M´Paria J-R and Mandel P (1981): Superoxide dismutase activity in rat brain during acute and chronic alcohol intoxication. Neurochem Res 6:385-390.

Lewohl JM, Wilson WR, Mayfield RD, Brozowski SJ, Morrisett RA and Harris RA (1999): G-protein-coupled inwardly rectifying potassium channels are targets of alcohol action. Nat Neurosci 2:1084-1090.

Li C, Peoples RW and Weight FF (1994): Alcohol action on a neuronal membrane receptor: evidence for a direct interaction with the receptor protein. Proc Natl Acad Sci USA 91:8200-8204.

Liang Q, Carlson EC, Borgerding AJ, Epstein PN (1999): A transgenic model of acetaldehyde overproduction accelerates alcohol cardiomyopathy. J Pharmacol Exp Ther 291:766-772.

Lieber CS (1994): Alcohol and the liver: 1994 update. Gastroenterology 106:1085-1105.

Lieber CS (2001): Alcoholic liver injury: pathogenesis and therapy in 2001. Pathol Biol 49:738-752.

Lieber CS, Jones DP, DeCarli LM (1965): Effects of prolonged ethanol intake: production of fatty liver despite adequate diets. J Clin Invest 44:1009-1021.

Lindros KO (1978): Acetaldehyde: its metabolism and role in the actions of alcohol. In: Research Advances in Alcohol and Drug Problems, pp. 111-176. Eds. Y Israel, FB Glaser and H Kalant, Plenum Press, New York.

Linnoila M (1987): Alcohol withdrawal and noradrenergic function. Ann Intern Med 107:875-889.

Litten RZ and Allen JP (1991): Pharmacotherapies for alcoholism: promising agents and clinical issues. Alcohol Clin Exp Res 15:620-633.

Lovinger DM (1997): Alcohols and neurotransmitter gated ion channels: past, present and future. Naunyn-Schmiedeberg's Arch Pharmac 356:267-282.

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.

Lundqvist C, Volk B, Knoth R and Alling C (1994): Long-term effects of intermittent versus continuous ethanol exposure on hippocampal synapses of the rat. Acta Neuropathol 87:242-249.

Lundqvist C, Alling C, Knoth R and Volk B (1995): Intermittent ethanol exposure of adult rats: hippocampal cell loss after one month of treatment. Alcohol Alcohol 30:737-748.

MacDermott AB, Mayer ML, Westbrook GL, Smith SJ and Barker JL (1986): NMDA-receptor activation increases cytoplasmic calcium concentration in cultured spinal cord neurones. Nature 321:519-522.

Mäkelä P (1998): Alcohol-related mortality by age and sex and its impact on life expectancy. Eur J Pub Health 8:43-51.

Mäkelä P, Valkonen T and Poikolainen K (1997): Estimated numbers of deaths from coronary heart disease "caused" and "prevented" by alcohol: an example from Finland. J Stud Alcohol 58:455-463.

Mann K, Batra A, Günthner A and Schroth G (1992): Do women develop alcoholic brain damage more readily than men? Alcohol Clin Exp Res 16:1052-1056.

Mantle D and Preedy VR (1999): Free radicals as mediators of alcohol toxicity. Adverse Drug React Toxicol Rev 18:235-352.

Marin-Garcia J, Ananthakrishnan R and Goldenthal MJ (1995): Heart mitochondria response to alcohol is different than brain and liver. Alcohol Clin Exp Res 19:1463-1466.

Marin-Garcia J, Ananthakrishnan R and Goldenthal MJ (1996): Mitochondrial dysfunction after fetal alcohol exposure. Alcohol Clin Exp Res 20:1029-1032.

Marques-Vidal P, Ducimetiere P, Evans A, Cambou J and Arveiler D (1996): Alcohol consumption and myocardial infarction: a case-control study in France and Northern Ireland. Am J Epidemiol 143:1089-1093.

Martin PR, Adinoff B, Weingartner H, Mukherjee AB and Eckardt MJ (1986): Alcoholic organic brain disease: nosology and pathophysiologic mechanisms. Prog Neuro-Psychopharmacol Biol Psychiatry 10:147-164.

Martinez SE, Vaglenova J, Sabria J, Martinez MC, Farres J and Pares X (2001): Distribution of alcohol dehydrogenase mRNA in the rat central nervous system. Eur J Biochem 268:5045-5056.

Matysiak-Butnik T, Jokelainen K, Kärkkäinen P, Mäkisalo H, Ohisalo J, Salaspuro M (1996): Hepatotoxicity and absorption of extrahepatic acetaldehyde in rats. J Pathol 178:469-474.

Mayhew TM and Gundersen HJG (1996): "If you assume, you can make an ass out of u and me": a decade of the disector for stereological counting of particles in 3D space. J Anat 188:1-15.

McDermott EW, O'Dwyer PJ and O'Higgins NJ (1992): Dietary alcohol intake does not increase the incidence of experimentally induced mammary carcinoma. Eur J Surg Oncol 18:251-254.

McElduff P and Dobson A (1997): How much alcohol and how often? Population bases case –control study of alcohol consumption and risk of a major coronary event. BMJ 314:1159-1164.

McQuilkin SJ and Harris RA (1990): Factors affecting actions of ethanol on GABA-activated chloride channels. Life Sci 46:527-541.

Mendenhall CL, Rouster SD, Roselle GA, Grossman CJ, Ghosn S and Gartside P (1993): Impact of chronic alcoholism on the aging rat: changes in nutrition, liver composition, and mortality. Alcohol Clin Exp Res 17:847-853.

Mennen LI, Balkau B, Vol S, Caces E and Eschwege E (1999): Fibrinogen. A possible link between alcohol consumption and cardiovascular disease? Arterioscler Tromb Vasc Biol 19:887-892.

Merikangas KR (1990): The genetic epidemiology of alcoholism. Psychol Med 20:11-22.

Mihic SJ and Harris RA (1996): Alcohol actions at rat the GABA(A) receptors chloride channel complex. In: Pharmacological Effects of Ethanol on the Nervous System, pp. 51-72. Eds. RA Deitrich and VG Erwin, CRC Press, Boca Raton.

Minghetti L and Levi G (1998): Microglia as effector cells in brain damage and repair: focus on prostanoids and nitric oxide. Prog Neurobiol 54:99-125.

Montoliu C, Vallés S, Renau-Piqueras J and Cuerri C (1994): Ethanol-induced oxygen radical formation and lipid peroxidation in rat brain: effect of chronic alcohol consumption. J Neurochem 63:1855-1862.

Muller P, Britton RS and Seeman P (1980): The effects of long-term ethanol on brain receptors for dopamine, acetylcholine, serotonin and norepinephrine. Eur J Pharmacol 65:31-37.

Murabe Y and Sano Y (1982): Morphological studies on neuroglia. V. Microglial cells in the cortex of rat, with special reference to their possible involvement in synaptic function. Cell Tiss Res 223:493-506.

Nachbar MA, Oppenheim JD and Thomas JO (1980): Lectins in the U.S. diet. Isolation and characterization of a lectin from tomato (lysopersicon esculentum). J Biol Chem 255:2056-2061.

Nakajima K and Kohsaka S (1993): Functional roles of microglia in the brain. Neuroscience Research 17:187-203.

Nakanishi N, Axel P and Shneider NA (1992): Alternative splicing generates functionally distinct N-methyl-D-asparate receptors. Proc Natl Acad Sci USA 89:8552-8556.

Nanji AA, Miao L, Thomas P, Rahemtulla A, Khwaja S, Zhao S, Peters D, Tahan SR and Dannenberg AJ (1997): Enhanced cyclooxygenase-2 gene expression in alcoholic liver disease in the rat. Gastroenterology 112:943-951.

Narahashi T, Kuriyama K, Illes P, Wirkner K, Fischer W, Muhlberg K, Scheibler P, Allgaier C, Minami K, Lovinger D, Lallemand F, Ward RJ, DeWitte P, Itatsu T, Takei Y, Oide H, Hirose M, Wang XE, Watanabe S, Tateyama M, Ochi R and Sato N (2001): Neuroreceptors and ion channels as target of alcohol. Alcohol Clin Exp Res Suppl 25:182-188.

Nestoros JN (1980): Ethanol selectively potentiates GABA-mediated inhibition of single feline cortical neurons. Life Sci 26:519-523.

Nevo I and Hamon M (1995): Neurotransmitter and neuromodulatory mechanisms involved in alcohol abuse and alcoholism. Neurochem Internat 26:305-336.

Niemelä O, Juvonen T and Parkkila S (1991): Immunohistochemical demonstration of acetaldehyde-modified epitopes in human liver after alcohol consumption. J Clin Invest 87:1367-1374.

Niemelä O, Parkkila S, Pasanen M, Iimuro Y, Bradford B and Thurman RG (1998): Early alcoholic liver injury formation of protein adducts with acetaldehyde and lipid peroxidation products, and expression of CYP2E1 and CYP3A. Alcohol Clin Exp Res 22:2118-2124.

Niemelä O, Parkkila S, Ylä-Herttuala S, Halsted C, Wiztum JL, Lanca A and Israel Y (1994): Covalent protein adducts in the liver as a result of ethanol metabolism and lipid peroxidation. Lab Invest 70:537-546.

Nissl F (1891): Über einige Beziehungen zwischen Nervenzellenerkrankungen und gliösen Erscheinungen bei verschiendenen Psychosen. Arch Psych 32:1-21.

Niwa K, Tanaka T, Sugie S, Shinoda T, Kato K, Tamaya T and Mori H (1991): Enhancing effect of ethanol or saké on methylazoxymethanol acetate-initiated large bowel carcinogenesis in ACI/N rats. Nutr Cancer 15:229-237.

Nordback IH, MacGowan S, Potter JJ, Cameron JL (1991): The role of acetaldehyde in the pathogenesis of acute alcohol pancreatitis. Ann Surg 214:671-678.

Nordmann R, Ribiére C and Rouach H (1992): Ethanol-induced lipid peroxidation and oxidative stress in extrahepatic tissue. Alcohol Alcohol 25:231-237.

Novak DJ and Viktor M (1974): The vagus and sympathetic nerves in alcoholic neuropathy. Arch Neurol 30:273-284.

Nutt DJ and Peters TJ (1994): Alcohol: the drug. Brit Med Bull 50:5-17.

Nutt DJ, Glue P, Molyneux S and Clark E (1988): Alpha2-adrenoceptor activity in alcohol withdrawal: a pilot study of the effects of i.v. clonidine in alcoholics and normals. Alcohol Clin Exp Res 12:14-18.

Oehmichen M (1983): Inflammatory cells in the central nervous system – an integrating concept based on recent research in pathology, immunology, and forensic medicine. Progr Neuropathol 5:235-277.

Ozer E, Sarioglu S and Gure A (2000): Effects of prenatal ethanol exposure on neuronal migration, neurogenesis and brain myelination in the mice brain. Clin Neuropathol 19:21-25.

Pajarinen J, Savolainen V, Perola M, Penttilä A and Karhunen PJ (1996): Polymorphism in the cytochrome P450 2E1 gene and alcohol-induced disorders of human spermatogenesis. Int J Androl 19:314-322.

Pajarinen J, Laippala P, Penttilä A and Karhunen PJ (1997): Incidence of disorders of spermatogenesis in middle aged finnish men, 1981-91: two necropsy series. BMJ 314:13-18.

Paula-Barbosa MM, Brandao F, Madeira MD and Cadete-Leite A (1993): Structural changes in the hippocampal formation after long-term alcohol consumption and withdrawal in the rat. Addiction 88:237-247.

Pentney RJ (1993): Alterations in the structure of the cerebellum after long-term ethanol consumption. In: Alcohol-induced Brain Damage, pp. 249-276. Eds. WA Hunt and SJ Nixon, National Institute of Health, Bethesda, MD.

Perlmutter LS, Barron E and Chui HC (1990): Morphologic association between microglia and senile plaque amyloid in Alzheimer's disease. Neurosci Lett 119:32-36.

Perry VH (1994): Macrophages and the Nervous System. RG Landes Company, CRC Press, Austin, Texas.

Perry VH and Gordon S (1988): Macrophages and microglia in the nervous system. Trends Neurosci 11:273-277.

Perry VH and Gordon S (1989): Resident macrophages of the central nervous system: modulation of phenotype in relation to a specialized microenvironment. In: Neuroimmune Networks: Physiology and Disease, pp. 119-125. Eds. E Goetyl and H Spector, Alan R Liss, New York.

Persson JO, Terelius Y and Ingelman-Sundberg M (1990): Cytochrome P450-dependent formation of oxygen radicals. Isoenzyme-specific inhibition of P450-mediated reduction of oxygen and carbon tetrachloride. Xenobiotica 20:887-900.

Pfefferbaum A, Sullivan EV, Rosenbloom MJ, Mahalon DH and Lim KO (1998): A controlled study of cortical gray matter and ventricular changes in alcoholic men over a 5-year interval. Arch General Psych 55:905-912.

Pfefferbaum A, Rosenbloom M, Deshmukh A and Sullivan EV (2001): Sex differences in the effects of alcohol on brain structure. Am J Psychiatry 158:188-197.

Phillips DE (1989): Effects of limited postnatal ethanol exposure on the development of myelin and nerve fibers in rat optic nerve. Exp Neurol 103:90-100.

Phillips S and Cragg B (1983): Does consumption of alcohol in the presence of disulfiram cause brain damage? Toxicol Appl Pharmacol 69:110-116.

Phillips SC and Cragg BG (1984): Alcohol withdrawal causes a loss of cerebellar Purkinje cells in mice. J Stud Alcohol 45:475-480.

Phillips SC, Harper CG and Kril J (1987): A quantitative histological study of the cerebellar vermis in alcoholic patients. Brain 110:301-314.

Piani D, Frei K, Do KQ, Cuenod M and Fontana A (1991): Murine brain macrophages induce NMDA receptor mediated neurotoxicity in vitro by secreting glutamate. Neurosci Lett 133:159-162.

Pignon JP and Hill C (1991): Estimation of alcohol-related deaths in France in 1985. Castroenterol Clin Biol 15:51-56.

Pohorecky L (1974): Effects of ethanol on central and peripheral noradrenergic neurons. J Pharmacol Exp Ther 189:380-391.

Pohorecky LA and Brick J (1988): Pharmacology of ethanol. Pharmacol Ther 36:335-427.

Ponnappa BC and Rubin E (2000): Modeling alcohol's effects on organs in animal models. Alcohol Res Health 24:93-104.

Price RW, Brew B, Sidtis J, Rosenblum M, Scheck AC and Cleary P (1988): The brain in AIDS: central nervous system HIV-1 infection and AIDS dementia complex. Science 239:586-592.

Puzziferri I, Signorile A, Guerrieri F, Papa S, Cuomo V and Steardo L (2000): Chronic low dose ethanol intake: biochemical characterization of liver mitochondria in rats. Life Sci 66:477-484.

Rabin RA, Wolfe BB, Dibner MD, Zahniser NR, Melchior C and Molinoff PB (1980): Effects of ethanol administration and withdrawal on neurotransmitter receptor systems in C57 mice. J Pharmacol Exp Ther 213:491-496.

Ramasarma T (1982): Generation of H2O2 in biomembranes. Biochim Biophys Acta 694:69-93.

Ravindranath V, Anandatheerthavarada HK and Shankar SK (1989): Xenobiotic metabolism in human brain –presence of cytochrome P-450 and associated mono-oxygenases. Brain Res 496:331-335.

Ridker PM, Vaughan DE, Stampfer MJ, Glynn RJ and Hennekens CH (1994): Association of moderate alcohol consumption and plasma concentration of endogenous tissue-type plasminogen activator. JAMA 272:929-933.

Riihioja P, Jaatinen P, Oksanen H, Haapalinna A, Heinonen E and Hervonen A (1997a): Dexmedetomidine alleviates ethanol withdrawal symptoms in the rat. Alcohol 14:537-544.

Riihioja P, Jaatinen P, Oksanen H, Haapalinna A, Heinonen E and Hervonen A (1997b): Dexmedetomidine, diazepam and propranolol in the treatment of ethanol withdrawal symptoms in the rat. Alcohol Clin Exp Res 21:804-808.

Riihioja P, Jaatinen P, Haapalinna A, Kiianmaa K and Hervonen A (1999a): Effects of dexmedetomidine on rat locus coeruleus and ethanol withdrawal symptoms during intermittent ethanol exposure. Alcohol Clin Exp Res 23:432-438.

Riihioja P, Jaatinen P, Oksanen H, Haapalinna A, Kiianmaa K and Hervonen A (1999b): Effects of aging and intermittent ethanol exposure on rat locus coeruleus and ethanol withdrawal symptoms. Alcohol Alcohol 34:706-717.

Rimm EB, Chan J, Stampfer MJ, Colditz GA and Willett WC (1995): Prospective study of cigarette smoking, alcohol use, and the risk of diabetes in men. BMJ 310:555-559.

Rimm EB, Klatsky A, Grobbee D and Stampfer MJ (1996): Review of moderate alcohol consumption and reduced risk of coronary heart disease: is the effect due to beer, wine, or spirits? BMJ 312:731-736.

Rimm EB, Williams P, Fosher K, Criqui M and Stampfer MJ (1999): Moderate alcohol consumption and lower risk of coronary heart disease: meta-analysis of effects on lipids and haemostatic factors. BMJ 319:1523-1528.

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.

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

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

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

Riveros-Rosas H, Julian-Sanchez A and Piña E (1997): Enzymology of ethanol and acetaldehyde metabolism in mammals. Arch Med Res 28:453-471.

Ronis MJ, Huang J, Crouch J, Mercado C, Irby D, Valentine CR, Lumpkin CK, Ingelman-Sandberg M and Badger TM (1993): Cytochrome P450 CYP 2E1 induction during chronic alcohol exposure occurs by a

two-step mechanism associated with blood alcohol concentrations in rats. J Pharmacol Exp Ther 264:944-950.

Rossetti ZL and Carboni S (1995): Ethanol withdrawal is associated with increased extracellular glutamate in the rat striatum. Eur J Pharmac 283:177-183.

Rouach H, Houze P, Gentil M, Orfanelli M-T and Nordmann R (1997): Changes in some pro- and antioxidants in rat cerebellum after chronic alcohol intake. Biochem Pharmacol 53:539-545.

Roy P, Roy S, Mitra A and Kulkarni A (1994): Superoxide generation by lipoxygenase in the presence of NADH and NADPH. Biochim Biophys Acta 1214:171-179.

Rubio S, Begega A, Santin LJ and Arias JL (1996): Ethanol- and diazepam-induced cytochrome oxidase activity in mammillary bodies. Pharmacol Biochem Behav 55:309-314.

Russ R, Abdel-Rahman AR and Wooles WR (1991): Role of the sympathetic nervous system in ethanol-induced hypertension in rats. Alcohol 8:301-307.

Salaspuro M (1996): Bacteriocolonic pathway for ethanol oxidation: characteristics and implications. Ann Med 28:195-200.

Salaspuro M (1997): Microbial metabolism of ethanol and acetaldehyde and clinical consequences. Addict Biol 2:35-46.

Salaspuro M (2003): Acetaldehyde, microbes and cancer of the digestive tract. Crit Rev Clin Lab Sci 40:183-208.

Sarviharju M, Jaatinen P, Hyytiä P, Hervonen A and Kiianmaa K (2001): Effects of lifelong ethanol consumption on drinking behavior and motor impairment of alcohol-preferring AA and alcohol-avoiding ANA rats. Alcohol 23:157-166.

Savage LM, Candon PM and Hohman H (2000): Alcohol-induced brain pathology and behavioral dysfunction: using an animal model to examine sex differences. Alcohol Clin Exp Res 24:465-475.

Savolainen VT, Liesto K, Männikkö A, Penttilä A and Karhunen PJ (1993): Alcohol consumption and alcoholic liver disease: evidence of a threshold level of effects of ethanol. Alcohol Clin Exp Res 17:1112-1117.

Schäfer K, Gretz N, Bader M, Oberbaumer I, Eckardt KU, Kriz W and Bachmann S (1994): Characterization of the Han:SPRD rat model for hereditary polycystic kidney disease. Kidney Int 46:134-152.

Schlicht I, Falk S, Krössin A and Mohnhaupt A (1992): Lifetime prolongation in voluntary alcohol-consuming rats (SHR) treated with chofibrate. Alcohol 9:139-148.

Seitz HK and Oneta CM (1998): Gastrointestinal alcohol dehydrogenase. Nutr Rev 56:52-60.

Seitz HK, Czygan P, Simanowski U, Waldherr R, Veith S, Raedsch R and Kommerell B (1985): Stimulation of chemically induced rectal carcinogenesis by chronic ethanol ingestion. Alcohol Alcohol 20:427-433.

Seitz HK, Egerer G, Oneta C, Krämer S, Sieg A, Klee F and Simanowski UA (1996): Alcohol dehydrogenase in the human colon and rectum. Digestion 57:105-108.

Seitz HK, Poschl G and Simanowski UA (1998): Alcohol and cancer. Rec Dev Alcoholism 14:67-95.

Seligman AM, Karnovsky MJ, Wasserkrug HL and Hanker JS (1968): J Cell Biol 38:1-14.

Shetty AK and Phillips DE (1992): Effects of prenatal ethanol exposure on the development of Bergmann glia and astrocytes in rat cerebellum: an immunohistochemical study. J Comp Neurol 321:19-32.

Shiratori Y, Teraoka H, Matano S, Matsumoto K, Kamii K and Tanaka M (1989): Kupffer cell function in chronic ethanol-fed rats. Liver 9:351-359.

Shultz JM, Rice DP and Parker DL (1990): Alcohol-related mortality and years of potential life lost – United States, 1987. Mor Mortal Wkly Rep CDC Surveill Summ 39:173-178.

Sillanaukee P and Koivula T (1990): Detection of a new acetaldehyde-induced hemoglobin fraction HbA1ach by cation exhance liquid chromatography. Alcohol Clin Exp Res 14:842-846.

Sinclair JD, Le AD and Kiianmaa K (1989): The AA and ANA rat lines, selected for differences in voluntary alcohol consumption. Experientia 45:798-805.

Single E, Robson L, Rehm J and Xiaodi X (1999): Morbidity and mortality attributable to alcohol, tobacco, and illicit drug use in Canada. Am J Pub Health 89:385-390.

Sippel HW (1974): The acetaldehyde content in rat brain during ethanol metabolism. J Neurochem 23:451-452.

Sjögren H, Eriksson A, Broström G and Ahlm K (2000): Quantification of alcohol-related mortality in Sweden. Alcohol Alcohol 35:601-611.

Smith DG, Learn JE, McBride WJ, Lumeng L, Li TK and Murphy JM (2001): Long-term effects of alcohol drinking on cerebral glucose utilization in alcohol-preferring rats. Pharmacol Biochem Behav 69:543-553.

Smith DG, Learn JE, McBride WJ, Lumeng L, Li TK and Murphy JM (2002): Local cerebral glucose utilization after relapse in ethanol drinking in alcohol-preferring (P) rats. Alcohol 27:115-126.

Sohal RS (1981): Age pigments. Elsevier, North-Holland Biomedical Press, Amsterdam.

Sohal RS and Brunk UT (1990): Lipofuscin as an indicator of oxidative stress and aging. Adv Exp Med Biol 266:17-29.

Soini SL, Hyytiä P and Korpi ER (2002): Brain regional μ-opioid receptor function in rat lines selected for differences in alcohol preference. Eur J Pharmacol 448:157-163.

Sterio DC (1984): The unbiased estimation of number and sizes of arbitrary particles using the disector. J Microsc 134:127-136.

Streit WJ and Graeber MB (1996): Microglia: a pictorial. Prog Histochem Cytochem 31:1-91.

Streit WJ, Graeber MB and Kreutzberg GW (1988): Functional plasticity of microglia: a review. GLIA 1:301-307.

Suzumura A, Marunouchi T and Yamamoto H (1991): Morphological transformation of microglia in vitro. Brain Res 545:301-306.

Suzumura A, Sawada M, Yamamoto H and Marunouchi T (1990): Effects of colony stimulating factors on isolated microglia in vitro. J Neuroimmunol 30:111-120.

Tabakoff B, Anderson RA and Ritzman RF (1976): Brain acetaldehyde after ethanol administration. Biochem Pharmacol 25:1305-1309.

Tabakoff B, Hellevuo K and Hoffman PL (1996): Alcohol, in pharmacological aspects of drug dependence. In: Handbook of Experimental Pharmacology, pp. 374-460. Eds. CR Schuster and MJ Kuhar, Springer, Berlin.

Tabbaa S, Dlugos C and Pentley R (1999): The number of granule cells and spine density on Purkinje cells in aged, ethanol-fed rat. Alcohol 17:255-260.

Takada A, Nei J, Takase S and Matsuda Y (1986): Effects of ethanol on experimental hepatocarcinogenesis. Hepatology 6:65-72.

Tan CY and Weaver DF (1997): Molecular pathogenesis of alcohol withdrawal seizures: the modified lipid-protein interaction mechanism. Seizure 6:255-274.

Tavares M and Paula-Barbosa M (1982): Alcohol-induced granule cell loss in the cerebellar cortex of the adult rat. Exp Neurol 78:574-582.

Tavares MA, Paula-Barbosa MM, Barroca H and Volk B (1985): Lipofuscin granules in cerebellar interneurons after long-term alcohol consumption in the adult rat. Anat Embryol 171:61-69.

Tavares MA, Paula-Barbosa MM and Cadete-Leite A (1987): Chronic alcohol consumption reduces the cortical layer volumes and the number of neurons of the rat cerebellar cortex. Alcohol Clin Exp Res 11:315-319.

Thaw HH, Brunk UT and Collins PV (1984): Influence of oxygen tension, pro-oxidants and antioxidants the formation of lipid peroxidation products (lipofuscin) in individual cultivated human glial cells. Mech Ageing Dev 24:211-223.

Thayer WS and Cummings JJ (1990): Effects of chronic alcohol consumption on the steady-state kinetics properties of cytochrome oxidase in rat liver. Biochim Biophys Acta 1016:333-338.

Thayer WS and Rottenberg H (1992): Comparative effects of chronic ethanol consumption on the properties of mitochondria from rat brain and liver. Alcohol Clin Exp Res 16:1-4.

Thomas WE (1990): Characterization of the dynamic nature of microglial cells. Brain Res Bull 25:351-354.

Thun MJ, Peto R, Lopez AD, Monaco JH, Henley SJ, Heath CW and Doll R (1997): Alcohol consumption and mortality among middle-aged and elderly U.S. citizens. N Engl J Med 337:1705-1714.

Thurman RG (1998): II. Alcoholic liver injury involves activation of Kupffer cells by endotoxin. Am J Physiol 275:605-611.

Tillonen J, Homann N, Rautio M, Jousimies-Somer H, Salaspuro M (1999): Ciprofloxacin decreases the rate of ethanol elimination in humans. Gut 44:347-352.

Tillonen J, Väkeväinen S, Salaspuro V, Zhang Y, Rautio M, Jousimies-Somer H, Lindros K, Salaspuro M (2000): Metronidazole increases intracolonic but not peripheral blood acetaldehyde in chronic ethanol-treated rats. Alcohol Clin Exp Res 24:570-575.

Tindberg N and Ingelman-Sundberg M (1996): Expression, catalytic activity, and inducibility of cytochrome P450 2E1 (CYP2E1) in the rat central nervous system. J Neurochem 67:2066-2073.

Torvik A and Torp S (1986): The prevalence of alcoholic cerebellar atrophy. A morphometric and histological study of an autopsy material. J Neurol Sci 75:43-51.

Torvik A, Lindboe CF and Rodge S (1982): Brain lesions in alcoholics. A neuropathological study with clinical correlations. J Neurol Sci 56:233-248.

Truelsen T, Gronbaek M, Schnohr P and Boysen G (1998): Intake of beer, wine and spirits and risk of stroke. The Copenhagen City Heart Study. Stroke 29:2467-2472.

Truelsen T, Thudium D and Gronbaek M (2002): Amount and type of alcohol and risk of dementia. The Copenhagen City Heart Study. Neurology 59:1313-1319.

Tuck RR and Jackson M (1991): Social, neurological and cognitive disorders in alcoholics. Med J Aust 155:225-229.

Tuominen K, Hilakivi LA, Päivärinta P, Korpi EA (1990): Behavior of alcohol-preferring AA and alcohol-avoiding ANA rat lines in tests of anxiety and aggression. Alcohol 7:349-353.

Uylings HBM and van Eden CG (1990): Qualitative and quantitative comparison of the prefrontal cortex in rat and in primates, including humans. In: The Prefrontal Cortex. Its Structure, Function and Pathology. Progress in Brain Research, pp. 31-62. Eds. HBM Uylings, CG van Eden, JPC De Bruin, MA Corner and MGP Feenstra, Elsevier, Amsterdam.

Uysal M, Kutalp G, Ozdemirler G and Aykac G (1989): Ethanol-induced changes in lipid peroxidation and glutathione content in rat brain. Drug Alcohol Depend 23:227-230.

Väkeväinen S, Tillonen J, Salaspuro M, Jousimies-Somer H, Nuutinen H, Färkkilä M (2000) Hypochlorhydria induced by a proton pump inhibitor leads to intragastric microbial production of acetaldehyde from ethanol. Aliment Pharmacol Ther 14:1511-1518.

Väkeväinen S, Mentula S, Nuutinen H, Salmela KS, Jousimies-Somer H, Färkkilä M, Salaspuro M (2002) Ethanol-derived microbial production of carcinogenic acetaldehyde in achlorhydric atrophic gastritis. Scand J Gastroenterol 37:648-655.

Valmadrid CT, Klein R, Moss SE, Klein BE and Cruickshanks KJ (1999): Alcohol intake and the risk of coronary heart disease mortality in persons with older-onset diabetes mellitus. JAMA 282:239-246.

Varlinskaya EI and Spear LP (2004): Acute ethanol withdrawal (hangover) and social behavior in adolescent and adult male and female Sprague-Dawley rats. Alcohol Clin Exp Res 28:40-50.

Victor M (1984): Polyneuropathy due to nutritional deficiency and alcoholism. In: Peripheral Neuropathy, p 1899. Eds. PJ Dick, PK Thomas, EH Lambert and RP Bunge, W.B. Saunders, Philadelphia.

Victor M, Adams R and Mancall EL (1959): A restricted form of cerebellar cortical degeneration occurring in alcoholic patients. Arch Neurol 1:579-688.

Victor M, Adams RD and Collins GH (1989): The Wernicke-Korsakoff syndrome and related neurological disorders due to alcoholism and malnutrition. FA Davis Co, Philadelphia.

Vittadini G, Buonocore M, Colli G, Terzi M, Fonte R and Biscaldi G (2001): Alcoholic polyneuropathy: a clinical and epidemiological study. Alcohol Alcohol 36:393-400.

Walker DW, Barnes DE, Zornetzer SF, Hunter BE and Kubanis P (1980): Neuronal loss in hippocampus induced by prolonged ethanol consumption in rats. Science 209:711-713.

Walker DW, Hunter BE and Abraham WC (1981): Neuroanatomical and functional deficits subsequent to chronic ethanol administration in animals. Alcohol Clin Exp Res 5:267-282.

Walker DW, King MA and Hunter BE (1993): Alterations in the structure of the hippocampus after long-term ethanol administration. In: Alcohol-induced Brain Damage, pp. 231-247. Eds. WA Hunt and SJ Nixon, National Institute of Health, Bethesda, MD.

Wang G, Volkow N, Fowler J, Pappas N, Wong C, Pascani K, Felder C and Hitzemann R (1998): Regional cerebral metabolism in female alcoholics of moderate severity does not differ from that of controls. Alc Clin Exp Res 22:1850-1854.

Watson PE (1989): Total body water and blood alcohol levels: undating the fundamentals. In: Human Metabolism of Alcohol, Vol. I: Pharmacokinetics, Medicolegal Aspects, and General Interest, p. 41. Eds. KE Crow and RD Batt, CRC Press, Boca Raton.

Wehr A, Rodo M, Lieber CS and Baraona E (1993): Acetaldehyde adducts and autoantibodies against VLDL and LDL in alcoholics. J Lipid Res 34:1237-1244.

West JR and Goodlett CR (1990): Teratogenic effects of alcohol on brain development. Ann Med 22:319-325.

West JR, Chen WJ and Pantazis NJ (1994): Fetal alcohol syndrome: the vulnerability of the developing brain and possible mechanisms of damage. Metab Brain Dis 9:291-322.

West JR, Goodlett CR, Bonthius DJ, Hamre KM and Marcussen BL (1990): Cell population depletion associated with fetal alcohol brain damage: mechanism of BAC-dependent cell loss. Alcohol Clin Exp Res 14:813-818.

West MJ (1993): New stereological methods for counting neurons. Neurobiol Aging 14:275-285.

Wetterling T, Veltrup C, Driessen M and John U (1999): Drinking pattern and alcohol-related medical disorders. Alcohol Alcohol 34:330-336.

Williams-Hemby L and Porrino LJ (1994): Low and moderate doses of ethanol produce distinct patterns of cerebral metabolic changes in rats. Alcohol Clin Exp Res 18:982-988.

Williams-Hemby L and Porrino LJ (1997): I. Functional consequences of intragastrically administered ethanol in rats as measured by the 2-[14C]deoxyglucose method. Alcohol Clin Exp Res 21:1573-1580.

Wirkner K, Poelchen W, Koles L, Muhlberg K, Scheibler P, Allgaier C and Illes P (1999): Ethanol-induced inhibition of NMDA receptor channels. Neurochem Internat 35:153-162.

Wong-Riley MTT (1979): Changes in the visual system of monocularly sutured or enucleated cats demonstrable with cytochrome oxidase histochemistry. Brain Res 171:11-28.

Wong-Riley MTT (1989): Cytochrome oxidase: an endogenous metabolic marker for neuronal activity. Trends Neurosci 12:94-101.

Worner TM and Lieber CS (1985): Perivenular fibrosis as precursor lesion of cirrhosis. J Am Med Assoc 254:627-630.

Yanez JL, Del Rio MC and Alvarez FJ (1993): Alcohol-related mortality in Spain. Alcohol Clin Exp Res 17:253-255.

Yearbook of Alcohol and Drug Statistics 2003 (Päihdetilastollinen vuosikirja 2003). SVT, Sosiaaliturva. Stakes, Gummerus Kirjapaino Oy, Saarijärvi.

Yokoyama A, Muramatsu T, Ohmori T, Yokoyama T, Okuyama K, Takahashi H, Hasegawa Y, Higuchi S, Maruyama K, Shirakura K, Ishii H (1998): Alcohol-related cancers and aldehyde dehydrogenase-2 in Japanese alcoholics. Carcinogenesis 19:1383-1387.

Zhu BCR and Laine RA (1989): Purification of acetellactosamine-specific tomato lectin by erythroglycan-sepharose affinity chromatography. Prep Biochem 19:341-350.

ORIGINAL COMMUNICATIONS