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Ethanol Withdrawal Symptoms and Ethanol-induced Neuropathology

Effects of Dexmedetomidine Treatment and Aging

ACADEMIC DISSERTATION
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To my lovely girl Sara

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This thesis is based on the following original articles, which are referred to in the text by the Roman numerals **I–V**:

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

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

III 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.

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

V Riihioja P, Jaatinen P, Haapalinna A, Kiianmaa K and Hervonen A (1999): Effects of ageing and intermittent ethanol exposure on rat locus coeruleus and ethanol withdrawal symptoms. Alcohol Alcohol 34:706–717.

Abbreviations

AA	Alko, alcohol (ethanol-preferring line of rats)
ABC	avidin-biotin-peroxidase complex
ANA	Alko, non-alcohol (ethanol non-preferring line of rats)
ANOVA	analysis of variance
ANCOVA	analysis of covariance
AUC	area under curve
BEC	blood ethanol concentration
BEC-0	blood ethanol concentration before the morning dose of ethanol
BEC-1h	blood ethanol concentration 1 h after the morning dose of ethanol
BSA	bovine serum albumin
Ca	calcium
CNS	central nervous system
CRF	corticotropin-releasing factor
CT	computerized tomography
DAB	diaminobenzidine
DEX	dexmedetomidine
DTI	diffusion tensor imaging
EtOH	ethanol
EtOH+SD	ethanol + single dose of dexmedetomidine
FIF	formaldehyde-induced fluorescence
GABA	gamma-aminobutyric acid
HPA	hypothalamic-pituitary-adrenal
i.p.	intraperitoneal
i.v.	intravenous
LC	locus coeruleus
MHPG	3-methoxy-4-hydroxyphenylglycol
MRI	magnetic resonance imaging
NGS	normal goat serum
NMDA	N-methyl-D-aspartate
PAP	peroxidase-antiperoxidase
PBS	phosphate-buffered saline
p.o.	per os
s.c.	subcutaneously
SCG	superior cervical ganglion
SEM	standard error of mean
TH	tyrosine hydroxylase
TH-IR	tyrosine hydroxylase immunoreactivity

Abstract

Chronic ethanol exposure has been shown to cause degenerative changes in several areas of the brain, including cerebral cortex, hippocampus, cerebellum and brainstem, and also in the peripheral nervous system. Ethanol-induced neuronal degeneration is multifactorial in origin, and changes in several neurotransmitter systems during chronic ethanol exposure have been reported. Overactivity of the noradrenergic neurotransmission has been reported in both the central and the peripheral nervous system. In previous studies prolonged noradrenergic overactivity has been supposed to contribute to the morphological and functional degeneration of rat peripheral sympathetic nervous system. Aging has been suggested to enhance the ethanol-induced degenerative changes in the CNS.

Ethanol withdrawal syndrome is the result of a cessation or reduction of a heavy, prolonged ingestion of alcohol. Alcoholics commonly undergo repeated withdrawal episodes. Even a single withdrawal episode has been reported to cause neurodegenerative alterations, and intermittent ethanol exposure with repeated withdrawal periods has been shown to be more harmful to neurons than continuous exposure. Overactivity of the noradrenergic nervous system has been shown to be one of the central mechanisms of the ethanol withdrawal syndrome. In the withdrawal phase, the noradrenergic overactivity may result, at least in part, from a reduced sensitivity of α_2-adrenergic receptors. Aging has been reported to increase the severity of ethanol withdrawal syndrome.

The aim of this study was to investigate the effects of dexmedetomidine (DEX), a selective and potent α_2-adrenergic agonist, on the ethanol withdrawal syndrome. The effects of DEX were compared with those of diazepam and propranolol, drugs presently used in the clinical treatment of ethanol withdrawal. The possible neuroprotective effects of DEX on ethanol-induced neuronal degeneration were studied in the peripheral sympathetic neurons of the superior cervical ganglion (SCG) during a 12-day ethanol exposure and in the central adrenergic neurons of the locus coeruleus (LC) during a five-week intermittent ethanol exposure. The effects of aging on the severity of ethanol withdrawal symptoms and ethanol-induced neurodegeneration in the rat were studied during a five-week intermittent ethanol exposure.

DEX significantly relieved the ethanol withdrawal syndrome in the rat, when measured as the sum score of the three most specific symptoms (rigidity, tremor, irritability). The efficiency of DEX in the treatment of ethanol withdrawal symptoms seemed to be comparable to the effect of diazepam. During the 5-week intermittent ethanol exposure, the effect of DEX on ethanol withdrawal symptoms seemed to remain unchanged or even improve during repeated withdrawal periods. DEX was shown to have neuroprotective effects on ethanol-induced degeneration in both the peripheral and the central nervous system. Aging did not significantly affect the severity of ethanol withdrawal symptoms or ethanol-induced loss of LC neurons.

In conclusion, DEX seems to be an interesting new alternative in the treatment of the ethanol withdrawal syndrome, especially with regard to its potential neuroprotective effects.

Review of the literature

1 Ethanol and the nervous system

1.1 Morphological and functional alterations of the human nervous system after chronic ethanol exposure

Neuropathological, as well as neuroradiological studies with computerized tomography (CT) or magnetic resonance imaging (MRI) have shown reduction in brain volumes, reflected on ventricular and sulcar enlargement of the brains of chronic alcoholics (Harper et al. 1988, Pfefferbaum et al. 1993, Mukamal 2004). A loss of cortical and subcortical white and gray matter has been shown (Pfefferbaum et al. 1992, Mann et al. 2001), although these changes have been reported to partly reverse during prolonged abstinence (O'Neill et al. 2001, Agartz et al. 2003). The frontal lobes have been reported to be most seriously affected, characterized by a significant volume loss (Kubota et al. 2001), a diminished glucose metabolic rate (Adams et al. 1993), as well as neuronal loss (Kril et al. 1997, Harper et al. 2003). In a study with magnetic resonance diffusion tensor imaging (DTI), also disruption of brain white matter microstructure has been reported (Pfefferbaum et al. 2000). The degeneration of frontal cortex may be associated with disturbances in working memory and attention (Kril et al. 1997, Pfefferbaum et al. 2000).

Wernicke's encephalopathy is a common disorder of alcoholics suffering from thiamine (B1-vitamin) deficiency, characterized by alterations of mental status, ataxia and ocular motility disturbances. Approximately 80% of patients suffer also from Korsakoff's syndrome with selective deficits in anterograde and retrograde memory (Victor et al. 1989). The characteristic necrotic lesions with typical microscopic changes occur symmetrically in structures surrounding the third ventricle, aqueduct and fourth ventricle (Victor et al. 1989). Neuronal loss has been reported in the diencephalic regions including mamillary bodies (Sheedy et al. 1999, Sullivan et al. 1999) and thalamus (Harding et al. 2000, Harper et al. 2003).

In the hippocampus, an important center for memory and learning, bilateral anterior volume deficits (Sullivan et al. 1995, Sullivan and Marsh 2003) have been reported in chronic alcoholics. However, in a study by Laakso et al. (2000) a significant volume loss was seen only in the right hippocampus. Studies using unbiased

stereological methods have found no loss of neurons but a significant loss of white matter in the hippocampus of alcoholics (Harding et al. 1997, Korbo 1999).

In chronic alcoholics, cerebellar atrophy, especially in the anterior vermis, has been reported (Phillips et al. 1987, Sullivan et al. 2000), characterized by typical signs like ataxia, dysarthria and impaired gait. Nicolás et al. (2000) showed that malnutrition and a high daily ethanol intake over ten years independently associated with the development of cerebellar shrinkage. Atrophy of the molecular layer and reduction in the number of cerebellar Purkinje cells have been reported in chronic alcoholics (Phillips et al. 1990, Karhunen et al. 1994). Baker et al. (1999) reported a 43% loss of Purkinje cells only in thiamine deficient alcoholics. In a recent study using stereological methods a decrease of the Purkinje cell volume in chronic alcoholics was seen without a concomitant loss of neurons (Andersen 2004).

The rare disorder of central pontine myelinolysis is seen in malnourished alcoholic patients suffering from progressive neurologic symptoms including paretic symptoms, dysarthria and eye movement disorders (Pirzada and Ali 2001, Afsari and Posin 2002). It is characterized by demyelination that involves the central portion of the base of the pons (Adams et al. 1959, Wright et al. 1979, Uchino et al. 2003). Marchiafava-Bignami syndrome is another rare disorder occurring predominantly in malnourished alcoholics, characterized by necrosis of the corpus callosum and the adjacent subcortical white matter (Charness 1993, Gabriel et al. 1999, Hund 2003). Typical symptoms include memory disturbances, spasticity, dysarthria, motor impairments and in severe cases this disorder may be fatal (Gabriel et al. 1999, Greenberg and Lee 2001).

Hepatic encephalopathy may develop as a consequence of alcohol-induced cirrhosis of the liver (Charness 1993, Greenberg and Lee 2001). Hepatic dysfunction may lead to increased concentrations of neurotoxins, including ammonia and manganese, in the blood and brain, where they can interfere with neurotransmitters and cause structural degeneration (Jalan et al. 2000, Butterworth 2003). Brains of patients with hepatic encephalopathy have been shown to have enlargement and proliferation of astrocytes in the basal ganglia, thalamus, red nucleus, pons and cerebellum (Victor et al. 1965, Norenberg 1987). There may be either acute hepatic encephalopathy with symptoms of delirium or a chronic form of hepatocerebral degeneration with symptoms of dementia, dysarthria, ataxia, intention tremor, choreoathetosis and corticospinal tract signs (Victor et al. 1965, Greenberg and Lee 2001).

Chronic alcohol abuse has been shown to cause degenerative changes also in the peripheral sensory and motor nervous system, and also in the autonomic nervous system (Monforte et al. 1995, Koike et al. 2001). Among the autonomic nervous system, the parasympathetic nerves have been reported to be more vulnerable to the degenerative changes of ethanol than the sympathetic ones (Gentile et al. 1994,

Ravaglia et al. 2004). Axonal degeneration involving both myelinated and unmyelinated fibers has been shown to be the main pathologic feature of alcoholic neuropathy (Walsh and McLeod 1970, Behse and Buchthal 1977). Studies have shown that the total lifetime dose of ethanol was the most important factor in the development of alcoholic neuropathy (Agelink et al. 1998, Ammendola et al. 2001, Nicolosi et al. 2005). In addition to the classic chronic neuropathy, also cases of acute alcohol-related axonal polyneuropathy have been reported (Wohrle et al. 1998, Vandenbulcke and Janssens 1999). Clinical manifestations of peripheral neuropathy include weakness or paresthesia in lower limbs, pain or painful burning sensation in the lower limbs, absent muscle tendon reflexes and sensory deficit on physical examination. Symptoms of autonomic neuropathy include erectile dysfunction, orthostatic hypotension and disturbances of gastrointestinal, urinary or sweating function (Monforte et al. 1995, Koike et al. 2001). In peripheral neuropathy slowing down nerve conduction velocities has been shown in electrophysiological studies (Behse and Buchthal 1977, Koike et al. 2001).

1.2 Pathophysiological mechanisms behind ethanol-induced neuronal degeneration

1.2.1 A review of general mechanisms

Ethanol-induced neuronal degeneration is multifactorial in origin (Charness 1993), and the pathophysiological mechanisms have been widely studied, mainly in experimental animals. However, the accurate mechanisms behind ethanol's neurotoxicity are not clear yet.

It has been supposed that thiamine deficiency has a central role in the development of cognitive and memory impairments in chronic alcoholics and especially in the pathogenesis of Wernicke-Korsakoff syndrome (Ciccia and Langlais 2000, Thomson 2000, Martin et al. 2003). Even in the presence of adequate dietary intake, chronic ethanol consumption can cause thiamine deficiency by inhibiting the intestinal absorption of thiamine (Gastaldi et al. 1989), and by decreasing the activity of thiamine metabolizing enzymes, and increasing the activity of enzymes breaking down the active form of thiamine (Laforenza et al. 1990). Deficiency of thiamine has been reported to reduce the activities of specific thiamine dependent enzymes and impair brain oxidative metabolism which can initiate a cascade leading to selective neuronal death (Singleton and Martin 2001, Ke et al. 2003).

Chronic ethanol exposure induces oxidative stress in brain by increased formation of reactive oxygen species as by-products of the oxidative metabolism of neurons and

glial cells (Montoliu et al. 1994, Sun et al. 2001, Huang et al. 2002). Reduced activities of antioxidant enzymes may also contribute to ethanol-induced oxidative stress (Sun et al. 2001, McDonough 2003). Increased oxidative stress has been shown to have a role in the generation of many neurodegenerative diseases including Alzheimer's and Parkinson's disease (Sun and Chen 1998), and is supposed to be involved in ethanol-induced neuropathology (Renis et al. 1996, Herrera et al. 2003, de Freitas et al. 2004, Gonthier et al. 2004).

Acetaldehyde, the first oxidative metabolite of ethanol, is supposed to have a role in the generation of ethanol-induced brain damage (Eriksson 2001). A small amount of peripheral acetaldehyde can reach the brain because of a highly efficient acetaldehyde oxidizing system at the blood brain barrier (Eriksson and Sippel 1977), but in experimental studies acetaldehyde has been shown to be formed locally in the brain tissue by specific enzymes (Kerr et al. 1989, Gill et al. 1992). Recent studies have provided evidence that acetaldehyde may be involved in ethanol-induced neurotoxicity through formation of adducts with brain proteins and other macromolecules (Rintala et al. 2000, Upadhya and Ravindranath 2002, Nakamura et al. 2003).

In brain tissue nonoxidative metabolism of ethanol has been shown to generate fatty acid ethyl esters, which have been supposed to act as mediators of ethanol-induced organ damage (Calabrese and Rizza 1999, Soderberg et al. 2003). The mechanisms are not clear, but these lipids have been reported to be able to interrupt the function of mitochondria and also to inhibit cholesterol esterification, which may lead to disrupted myelin metabolism (Bora and Lange 1993).

1.2.2 Neurotransmitters in ethanol-induced neuronal degeneration

Several mechanisms of ethanol-induced neurotoxicity are associated with over-activation of glutamatergic receptors (Fadda and Rossetti 1998, Dodd et al. 2000, Davis and Wu 2001). In animal models, chronic ethanol exposure leads to an upregulation of glutamatergic NMDA receptors (Hu and Ticku 1995, Wirkner et al. 1999, Nagy 2004). Excessive glutamatergic transmission related to chronic ethanol exposure has been shown to result in excitotoxicity and delayed neuronal cell death (Choi 1992, Lovinger 1993, Hoffman et al. 1995, Crews et al. 1999). The glutamatergic mechanisms of ethanol-induced neurotoxicity may include the formation of reactive oxygen species, excessive amounts of nitric oxide and excitotoxic polyamines, and they have also been supposed to be involved in the cascade of events leading to apoptosis (see Fadda and Rossetti 1998 for review).

Reduced plasma GABA levels, reflecting reduced brain GABAergic activity, have been reported in chronic alcoholics (Petty et al. 1993). Down-regulation of the

inhibitory $GABA_A$ –receptors is supposed to have an important role in the development of ethanol dependence and tolerance (Faingold et al. 1998, Davis and Wu 2001, Kumar et al. 2004). However, chronic ethanol exposure has been reported to affect diffenrently the expression of $GABA_A$ receptor subunits depending on the brain area (Grobin et al. 2000, Petrie et al. 2001). Glutamatergic NMDA receptors and $GABA_A$ receptors have been reported to modulate each other's function (Isokawa 1998, Stobbs et al. 2004). A reduced GABAergic neurotransmission during chronic ethanol exposure may lead to an increase in glutamatergic neurotransmission and thus to excitotoxicity and neuronal cell death (Nutt and Glue 1990, Lovinger 1993).

Prolonged exposure to ethanol has been shown to lead to increased activity of voltage-operated calcium channels, particularly due to an increasing number of dihydropyridine binding sites (Kostowski and Bienkowski 1999, Walter et al. 2000, Newton et al. 2005). Voltage-operated calcium channels have also been reported to be involved in the regulation of ethanol consumption and preference (De Beun et al. 1996, Kuzmin et al. 1999, Newton et al. 2004). Excessive cellular influx of $Ca^{2+}$is involved in glutamate-induced excitotoxicity and may activate several Ca-dependent enzymes (Arundine and Tymianski 2003, Choi 2005). Calcium antagonists have been reported to have neuroprotective properties against NMDA-induced cell death in rat nucleus basalis (Luiten et al. 1995), and in a model of global cerebral ischemia in the gerbil (Zapater et al. 1997). Nimodipine, a voltage-gated Ca^{2+} channel blocker, has been shown to reduce the neuronal damage in the dentate gyrus in the rat during a 4-day ethanol exposure by reducing excitotoxic alterations (Corso et al. 1998).

Chronic ethanol exposure has been reported to cause overactivity of the noradrenergic neurons both in the central (Hunt and Majchrowicz 1974, Sjöquist et al. 1983; Hoffman and Tabakoff 1985, Nutt and Glue 1990) and the peripheral nervous system (Perec et al 1979, Pohorecky 1982, Jaatinen and Hervonen 1994). Increased levels of norepinephrine metabolites, like 3-methoxy-4-hydroxyphenylglycol (MHPG), have been reported in the brain and the cerebrospinal fluid of chronic alcoholics (Borg et al. 1983, Sjöquist et al. 1983, Valverius et al. 1993). A recent study by Patkar et al. (2004) showed significantly higher plasma noradrenaline concentrations in actively drinking alcohol-dependent subjects compared to abstinent ones and non-alcohol dependent controls. A study using the technique of brain microdialysis showed that long-term alcohol treatment resulted in an increased release of noradrenaline from rat hippocampus (Huttunen 1991). However, in a recent study of Sabria et al. (2003), chronic ethanol administration of 5 weeks significantly decreased the release of noradrenaline in rat cortical synaptosomes and had no effect to the release of noradrenaline in rat hippocampus. In rat peripheral sympathetic nervous system the prolonged noradrenergic overactivity caused by chronic ethanol exposure has been

supposed to contribute on the morpho-functional degeneration (Jaatinen et al. 1992, 1993; Jaatinen and Hervonen 1994).

1.3 Neurotransmitters in chronic ethanol exposure, ethanol preference and dependence

The mesolimbic dopamine system has an important role in mediating the reinforcing effects of ethanol (Kiianmaa et al. 2003, Bowirrat and Oscar-Berman 2005). In human alcoholics, an association between low levels of dopamine neurotransmission and addiction severity has been shown (Schmidt et al. 1996). Studies to assess effects of chronic alcohol use on the dopaminergic system have produced inconsistent results possibly due to differences in the durations of alcohol administered, the model assessed and the route of alcohol exposure (Fadda and Rossetti 1998). Diana et al. (1995) reported a decrease in the mean firing rate of dopaminergic ventral tegmental area neurons but no difference in the number of spontaneously active cells after chronic ethanol treatment in rats. Brodie (2002) showed increased ethanol excitation of mouse dopaminergic neurons of the ventral tegmental area after 21 days of ethanol treatment. In a study by Rothblat et al. (2001) decreased dopamine levels in the rat mesostriatal system were seen after 1 year of chronic alcohol use. Receptor studies have shown reduction of dopamine D2 receptor density and function in the brain of chronic alcoholics (Hietala et al. 1994, Volkow et al. 1996, Tupala and Tiihonen 2004). Dysregulation of dopaminergic activity in prefrontal cortex and ventral tegmental area may contribute to the cognitive impairments seen in alcoholics (Fadda and Rossetti 1998).

Serotonergic neuronal system has been shown to play an important role in the regulation of ethanol intake, preference and dependence (LeMarquand et al. 1994, Buck et al. 2004, McBride et al. 2004). Decreased cerebrospinal fluid concentrations of serotonin and its metabolites have been shown in chronic ethanol abusers (Borg et al. 1985, Fils-Aime et al. 1996). A study of Uzbay et al. (2000) showed decreased serotonin levels in rat cerebral cortex and corpus striatum during chronic ethanol exposure. Compared with the ethanol-nonpreferring rats, ethanol-preferring rats have shown in several brain areas lower concentrations of serotonin and its metabolites (Murphy et al. 1987, Gongwer et al. 1989), a reduced number of serotonergic fibers and neurons (Zhou et al. 1991; 1994), showing an association between low levels of serotonin and higher ethanol preference. In human alcoholics a reduction of central serotonergic neurotransmission (Berggren et al. 2002), but also a loss of brainstem serotonergic neurons (Halliday et al. 1993; 1995) has been reported, indicating neurotoxic effects of chronic alcohol exposure on the serotonergic neuronal system.

Studies with laboratory animals have shown that chronic ethanol exposure impairs central cholinergic neurotransmission with a parallel impairment in memory and learning (Hodges et al. 1991, Melis et al. 1996). Arendt et al. (1989) showed that severe memory impairments after 28 weeks ethanol administration in rats could be ameliorated with cholinergic-rich fetal basal forebrain transplants.

Chronic alcohol has been shown to stimulate the activity of the hypothalamic-pituitary-adrenal (HPA) axis (Spencer and McEwen 1990, Madeira and Paula-Barbosa 1999), characterized by elevated levels of adrenal steroids. Chronic ethanol has been reported to increase hypothalamic CRF secretion (Rivier 1996, Ogilvie et al. 1998). The HPA axis has been reported to develop tolerance to the effects of chronically binge administered ethanol (Ogilvie et al. 1998, Zhou et al. 2000, Lee and Rivier 2003). Roy et al. (2002) showed that decreased glucocorticoid receptor protein levels in brain structures may be associated with neuroadaptational mechanisms to chronic ethanol exposure.

The response of vasopressin-producing neurons to long-term ethanol exposure has been reported to be region specific (Madeira and Paula-Barbosa 1999). Chronic ethanol exposure has been shown to reduce the number of vasopressin-producing neurons in the rat supraoptic nucleus (Madeira et al. 1993), and has also been shown to be toxic to human hypothalamic vasopressin-producing neurons in a concentration- and time-dependent manner (Harding et al. 1996).

Endogenous opioid peptides have been shown to play a central role in the development of alcohol addiction (Herz 1997, Gianoulakis 2004). Chronic heavy ethanol exposure has been reported to induce a central opioid deficiency, which may be perceived as opioid withdrawal and may promote ethanol consumption through the mechanisms of negative reinforcement (Gianoulakis 2001). Neuropeptide Y (NPY) has been reported to have a role in alcohol preference and dependence (Pandey et al. 2003, Thiele and Badia-Elder 2003). NPY has recently been reported to reduce ethanol intake in rats with a history of prolonged ethanol vapour exposure (Thorsell et al. 2005). Rats selectively bred for ethanol preference have also been shown to have altered levels of NPY in several brain regions compared with ethanol-nonpreferring rats (Ehlers et al. 1998, Hwang et al. 1999).

1.4 The ethanol withdrawal syndrome

Ethanol withdrawal syndrome results from cessation or a reduction of a heavy, prolonged ingestion of alcohol. The ethanol withdrawal symptoms start within 6–48 hours after the last dose of ethanol, and the severity of the symptoms varies according to the amount and duration of ethanol exposure, the history of previous withdrawal

episodes and individual properties (Romach and Sellers 1991, Özdemir et al. 1993, Hall and Zador 1997). The typical withdrawal symptoms in human include tremor, weakness, nausea, vomiting, diarrhea, hyperreflexia, fever, autonomic hyperreactivity including tachycardia, sweating and elevated blood pressure, anxiety, irritability, orthostatic hypotension, and headache, and in severe cases seizures, delirium and hallucinations (Gross et al. 1974, Adinoff et al. 1988, Holbrook et al. 1999a). Transient auditory or visual hallucinations may occur within the first 2 days of withdrawal, delirium tremens within 2 to 4 days after the last drink (Holbrook et al. 1999a, Asplund et al. 2004). Delirium tremens is characterized by disorientation, hallucinations, agitation and tremulousness and autonomic signs like tachycardia, hypertension and fever, and in 2–5% of patients even death due to the metabolic, cardiovascular and infectious complications (Trevisan et al. 1998, Asplund et al. 2004). Seizures, usually of the grand mal type, have been reported to occur generally within 1 to 4 days after withdrawal in up to 25% of withdrawn patiens (Saitz 1998).

In rodents, withdrawal from ethanol is followed by withdrawal signs such as piloerection, tail stiffening and arching, teeth chattering, wet shakes, abnormal posture and gait, spontaneous vocalizations, bizarre behavior with stereotypical body movements, whole body rigidity, general hyperreactivity, but also locomotor hypoactivity, and spontaneous or handling-induced convulsions (Freund 1980, Friedman 1980, Clemmesen et al. 1988).

The basic mechanisms of the alcohol withdrawal syndrome are still at least partly unknown. Removal of ethanol after chronic exposure leads to “hyperexcitability” of the central nervous system (Lovinger 1993). In general, changes during ethanol withdrawal are similar to those seen during chronic ethanol exposure, in contrast to the acute effects of ethanol (Nutt and Glue 1990). The changes occurring in major neurotransmitter systems during chronic ethanol exposure and ethanol withdrawal are summarized in Table 1.

Table 1. Changes in the activity of different neurotransmitter systems during chronic ethanol exposure and during ethanol withdrawal.

Neurotransmitter		Chronic ethanol exposure		Ethanol withdrawal
Biogenic amines				
Noradrenaline	↑	Hunt and Majchrowicz 1974, Hoffman and Tabakoff 1985, Jaatinen and Hervonen 1994, Patkar et al. 2004	↑	Pohorecky et al. 1974, Linnoila et al. 1987, Bailey et al. 2000, Kovacs et al. 2002
Dopamine	↑↓	Diana et al. 1995, Rothblat et al. 2001, Brodie 2002, Tupala and Tiihonen 2004	↓	Diana et al. 1996, Rossetti et al. 1999b, Shen 2003
Serotonin	↓	Borg et al. 1985, Uzbay et al. 2000	↓	Pandey and Pandey 1996, Uzbay et al. 1998, Patkar et al. 2003
Glutamate	↑	Hu and Ticku 1995, Wirkner et al. 1999, Nagy 2004	↑	Hu and Ticku 1997, Dodd et al. 2000, De Witte 2004
GABA	↓	Petty et al. 1993	↓	Faingold et al. 2000, Cagetti et al. 2003
Acetylcholine	↓	Hodges et al. 1991, Melis et al. 1996	↑	Imperato et al. 1998
Corticotropin-releasing factor	↑	Ogilvie et al. 1998, Rivier 1996	↑	Adinoff et al. 1996
Vasopressin	↓	Gulya et al. 1991, Silva et al. 2002	↑	Wiese et al. 2000
Neuropeptide Y			↓	Roy and Pandey 2002
Endogenous opioid peptides	↓	Gianoulakis 2001	↑↓	Esel et al. 2001 Przewlocka et al. 1997

The overactivity of the noradrenergic nervous system has been reported to be one of the central mechanisms of the ethanol withdrawal syndrome (Pohorecky et al. 1974, Linnoila 1987, Adinoff et al. 1988, Hawley et al. 1994). Increased noradrenaline concentrations of the brainstem and hypothalamus (Wang et al. 1993), and increased turnover of noradrenaline in the brainstem (Eisenhofer et al. 1990) have been reported during the acute withdrawal phase. Bailey et al. (2000) reported an increase in the noradrenaline levels of mouse ventral tegmental area at 24 h after cessation of chronic ethanol treatment. During acute ethanol withdrawal, elevated concentrations of noradrenaline and its main metabolite MHPG have been shown in the cerebrospinal fluid (Borg et al. 1981, Linnoila 1987, Hawley et al. 1994), and also in the blood of detoxified patients (Eisenhofer et al. 1985, Hawley et al. 1985, Smith et al. 1990, Patkar et al. 2003), as well as laboratory animals (Kovacs et al. 2002). A significant correlation between the cerebrospinal fluid noradrenaline concentration and the severity of the withdrawal symptoms has been reported (Fujimoto et al. 1983, Hawley et al. 1985, Kovacs et al. 2002). Many typical withdrawal symptoms, like anxiety, tremor, sweating, increased blood pressure and heart rate, are also classic symptoms of sympathetic overactivity (Linnoila 1987, Nutt and Glue 1990).

Two major subtypes of adrenergic receptors are α- and β-receptors, which are further divided into subtypes 1 and 2. α_1- and β-receptors are post-synaptic excitatory receptors, whereas α_2-receptors are inhibitory presynaptic autoreceptors. In the withdrawal phase, the noradrenergic overactivity may result, at least in part, from a reduced sensitivity of α_2-adrenergic receptors (Hawley et al. 1985, Nutt et al. 1988).

A reduction in the activity of mesolimbic dopaminergic system during ethanol withdrawal has been reported in several studies (Diana et al. 1996, Rossetti et al. 1999b, Shen 2003). The inhibition of dopaminergic activity has been considered a neuro-biological correlate of the dysphoric and depressive symptoms of ethanol withdrawal (Diana et al. 1993, Weiss et al. 1996). A recent study of Bailey et al. (2000) reported changes in dopamine turnover in the mouse ventral tegmental area as late as 2 months after cessation of chronic ethanol consumption. Changes in the activity of dopaminergic receptors during ethanol withdrawal have also been reported (Djouma and Lawrence 2002, Diana et al. 2003).

A decreased serotonergic activity in several areas of the CNS has been reported during the ethanol withdrawal phase (Pandey and Pandey 1996, Weiss et al. 1996, Uzbay et al. 1998), also reflected by reduced levels of cerebrospinal fluid and plasma serotonin and its metabolite 5-hydroxyindoleacetic acid (5-HIAA; Borg et al. 1985, Patkar et al. 2003). Decreases in serotonin release contribute to the negative affective consequences of ethanol withdrawal (Weiss et al. 1996).

Ethanol withdrawal is associated with overactivity of the excitatory glutamatergic nervous system (Hu and Ticku 1997, Dodd et al. 2000, De Witte 2004). Both the number and the function of glutamatergic NMDA-receptors have been shown to increase (Rossetti et al. 1999a, Yamamoto et al. 1999, Haugbol et al. 2005). Excessive NMDA receptor activation has been shown to have a role in the development of ethanol withdrawal seizures (Gulya et al. 1991, Kosobud and Crabbe 1993). A reduction in the central inhibitory GABAergic transmission has been reported, which together with the enhanced activity of the glutamatergic system leads to the hyperexcitability seen during ethanol withdrawal (Faingold et al. 2000; Davis and Wu 2001, Cagetti et al. 2003). Increased activity of the NMDA receptors and excitotoxic mechanisms related to it are supposed to have a central role in the development of withdrawal-induced neuronal degeneration, as well (Thomas and Morrisett 2000, Nagy 2004).

Calcium transmission has been supposed to have a role in the generation of ethanol withdrawal hyperexcitability (Colombo et al. 1995, Watson and Little 2002). Up-regulation of L-type calcium channels during ethanol withdrawal has been reported (Walter and Messing 1999), and this up-regulation has been shown to be associated with seizure susceptibility (N'Gouemo and Morad 2003).

Cholinergic mechanisms have also been shown to have a role in the susceptibility to epileptiformic seizures, and are also supposed to be involved in the generation of ethanol withdrawal-induced convulsions (Mark and Finn 2002). Imperato et al. (1998) have shown a rapid increase in acetylcholine release in the rat hippocampus during ethanol withdrawal.

Ethanol withdrawal has been shown to reduce serum magnesium levels (Elisaf et al. 1995, Mayo-Smith 1997), and a correlation between the severity of withdrawal syndrome and magnesium level has been shown (Flink 1986). However, a double-blind placebo-controlled trial studying the effect of intramuscular administration of magnesium in the treatment of ethanol withdrawal reported no effect on the severity of the withdrawal symptoms (Wilson and Vulcano 1984).

Several studies have shown increased activation of the hypothalamic-pituitary-adrenal (HPA) axis after withdrawal from chronic ethanol (Costa et al. 1996, Hundt et al. 2001). Increased plasma cortisol levels (Esel et al. 2001, Zimmermann et al. 2003) and increased cerebrospinal fluid CRF levels (Adinoff et al. 1996) have been reported during ethanol withdrawal. CRF has been assumed to be involved in the anxiogenic symptoms of ethanol withdrawal (Baldwin et al. 1991). Increased serum level of vasopressin has been reported during ethanol withdrawal (Wiese et al. 2000).

Decreased cellular expression of neuropeptide Y has been reported during ethanol withdrawal in several brain structures including amygdala, cortex and hypothalamus (Roy and Pandey 2002). Ethanol withdrawal symptoms including irritability, tremor

and rigidity have been shown to be attenuated by intracerebroventricular administration of neuropeptide Y in rats (Woldbye et al. 2002).

There are only a few studies on the effects of ethanol withdrawal on endogenous opioid peptides. A decrease in plasma levels of beta-endorphin has been reported during ethanol withdrawal. Beta-endorphin deficiency was shown to continue despite withdrawal symptoms subsided in the patients, thus suggesting that their beta-endorphin deficiency was independent of the withdrawal syndrome and may contribute to alcohol craving (Esel et al. 2001). Ethanol withdrawal has been reported to up-regulate the prodynorphin system activity in the rat nucleus accumbens (Przewlocka et al. 1997), indicating that the prodynorphin-system may be involved in the neurochemical mechanisms of ethanol dependence.

1.5 The adrenergic neuron as a model of ethanol-induced neuronal degeneration

As described previously, the overactivity of the noradrenergic nervous system is one of the central mechanisms in the generation of ethanol withdrawal syndrome, and it is also likely to contribute to the pathogenesis of ethanol-induced neurodegeneration. The well-defined population of noradrenergic neurons in the superior cervical ganglion (SCG) has provided a comparatively simple model for research on aging and ethanol-induced neurodenenerative changes in the nervous system (Hervonen et al. 1986, Jaatinen et al. 1992, 1993; Jaatinen and Hervonen 1994). In previous studies, significant morphological and histochemical alterations in the rat SCG have been reported after 4 weeks of ethanol feeding. Chronic ethanol exposure resulted in a cytoplasmic vacuolation of a population of SCG neurons (Jaatinen et al. 1993), which may represent a specific reaction to increased sympathetic activity. Both the lifelong voluntary (Jaatinen et al. 1992) and the shorter, forced ethanol exposure (Jaatinen et al. 1993, Jaatinen and Hervonen 1994) have been shown to decrease the neuronal packing density and induce a loss of neurotransmitter synthetic capacity in a subpopulation of neurons. However, in a later study applying unbiased stereological methods, no ethanol-induced neuronal loss was seen in the SCG after lifelong ethanol exposure (Riikonen et al. 1999b).

The locus coeruleus (LC), the main noradrenergic nucleus in the central nervous system, is a pontine group of neurons that lie in a compact cluster within the periventricular gray in the dorsolateral corner of the fourth ventricle (Foote et al. 1983, Loughlin and Fallon 1985). The locus coeruleus provides most of the noradrenergic innervation to almost every region of the brain, sending efferent inputs to, e.g., the cerebral cortex, brainstem, thalamus, amygdala, hippocampus, cerebellum,

hypothalamus and the spinal cord (Foote et al. 1983, Berridge and Waterhouse 2003). It also receives highly convergent inputs from diverse areas of the central nervous system (Grzanna and Molliver 1980, Pieribone and Aston-Jones 1991). In addition to noradrenaline, other neurotransmitters have been reported to coexist in LC neurons, including GABA, serotonin, substance P, cholecystokinin, galanin, thyrotropin-releasing hormone and encephalin (Fodor et al. 1992, Singewald and Philippu 1998)

The LC is an important center for maintaining homeostasis within the CNS, and it is known to regulate a variety of physiological processes, including regulation of sleep, wakefulness, attention, orientation, learning, memory, nociception, stress, and autonomic and endocrine functions (Vermetten and Bremner 2002, Berridge and Waterhouse 2003, Kayama and Koyama 2003). Alterations in LC function, neuronal number and morphology have been reported in anxiety and depressive disorders (Tanaka et al. 2000, Harro and Oreland 2001), and in certain neurodegenerative diseases, like dementia of Alzheimer-type and Parkinson’s disease (German et al. 1992, Zarow et al. 2003).

Vijayashankar and Brody (1979) have reported a 40% decrease in the number of human LC neurons in the age group over 63 years compared with the age group of 14-63 years. A LC neuron loss of 27-37% in brains of old humans compared to young adults was seen in a study of Chan-Palay and Asan (1989). Marcyniuk et al. (1989) have reported a significant LC neuron loss in humans over 79 years of age. However, in a study of Mouton et al. (1994) no age-related neuronal loss was found in humans up to 72 years of age. A study using unbiased morphometric methods showed no change in the human LC neuronal number during aging (Ohm et al. 1997). A study by Kubis et al. (2000) showed a preservation of midbrain catecholaminergic LC neurons in very old human subjects. In animals, no age-related loss of LC neurons was found in Fischer 344 rats up to 30 months of age (Goldman and Coleman 1981), or in alcohol-preferring AA (Alko, alcohol, alcohol-preferring line of rats) rats up to 24 months of age (Lu et al. 1997, Rintala et al. 1998).

Results of studies concerning the effects of chronic ethanol exposure on the LC neurons have also been somewhat controversial. In human alcoholics, a 23 to 27% reduction in the total number of pigmented LC neurons has been reported (Arango et al. 1994, 1996). Between ages 30 to 70, alcoholics have been shown to have 12-51% fewer LC neurons than controls of the same age (Arango et al. 1996). Also the synapse-to-neuron ratio in rat LC has been shown to decrease after chronic ethanol exposure (Kjellström et al. 1993). However, Baker et al. (1994) found no significant loss of TH-positive neurons in the LC of chronic alcoholics compared with age-matched controls. Another study found no difference in the number of LC neurons between chronic alcoholics with Wernicke's encephalopathy and age-matched controls (Halliday et al.

1992). Secular trends, species and strain differences as well as the lack of unbiased methods for counting neurons could contribute to the controversy of the results. In studies using the unbiased disector method lifelong ethanol exposure has been shown to reduce the total neuron number of the LC of AA rats by 26–30% (Lu et al. 1997, Rintala et al. 1998).

1.6 Interactions of ethanol and aging on the nervous system

Several neuropsychological, neuroanatomical and neurophysiological studies have suggested that chronic ethanol exposure may enhance the age-related changes in the central nervous system (see Ryan and Butters 1984 for review). In neuropsychological tests, the impaired performance of chronic alcoholics has been shown to be comparable with the performance of 10 to 20 years older non-alcoholics, especially in the tests of short-term memory (Ryan and Butters 1980; 1984), abstract reasoning and concept formation (Parker and Noble 1980, Parker et al. 1982). However, in a study by Becker et al. (1983) there was only partial overlap between the effects of alcohol and aging on the performance of tasks of verbal and nonverbal divided attention. Studies with computerized tomography have reported chronic alcoholics to have cortical brain atrophy comparable with 20 years older non-alcoholics (Cala and Mastaglia 1981, Carlen et al. 1981).

Studies have demonstrated an additive effect of chronic ethanol exposure on age-related lipofuscin accumulation in several areas of rat brain (Tavares and Paula-Barbosa 1983, Borges et al. 1986). However, experimental studies have mainly not supported the idea of a general ethanol-induced premature aging effect. Samorajski et al. (1982) reported opposite effects of chronic ethanol exposure and aging on mice brain muscarinic receptor density, and showed no interaction of aging and ethanol exposure on behavioral measures. Pietrzak et al. (1990) showed that aging reduced the density of muscarinic receptors in rat cortex, hippocampus and striatum, whereas chronic ethanol exposure increased muscarinic receptor density. Pentney and Quackenbush (1991) found the effects of long-term ethanol consumption distinct from the effects of concurrent aging processes in the cerebellar Purkinje cell dendritic networks. Age-dependent changes in adenosine receptors of rat brain were not modified by lifelong intermittent ethanol exposure (Fredholm et al. 1998). The studies of Lu et al. (1997) and Rintala et al. (1998) showed that the total number of LC neurons did not change with age in the AA or ANA rats, but lifelong ethanol consumption caused a significant loss of LC neurons in the AA rats. The effects of lifelong ethanol exposure on rat cerebellar layer volumes were shown to be rather different from those produced by aging (Rintala et al. 1997), providing no support for the premature aging theory. Studies on the rat

peripheral noradrenergic nervous system have reported partly different patterns of histological alterations observed during aging and chronic ethanol exposure (Jaatinen et al. 1992, Jaatinen and Hervonen 1994). In a morphometric study by Riikonen et al. (1999b) the total number of SCG neurons did not change with age or lifelong ethanol exposure.

Althought there is little support for an ethanol-induced premature aging of the nervous system, aging has been reported to increase the vulnerability of the CNS to ethanol-induced neurodegenerative changes. Magnetic resonance imaging has shown more pronounced brain abnormalities in the frontal cortex, white matter and hippocampus in aged alcoholics compared with younger ones (Hayakawa et al. 1992, Sullivan et al. 1995, Pfefferbaum et al. 1997). Chronic alcohol intake has been shown to cause a stress response with increased levels of heat shock proteins in the aged but not in the younger rat brain (Unno et al. 2002). Aging has also been reported to increase the vulnerability of the peripheral nervous system to ethanol-induced degeneration (Jaatinen and Hervonen 1994). Ethanol-induced neuronal loss was most prominent in the rat SCG of the 24-25 months old group compared with the younger ones.

1.7 Effects of aging on ethanol withdrawal symptoms

Results of the studies concerning the effects of aging on ethanol withdrawal symptoms have been contradictory. Some clinical studies have reported more severe ethanol withdrawal symptoms in older persons compared with younger ones (Liskow et al. 1989, Brower et al. 1994). Kraemer et al. (1997) found that patients over 60 years seemed to have an increased risk for delirium and for cognitive and functional impairment during withdrawal compared with younger patients. Foy et al. (1997) reported that patients over 70 years have a higher risk of complications during alcohol withdrawal, including delirium and hallucinations. Feuerlein and Reiser (1986) found a significantly raised mortality rate in delirium patients of 55 years or older compared with younger patients.

Other studies have found no differences in the severity of ethanol withdrawal syndrome between different age groups. Wojnar et al. (2001) reported no differences between five age groups (< 30, 30–39, 40–49, 50–59 and ≥ 60 years old) in the duration or severity of alcohol withdrawal symptoms. Kasahara et al. (1996) did not find any difference in the frequency of delirium tremens between the aged (≥ 60 years old) group and the younger (35–35 years old) group. A possible explanation for the increased withdrawal severity of aged patients may be a longer history of alcohol abuse, which may lead to the “kindling”, i.e. an increased severity of withdrawal symptoms with an increasing number of withdrawal episodes (Ballenger and Post 1978). Aged patients

have also been shown to have increased blood ethanol levels and intoxication levels compared with the younger subjects, when the same doses of ethanol (g/kg of body weight) are administered (Wiberg et al. 1970, York 1982). Therefore, if an alcohol-dependent aging subject continues to consume similar amounts of ethanol he or she used to consume when younger, this may result in more severe intoxication and eventually, more severe withdrawal symptoms.

2 Treatment of the ethanol withdrawal syndrome

A proper management of the ethanol withdrawal syndrome is important not only for relieving the symptoms, but also for the prevention of serious complications including withdrawal seizures, delirium tremens, hallucinations or cardiac arhytmias, and for motivating patients to remain in abstinence (Romach and Sellers 1991, Özdemir et al. 1993, Mayo-Smith 1997). An inadequate treatment of the ethanol withdrawal syndrome has been suggested to lead to an increased severity of withdrawal symptoms including withdrawal seizures during the next withdrawal episode (Ballenger and Post 1978, Gonzalez et al. 2001, Duka et al. 2004). A history of epileptic seizures and delirious episodes in previous withdrawal periods have been shown to be risk factors for the development of alcohol withdrawal delirium (Palmstierna 2001, Fiellin et al. 2002). A proper treatment of ethanol withdrawal is important also in view of ethanol withdrawal possibly having a role in the pathophysiology of ethanol-induced neuronal degeneration (Thomas and Morrisett 2000, Gibson et al. 2003).

Controlled studies have demonstrated the efficacy of non-pharmacological interventions in the treatment of alcohol withdrawal syndrome. Supportive care including correction of fluid and electrolyte imbalances and treatment of concurrent medical illness has been shown to improve the prognosis of alcohol withdrawal syndrome (Shaw et al. 1981, Naranjo et al. 1983). However, specific pharmacotherapy is necessary for patients with moderate to severe alcohol withdrawal symptoms, or in the presence of either a past history of severe withdrawal symptoms or existing complications (Romach and Sellers 1991, Özdemir et al. 1993). In the past 50 years over 150 different drugs and drug combinations have been described in the literature for the treatment of ethanol withdrawal symptoms (Elton 1986, Mayo-Smith 1997).

Thiamine has been reported to be ineffective in the treatment of ethanol withdrawal symptoms including delirium and seizures (Kaim et al. 1969, Mayo-Smith 1997). However, thiamine is recommended for the prevention of Wernicke-Korsakoff syndrome (Mayo-Smith 1997, Day et al. 2005). There is no clinical evidence supporting

the efficacy of magnesium in the treatment of alcohol withdrawal syndrome, either (Wilson and Vulcano 1984).

Benzodiazepines are currently the drugs of choice in the treatment of alcohol withdrawal (Romach and Sellers 1991, Özdemir et al. 1993, Daeppen et al. 2002). Meta-analyses have shown that benzodiazepines significantly relieve the severity of ethanol withdrawal and reduce the incidence of delirium and seizures during the withdrawal period (Mayo-Smith 1997, Holbrook et al. 1999b, Bråthen et al. 2005). None of the drugs commonly used in the treatment of ethanol withdrawal was found to be more effective than benzodiazepines (Holbrook et al. 1999b). Benzodiazepines have been shown to have a wide margin of safety, to be rapidly absorbed and to be quite long-acting (Nutt et al. 1989, Litten and Allen 1991). Beyond acting on the GABA/ benzodiazepine receptors, benzodiazepines have also been reported to interact with the central noradrenergic system (Grant et al. 1980, Ida et al. 1985). Long-acting benzodiazepines, most commonly diazepam (half-life approximately 33 hours), are currently administered during ethanol withdrawal syndrome by the 'benzodiazepine loading' method: 10–20 mg of diazepam is administered orally every 1 or 2 hours until the patient shows clinical improvement or becomes sedated (Sellers et al. 1983, Heinälä et al. 1990). In elderly patients and those with liver disease a shorter-acting benzodiazepine with less oxidation in the liver should be considered (Naik and Lawton 1993, Peppers 1996).

However, benzodiazepines can be used only in short-term therapy, because of the risk of development of dependence and future abuse (Schuster and Humphries 1981, O'brien 2005). In a study by Martijena et al. (2001) chronic benzodiazepine administration facilitated the subsequent development of ethanol dependence in rats. Other reported adverse effects of benzodiazepines include memory impairment, drowsiness, lethargy, ataxia, diplopia and confusion (George and Dundee 1977, Lister 1985, Heinälä et al. 1990). When used in high doses, cardiovascular and respiratory depression may also occur (Baldessarini 1985). However, the adverse effects usually tend to subside quickly, and can be minimized by careful monitoring of the patient during benzodiazepine loading (Romach and Sellers 1991).

Beta-adrenergic antagonists have been reported to reduce the autonomic manifestations of alcohol withdrawal (Mayo-Smith 1997). Propranolol has been shown to prevent the development of arrhythmias, and to relieve the symptoms of sympathetic overactivity, like tremor, tachycardia and high blood pressure during ethanol withdrawal (Sellers et al. 1977, Zilm et al. 1980, Worner 1994, Charles et al. 1999). Kraus et al. (1985) found a more rapid resolution of alcohol withdrawal symptoms and shorter hospitalization with atenolol treatment compared with placebo; however, oxazepam was also administered to the patients when needed. In another study atenolol

in combination with oxazepam returned the vital signs (blood pressure, heart rate, body temperature) to normal more rapidly compared to placebo and oxazepam, but also relieved anxiety, agitation and hallucinations associated with alcohol withdrawal and reduced the craving for alcohol (Horwitz et al. 1989). However, beta-blockers have no anticonvulsant activity (Mayo-Smith 1997), and propranolol has been reported to cause confusion as a side-effect (Zilm et al. 1980, Jacob et al. 1983). Congestive heart failure or symptomatic obstructive pulmonary diseases are also contraindicative to the use of beta-blockers in the treatment of ethanol withdrawal syndrome (Romach and Sellers 1991, Özdemir et al. 1993).

Several studies have demonstrated the effectiveness of centrally acting α_2-adrenergic agonists, such as clonidine and lofexidine, in alleviating the symptoms of ethanol withdrawal (Mayo-Smith 1997, Bayard et al. 2004, Stanley et al. 2005). Alpha$_2$-adrenergic agonists have been shown to decrease the symptoms of sympathetic overactivity like tremor, tachycardia and hypertension (Manhem et al. 1985, Cushman and Sowers 1989, Adinoff 1994, Fahlke et al. 2000), but also to relieve the anxiety and depression related to alcohol withdrawal (Björkqvist 1975, Baumgartner and Rowen 1991). However, some adverse effects including sedation, orthostatic hypotension, drowsiness, dry mouth and fatigue, have been reported related to the use of α_2-adrenergic agonists (Baumgartner and Rowen 1987, Cushman et al. 1985, Robinson et al. 1989). There is no experimental evidence to support their efficacy in the treatment or prevention of withdrawal seizures or delirium (Mayo-Smith 1997).

Anticonvulsants have been shown to be effective in the treatment of alcohol withdrawal syndrome, especially in the treatment of withdrawal seizures (Malcolm et al. 2001, Hillbom et al. 2003). Carbamazepine has been shown to be superior to placebo, and equal in efficacy to lorazepam and oxazepam in the treatment of alcohol withdrawal (Björkqvist et al. 1976, Stuppaeck et al. 1992, Malcolm et al. 2002). Carbamazepine has been shown to prevent alcohol withdrawal seizures (Butler and Messiha 1986, Sternebring 1990). Phenytoin in combination with benzodiazepines has been reported to have some effectiveness in the prophylaxis and treatment of alcohol withdrawal seizures (Sampliner and Iber 1974, Willbur and Kulik 1981). Several studies have shown the effeciency of valproate in the treatment of alcohol withdrawal syndrome (Reoux et al. 2001, Myrick and Brady 2003). Among the newer anti-convulsants gabapentin (Voris et al. 2003, Rustembegovic et al. 2004) and topiramate (Rustembegovic et al. 2002) have shown encouraging results in the treatment of ethanol withdrawal, especially in the treatment of withdrawal seizures. Anticonvulsants have been reported to lack abuse potential and have minimal interactions with alcohol (Wilbur and Kulik 1981, Butler and Messiha 1986, Malcolm et al. 2001, Longo et al. 2002), but some side effects like pruritus, dizziness, incoordination, light-headedness,

vomiting and nausea have been reported (Butler and Messiha 1986, Hillbom et al. 1989, Malcolm et al. 2002).

Neuroleptic agents, including the butyrophenone haloperidol, have been shown to have some effectiveness in the treatment of alcohol withdrawal syndrome, but their use is limited because they may lower the seizure threshold (Wilbur and Kulik 1981, Mayo-Smith 1997). Haloperidol, in conjunction with benzodiazepines or anticonvulsants, has been used in the treatment of agitated patients such as in alcohol withdrawal delirium (Rosenbloom 1988, Jacobson and Schreibman 1997).

Chlormethiazole is a sedative-hypnotic agent which has been shown to be effective in the therapy of alcohol withdrawal symptoms (Williams and McBride 1998, Seifert et al. 2004), in the treatment and prevention of delirium tremens (Majumdar 1991) and in the prevention of withdrawal seizures (Franz et al. 2001). However, it has been reported to have adverse effects including sedation, respiratory depression, hypotonia, and addiction (Pentikäinen et al. 1976, Franz et al. 2001).

Baclofen, a $GABA_B$ receptor agonist, has been reported to relieve alcohol withdrawal symptoms, including delirium tremens (Colombo et al. 2004). Gamma-hydroxybutyric acid has also been shown to have some effectiveness in the treatment of alcohol withdrawal (Addolorato et al. 1999, Korninger et al. 2003).

Several studies have reported nitrous oxide to be effective in the treatment of the alcohol withdrawal syndrome (Ojutkangas and Gillman 1994, Gillman and Lichtigfeld 2004). However, in the recent double-blind placebo-controlled randomized studies nitrous oxide was not superior to placebo in the treatment of ethanol withdrawal (Alho et al. 2003), and did not decrease craving for alcohol (Alho et al. 2002).

In summary, a large variety of drugs have been used in the treatment of the ethanol withdrawal syndrome, but the optimal, well-tolerated, safe and non-addictive medicine or combination of medicines, for the treatment of withdrawal symptoms is yet to be found.

3 Dexmedetomidine, a potent and selective alpha-2-adrenergic agonist

Dexmedetomidine (DEX) (Fig. 1) is the pharmacologically active dextro enantiomer of medetomidine (MacDonald et al. 1991, Savola and Virtanen 1991). Both DEX and medetomidine have been used in veterinary practice for their hypnotic, sedative, and analgesic effects (MacDonald et al. 1988, Murrell and Hellebrekers 2005). DEX is a highly selective, spesific and potent alpha-2-adrenergic agonist (Savola et al. 1986, Scheinin et al. 1992b). The α_2: α_1 - binding selectivity ratio of medetomidine has been

shown to be 1620:1, compared to 220:1 for clonidine (Virtanen et al. 1988). In receptor binding experiments medetomidine had no affinity or effects on other receptors including β_1-, β_2-, 5-HT_1-, 5-HT_2-, muscarine, dopamine, GABA and benzodiazepine receptors. The potency of medetomidine has been reported to be superior to clonidine in central and peripheral actions on α_2-adrenoceptors (Savola et al. 1986).

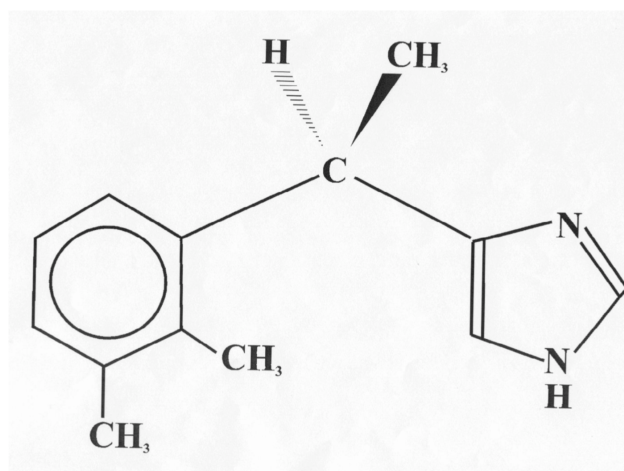


Figure 1. Structure of dexmedetomidine.

DEX has been introduced into the clinical practice of anesthesiology for its anxiolytic, sedative, analgesic, anesthetic-sparing, and hemodynamic-stabilizing properties (Aho et al. 1992, Belleville et al. 1992, Bloor et al. 1992, Esmaoglu et al. 2005). Pretreatment with DEX has been shown to attenuate haemodynamic responses to intubation, to decrease plasma catecholamine concentrations during subsequent anaesthesia, and to reduce the perioperative requirements for opioids (Scheinin et al. 1992a, 1993; Unlugenc et al. 2005). The LC has been suggested to be an important site in mediating the hypnotic, sedative and antinociceptive responses to DEX (Correa-Sales et al. 1992, Jorm and Stamford 1993, Guo et al. 1996).

Only a couple of studies have been published on the effects of DEX on ethanol-induced reactions in the nervous system. The interactions of DEX and ethanol have been previously studied in terms of acute effects on motor performance, body temperature and brain monoamine metabolites (Seppälä et al. 1994, Idänpään-Heikkilä et al. 1995). DEX, at the dose of 300 μg/kg i.p., was found to enhance the ethanol-induced hypothermia, sedation and motor impairment of mice. Contrary to the enhanced behavioral effects of ethanol, DEX inhibited the ethanol-induced increase in brain MHPG levels (i.e., noradrenaline turnover remained at the control level) when

ethanol and DEX were administered together (Seppälä et al. 1994, Idänpään-Heikkilä et al. 1995). There are no previous studies on the use of DEX during the ethanol withdrawal phase.

The aims of the study

Alcoholics commonly undergo repeated withdrawal episodes. Even a single withdrawal episode has been reported to cause neurodegenerative alterations (Collins et al. 1996), and intermittent ethanol exposure with repeated withdrawal periods has been shown to be more harmful to neurons than continuous exposure (Phillips and Cragg 1984, Lundqvist et al. 1995, Riikonen et al. 1999a, Obernier et al. 2002). Overactivity of the noradrenergic nervous system has been shown to be one of the central mechanisms of the ethanol withdrawal syndrome (Linnoila 1987, Hawley et al. 1994, Jaatinen and Hervonen 1994, Bailey et al. 2000, Kovacs et al. 2002, Patkar et al. 2003).

The aim of this study was to investigate the effects of dexmedetomidine, a selective and potent α_2-adrenergic agonist, on the ethanol withdrawal syndrome. The effects of DEX were compared with those of diazepam and propranolol, drugs presently used in the clinical treatment of ethanol withdrawal. The possible neuroprotective effects of DEX against ethanol- or withdrawal-induced neuronal degeneration were studied in the peripheral sympathetic neurons of the SCG and in the central adrenergic neurons of the LC.

Aging has been reported to increase the vulnerability of the nervous system to ethanol-induced degenerative changes (Hayakawa et al. 1992, Jaatinen and Hervonen 1994, Sullivan et al. 1995, Pfefferbaum et al. 1997, Unno et al. 2002). The results of the studies concerning the effects of aging on ethanol withdrawal symptoms have been contradictory. Some clinical studies have reported more severe ethanol withdrawal symptoms in older persons compared with younger ones (Liskow et al. 1989, Brower et al. 1994, Kraemer et al. 1997, Foy et al. 1997). Other studies have found no difference in the severity of ethanol withdrawal syndrome between different age groups (Kasahara et al. 1996, Wojnar et al. 2001). As the effects of aging on ethanol withdrawal symptoms and ethanol- or withdrawal-induced neurodegeneration were somewhat controversial in previous literature, we designed an experimental study of intermittently ethanol-exposed young and old rats to clarify these questions.

More specifically, the aims were:

1. To study the effects of dexmedetomidine on the severity of the ethanol withdrawal symptoms in the rat, and to compare its effects with those of diazepam and propranolol.

2. To study the possible neuroprotective efficacy of dexmedetomidine against ethanol- and withdrawal-induced degeneration in the rat superior cervical ganglion and locus coeruleus.

3. To study the effects of aging on the severity of the ethanol withdrawal symptoms and on ethanol-induced neurodegeneration in the locus coeruleus of the rat.

Materials and methods

1 Experimental animals

A total of 158 young (3–4 months) and 24 old (29–30 months) male Wistar rats were used for the study. The rats were housed individually under standard conditions with a constant 11:13 h or 12:12 h light-dark cycle (according to the ethanol feeding regimen) and temperature (21 ± 1 ^{o}C or 22 ± 1 ^{o}C). The animals were carefully habituated to handling before the experiment was started.

2 Ethical considerations

Every effort was made to save the animals from unnecessary suffering. Ethanol itself was an anaesthetic during the experiments, and during the ethanol withdrawal phase the animals were given medication to relieve the severity of symptoms. The control animals, which were treated by ethanol only, however, could not be given any kind of medical relief. For histological studies, the rats were killed by decapitation either under ether anaesthesia or under sodium pentobarbital anaesthesia (60 mg/kg i.p.). All the experimental procedures were approved by the local committee for animal research and county veterinary surgeon.

3 Ethanol exposures and medications

3.1 Ethanol feedings (I, II, III, IV, V)

25% ethanol (+ 5% sucrose) or isocaloric sucrose was given to the animals by intragastric intubations three or four times a day. Immediately before each ethanol feeding, the severity of intoxication of each rat was evaluated by using the following intoxication scale (Clemmesen and Hemmingsen 1984, Clemmesen et al. 1988): (0) neutrality, no signs of intoxication; (1) sedation, reduced muscle tone and motor activity, no impairment of gait or coordination; (2) ataxia 1: walking is slightly impaired, but the rats is able to elevate the abdomen and pelvis; (3) ataxia 2: clearly impaired walking, impaired elevation of abdomen and pelvis; (4) ataxia 3: slowed

righting reflex, no elevation of abdomen and pelvis; (5) loss of righting reflex, response to pain stimuli; (6) general anaesthesia/coma, no response to pain stimuli but spontaneous breathing. The dose of ethanol varied according to the level of intoxication as follows: 0 = 4.0–4.5 g/kg, 1 = 3.5 g/kg, 2 = 3.0 g/kg, 3 = 2.5 g/kg, 4 = 2.0 g/kg, 5 = 1.0 g/kg, 6 = 0 g/kg. The aim was to keep the animals in a state of intoxication graded 3 or 4 during most of the day, depending on the duration of the ethanol exposure.

3.2 The four-day ethanol exposure (I, II)

In the dose-response study (I) the rats (n = 60, 3–4 months old) were kept in a state of continuous, heavy ethanol intoxication for 4 days. The intragastric feedings of 25% ethanol were given to the rats every 6 hours for the first day and every 8 hours for the next 3 days (the first dose of ethanol was administered at 8 am). The rats had free access to standard laboratory rat food and tap water during the experiment. After the intoxication period the rats were divided into four groups (n = 7–8/group, the average weight was the same in all groups): an ethanol-fed control group (EtOH) with no medication (saline injections) in the withdrawal phase, and three DEX-treated groups (EtOH+DEX3, EtOH+DEX10 and EtOH+DEX30) with different doses of medication (3, 10 and 30 µg/kg respectively). DEX was given to the treatment groups by s.c. injections 10, 16, 22, and 39 h after ethanol withdrawal. Dexmedetomidine hydrochloride (Orion Corporation Pharma, Finland) was dissolved in sterile physiological saline and the injection volume was 0.5 ml/kg. Correspondingly, the control animals were given saline injections. The study was carried out in two equal trials (30 animals in each trial), as the laboriousness of the methods used did not allow a larger number of animals to be studied simultaneously.

In the second study (II) the rats (n = 30, 3–4 months old) were kept in a state of continuous, heavy ethanol intoxication for 4 days. After the intoxication period, rats were divided into four groups (n = 7–8/group): a DEX-treated group (30 µg/kg s.c.), a diazepam-treated group (2 mg/kg s.c.), a propranolol-treated group (5 mg/kg s.c.) and an ethanol-fed control group with placebo medication (Intralipid®-injections s.c.). Medication was given in the withdrawal phase 2, 8, 14 and 20 hr after the onset of the ethanol withdrawal symptoms. In most of the animals, the first symptoms were seen 8 to 11 hr after the last dose of ethanol. For this study, the optimal dose of propranolol and diazepam was determined in a previous open dose-finding study (with doses 2, 5, 10 mg/kg s.c. for propranolol, and 1, 2, 3, 5 mg/kg s.c. for diazepam). The aim was to find for each drug a dose that would efficiently relieve ethanol withdrawal symptoms, without causing severe sedation or ataxia. For DEX, 30 µg/kg was chosen as an optimum dose according to the previous dose-response study (I).

3.3 The 12-day ethanol exposure (III)

Before the 12-day ethanol exposure the rats (n = 30, 4 months old) were divided into five experimental groups: an ethanol-fed group without medication (EtOH, n = 6), an ethanol-fed chronically DEX-treated group (EtOH + DEX, 100 μg/kg x 2 p.o. throughout the experiment, n = 6), an ethanol-fed group with a single dose of DEX (EtOH + SD, 300 μg/kg p.o. 2–4 h before sacrifice, n = 6), a sucrose-fed control group (Sucrose, n = 6), and a chronically DEX-treated sucrose-fed control group (Sucrose + DEX, 100 μg/kg x 2 p.o. throughout the experiment, n = 6). The ethanol-exposed rats were given highly intoxicating doses of ethanol for a total of 12 days. The daily dose of ethanol was gradually increased to 10 g/kg as absolute alcohol. The sucrose-fed control rats were given isocaloric sucrose doses according the respective ethanol-exposed rats. The ethanol and sucrose intubations were given to the rats three times a day (8 am, 2 pm and 8 pm). During the last 5–6 days of the experiment, the rats were severely intoxicated for most of the day, and in the morning they exhibited withdrawal symptoms of varying severity. The ethanol-exposed rats had free access to standard laboratory rat food and tap water, and the sucrose-fed control rats were given food according to the consumption of the respective ethanol-exposed rats. Dexmedetomidine hydrochloride was given in connection with the ethanol/sucrose intubations.

3.4 The 5-week intermittent ethanol exposure (IV, V)

During the five-week intermittent ethanol exposure (IV, V) a total of 58 rats were used. The young rats (n = 34, 3–4 months old) were divided into five groups (6–11/group): a non-treated control group, a sucrose-fed control group, a sucrose-fed DEX-treated control group, an ethanol-fed group, and an ethanol-fed DEX-treated group. The old rats (n = 24, 29–30 months old) were divided into three groups (6–11/group): a non-treated control group, a sucrose-fed control group, and an ethanol-fed group. The ethanol-fed rats were given highly intoxicating doses of ethanol for four days, which was followed by a three-day withdrawal period. This 7-day cycle of ethanol exposure and withdrawal was repeated five times. The old animals were given slightly smaller ethanol doses per kg compared with the young animals, because of their decreased tolerance to ethanol compared with the younger ones. Compared with the young EtOH group, in the old EtOH group the total ethanol dose was 6.1 g/kg/week smaller during the first week of ethanol exposure, and 4.0 g/kg/week smaller during the last week of ethanol exposure. The sucrose-fed control animals were given isocaloric sucrose doses according to the respective ethanol-fed animals. The ethanol-fed animals had free

Table 2. The different ethanol exposures and the parameters investigated in this study.

Animals	Age	Ethanol exposure	Medical treatments	Measurements	n
Wistar male rats	3–4 mo	Exposure to 25% ethanol for 4 days	DEX at doses 3, 10 and 30 µg/kg s.c. in the withdrawal phase	Severity of the ethanol withdrawal symptoms	60
Wistar male rats	3–4 mo	Exposure to 25% ethanol for 4 days	DEX (30 µg/kg s.c.), diazepam (2mg/kg s.c.), propranolol (5 mg/kg s.c.) in the withdrawal phase	Severity of the ethanol withdrawal symptoms	30
Wistar male rats	4 mo	12-day exposure to 25% ethanol	DEX (100 µg/kg p.o.) througout the experiment, dex (300 µg/kg p.o.) as a single dose 2–4 h before sacrifice	Morphology of peripheral sympathetic neurons (SCG)	30
Wistar male rats	3–4 mo, 29–30 mo	Intermittent 5-week exposure to 25% ethanol (Four days of ethanol exposure followed by three days of withdrawal, repeated for five times)	DEX (at the dose decreasing from 30 µg/kg to 10 µg/kg s.c.) in withdrawal phases	Severity of the ethanol withdrawal symptoms, morphology of LC	young 38 old 24

access to laboratory rat food and tap water. The sucrose-fed control animals were given food according to the consumption of the respective ethanol-exposed animals. The non-treated control animals had free access to food and water. DEX (at a dose decreasing from 30 µg/kg to 10 µg/kg s.c.) was given to the treatment animals in every withdrawal phase 13, 17, 21, 25 and 39 hr after the last dose of ethanol. The different ethanol exposures and the parameters studied are seen in Table 2.

4 Measurement of the ethanol withdrawal symptoms (I, II, IV, V)

The severity of the ethanol withdrawal syndrome was measured in the withdrawal phase, blind to the treatments (I, II, IV, V). The rating of the withdrawal symptoms was done by using a standardized four-item scale (Clemmesen and Hemmingsen 1984, Clemmesen et al. 1988, Ulrichsen et al. 1995):

1) RIGIDITY, based on the position of the tail, the gait and the stance

0 = not present
1 = only tail stiffness observable
2 = some extension and abduction of extremities, somewhat broad based stance or gait
3 = rigid posture with maximally extended and abduced extremities

2) INTENTIONAL TREMOR, tremulous ataxia occurring during movement and intended movement

0 = not present
1 = slight
2 = moderate
3 = severe

3) IRRITABILITY, increased response to external stimuli (e.g. enhanced startle reflex, aggressiveness on handling)

0 = not present
1 = slight
2 = moderate
3 = severe

4) HYPOACTIVITY

0 = normal or increased activity
1 = slightly reduced level of activity
2 = clear hypoactivity
3 = almost complete hypoactivity

When the effects of DEX on the ethanol withdrawal symptoms were studied, the sum score of the three most specific symptoms (rigidity, tremor and irritability) was used as a general measure of the severity of the withdrawal reaction (I, II, IV). The rating of the withdrawal reaction was always done by the same investigator, blind to the treatments. The animals were carefully observed each in its turn, placed on an empty table. All disturbing voices, movements and lights were avoided during the observation. The withdrawal syndrome was termed mild (grade 1) when tremor, irritability and rigidity were only just observable, the term moderate (grade 2) represented a clinical state between mild and severe, and severe (grade 3) was used in cases where the symptoms were intense.

The assessment of rigidity was based on the position of the tail, the gait and the stance. When rigidity was severe the extremities were maximally extended and abduced. Irritability was expressed as an increased response to external stimuli, such as an enhanced reaction to sudden noise, hissing respiration, squealing, chattering teeth, opistotonus and unusual aggressiveness on handling. The animals had also atypical or aimless locomotor activity, e.g. stereotyped body movements or sprawling episodes. Intentional tremor was expressed as tremulous ataxia occurring during movement or intended movement, for example when the rat was held by the tail. Tremor involved tail, caudal region or head, and in severe cases the whole body was shivering. Hypoactivity appeared as the decrease of the general level of locomotor activity, both normal purposive behavior and bizarre inadequate behavior. Furthermore, the animals were observed for possible epileptiformic seizures.

5 Blood ethanol concentrations (III, IV, V)

Blood ethanol concentrations (BEC) were determined by gas chromatography (Eriksson 1973) from capillary blood samples (0.05 ml), which were taken from the tip of the tail (III, IV, V). The blood samples were taken from the rats two days before the 12-day exposure was finished (III), or during the last two days of the fourth week of ethanol exposure (IV, V). The samples were taken before and 1 h after the morning dose of ethanol. During the five-week ethanol exposure, for technical reasons, the blood samples were taken on two consecutive days (IV, V).

6 Tissue preparation and histological methods (III, IV, V)

6.1 Histological procedures of the superior cervical ganglion (III)

After the 12-day ethanol exposure the rats were killed by decapitation under light ether anaesthesia. The superior cervical ganglia (SCG) were rapidly prepared and frozen in liquid nitrogen. After storing in liquid nitrogen, the ganglia were processed for histochemical detection of catecholamines by the standardized formaldehyde-induced fluorescence (FIF) method (Eränkö 1967). The samples were freeze-dried for seven days at –40 ^{o} C using phosphorous pentoxide as water trap under a vacuum of 0.1 Pa or less (10^{-3} – 10^{-4} Torr). The specimens were then exposed to paraformaldehyde vapour, generated from paraformaldehyde powder previously equilibrated at 80% relative humidity. The duration of the exposure was 60 minutes at +60 ^{o} C, after which the specimens were embedded in paraffin and sectioned serially at 10 μm. The control samples were always processed simultaneously with the experimental ones.

Every tenth section was viewed through an Olympus Vanox-T-fluorescence microscope (Olympus, Inc., Tokyo, Japan) equipped with a special filter combination for the detection of monoamines (filter block "V", excitation light wavelength 395 to 415 nm, emission light 455 nm and up). Two randomly selected sections from the middle part of the ganglion were processed for pseudo color video prints by a Mitsubishi CP 100E video printer (Mitsubishi Electric Corporation, Tokyo, Japan) to attain an objective image of the neuronal FIF-intensity. For a semiquantitation of the FIF intensity, pseudo color video prints were processed by a standardized method (the same adjustments of the microscope, the video camera and the image processor throughout the series) from comparable sections of the ethanol-exposed and control rats.

Tyrosine hydroxylase immunoreactivity (TH-IR) in the sympathetic ganglia was demonstrated by using the peroxidase-antiperoxidase (PAP) technique (Sternberger 1974) on the sections previously studied by fluorescence microscopy. First, the sections were deparaffinized by three rinses in xylene and two rinses in absolute ethanol, after which the endogenous peroxidase activity was blocked by incubation with 0, 3% hydrogen peroxide in absolute methanol for 20 minutes. After two phosphate-buffered saline (PBS) rinses the sections were incubated with 5% normal goat serum (NGS) in PBS for 30 minutes at room temperature, to block the non-specific binding of antibodies. The polyclonal TH-antibody (Eugene Tech. International, Allendale, NJ) was raised up in rabbits against purified TH from bovine adrenal medulla, and its specifity was confirmed by Western blot. The dilution of the primary antibody was

1:1000, diluted in 1% NGS/PBS, and the incubation time was 24 hours at +4°C. After the incubation, the sections were rinsed with 1% NGS/PBS three times. In the control sections the specific antiserum was replaced by normal rabbit serum diluted 1:100. Goat anti-rabbit serum (Sigma, St. Louis, MO) diluted 1:80 in 1% NGS was used as a secondary antibody for 30 minutes at room temperature. After two rinses in 1% NGS the sections were incubated with PAP complexes (Jackson Immunoresearch, West Grove, PA) diluted 1:80 in PBS for 30 minutes at room temperature. Diaminobenzidine (DAB) was used as a chromogen. The reaction solution contained 0.006% hydrogen peroxide and 0.02% DAB in 0.5M Tris-Water (pH 7.6). The reaction was terminated with PBS, and the sections were rinsed twice in PBS and twice in distilled water. Finally, the sections were embedded in Aquamount and coverslipped.

6.2 Histological procedures of the locus coeruleus (IV, V)

The rats of the five-week ethanol exposure (IV, V) were killed by decapitation under deep sodium pentobarbital anaesthesia (Mebunat®, 60 mg/kg i.p.). After decapitation, the brains were quickly removed. The cerebellar peduncules were transected and the brainstem was separated by a transverse cut rostral to the inferior colliculi. The brainstem was further cut midsagittally in two halves. The tissue was fixed in 4% paraformaldehyde in PBS (pH 7.4) at 4 ^{o}C for 24 h, and then incubated in 10%, 20% and 30% sucrose in PBS to avoid cryodamage. The right side of the LC of each brainstem was taken to the morphometric analysis. The brainstem was sectioned sagittally at 8 μm in a cryostat. Six serial sections were collected on one slide for cresyl violet staining and the next two sections on another slide for immunohistochemistry. A total of 8 to 12 series of eight sections were obtained from each LC. For the morphometric analysis, the sections were stained in 0.5% cresyl violet acetate, diluted in distilled water. After the cresyl violet staining, the sections were dehydrated through graded ethanol. After dehydration, the sections were cleared with xylene for 30 seconds and finally coverslipped with Entellan®.

The noradrenergic nature of the neurons was ensured by immunohistochemical demonstration of tyrosine hydroxylase by the avidin-biotin-peroxidase complex (ABC) reaction (Hsu et al. 1981). First, the sections were incubated for 24 hours at +4°C with the primary antibody, TH antiserum (Eugene Tech. International, Allendale, NJ), diluted 1:1000 in PBS containing 1% bovine serum albumin (BSA) (Boehringer-Mannheim, Mannheim, Germany) and 0.3% Triton X-100 (Sigma, St. Louis, MO). Between every incubation step, the sections were rinsed three times with PBS. Incubation in biotinylated goat anti-rabbit antibody (Vectastain® ABC kit, Vector Labs, Burlingame, CA) diluted 1:200 in 1% BSA and 0.3% Triton X-100 in PBS was

performed at +37°C for 30 minutes. The samples were then incubated in avidin-biotin-label complex (Vectastain® ABC kit: 10 μl of reagent A (Avidin), 10 μl of reagent B (Biotin) and 980 μl of PBS) for 30 minutes at +37°C. DAB was used as a chromogen. The reaction was terminated with PBS, and after two rinses with PBS and distilled water the sections were embedded in Aquamount and coverslipped.

7 Morphometric analyses (III, IV, V)

7.1 Morphometric analyses of the superior cervical ganglion (III)

All the SCG were cut longitudinally to 10 μm sections, and the sections for the morphometric measurements were randomly selected from a corresponding set of sections in the middle part of each SCG. Neuronal density, and the proportions of TH-positive, TH-negative and vacuolated neurons were measured from two randomly selected TH-immunostained sections of each ganglion. All nucleated neuronal profiles on each section were included in these measurements. Neuronal size (cross-sectional area) was measured from 50 nucleated neuronal profiles at both the cranial and the caudal end of one randomly selected section per animal (i.e., from 600 neurons in each group). The morphometric measurements were carried out with the aid of a Hamamatsu ARGUS-10 image processor (Hamamatsu Photonics, Hamamatsu City, Japan).

7.2 Morphometric analyses of the locus coeruleus (IV, V)

The stereological study of the locus coeruleus (IV, V) was done by an Olympus Vanox-t microscope (Olympus Inc., Tokyo, Japan), a Hamamatsu ARGUS-10 image processor and a video monitor. From the cresyl violet-stained sections, the first and the last section containing LC proper neurons were identified. The reference section was chosen randomly on the first slide (drawing by lot a number between 1 and 5) and the next section was the look-up section. The next section was used if the chosen section was damaged. The boundaries of the LC proper were determined by the cresyl violet-stained sections and the volume of the LC proper was calculated by using the point counting method (Cavalieri 1966, Gundersen and Jensen 1987). A systematic set of points was placed over the video image and grid points hitting the LC proper were counted semi-automatically. The measurements were performed by using the 10 x objective. The nearest TH-IR section was used to define the boundaries of the LC, when the boundaries were not clear on the cresyl violet section. The volume of the LC ($V_{(ref)}$) was obtained from the following equation:

$V_{(ref)}$ (mm^3) = the total number of grid points hitting the LC proper x the area associated with each point corrected for magnification (0.0088 mm^2) x section thickness (0.008 mm) x the number of sections in each series (eight sections).

The total number of LC neurons was estimated by the disector method (Gundersen et al. 1988a, West 1994) on the cresyl violet-stained sections by using the 30 x objective. Starting at a random point outside the LC, a counting frame (area 2500 mm^2) was moved in a systematic, stepwise manner through the entire LC area. The frame was placed on the reference section in a systematic random manner: the coordinates of the square to be measured were drawn by lot. The origo was the lower left corner of the locus coeruleus. Then the frame was moved through the LC proper stepwise, using the photo grid as a measure of distance (one step was the length/height of the photo grid). When the frame couldn't move further to the right, it was turned to the left. Neurons were counted when their nuclei fell within the disector or touched the inclusion lines and appeared on the reference section, but not on the look-up section (Sterio 1984). If the neuron touched the exclusion line, it was not calculated. If the disector probe was not entirely on the locus coeruleus, the proportion of the counting frame area which was on the LC, was measured.

The numerical density (N_v) of neurons within the LC was calculated as follows: N_v (cells/mm^3) = neurons counted/ [total number of disectors counted x volume of the disector (0.0025 mm^2 x 0.008 mm)].

The total number of neurons within LC proper was calculated as follows: Total number of neurons = $V_{(ref)}$ x N_v.

8 Statistical methods

Area under curve (AUC) of the individual ethanol withdrawal symptoms and of the sum scores, plotted against time, was used as a measure of the overall severity of the withdrawal reaction after the 4-day ethanol exposure (I, II). Two-way analysis of variance (ANOVA) with dose and trial as factors (I) or one-way ANOVA (II) was used to analyse the overall treatment effects (AUC differences). The comparison of each treatment group with the control group was done by Bonferroni-corrected *t*-tests (I, II). The effect of DEX on seizures was tested by using χ^2-test. In the five-week ethanol exposure of young animals (IV), ANOVA for repeated measurements was used to analyse the group differences in ethanol withdrawal symptoms. One-way ANOVA and Bonferroni-corrected t-tests were used to measure the differences between the control and DEX-treated groups in the severity of the ethanol withdrawal reaction (IV). Analysis of covariance (ANCOVA) was used to analyse differences between the age

groups in the severity of ethanol withdrawal symptoms with ethanol dose and intoxication score as covariates (V).

When studying the effects of DEX on rat sympathetic neurons (III), one-way ANOVA was used to analyse the overall effects, treatment as a factor. The effects of the treatments were further analysed by Bonferroni-corrected *t*-tests, by comparing all groups with the control group and by comparing the dex-treated ethanol-exposed groups with the EtOH group.

During the 5-week ethanol exposure, the differences between the young ethanol-treated groups in BECs were analysed by Student's t-test (IV). One-way ANOVA was used to analyse the overall differences between the treatment groups, and when ANOVA showed a significant treatment effect, the groups were further compared by Bonferroni-corrected *t*-tests (IV). The effect of cumulative ethanol dose (g/kg), intoxication score and withdrawal symptom score on the morphometric variables was tested with analyses of covariance and Pearson's correlation analysis. ANOVA for repeated measurements was used to analyse group differences in the intoxication score/ethanol dose ratios (IV).

The overall effects of age and the treatments on LC morphometry (V) were studied by a standard two-way ANOVA (three treatment categories and two age categories). The group differences were further studied by Bonferroni-corrected t-tests. The post hoc comparisons were performed between the young and old controls (age effect), between the control and sucrose groups for each age (sucrose treatment effect) and between the sucrose and EtOH groups for each age (ethanol treatment effect). If only ethanol-treated rats were studied, one-way ANOVA was used. Age differences in the vulnerability to ethanol-induced neurodegeneration (V) were analysed by ANCOVA with ethanol dose, intoxication score, and ethanol withdrawal symptom score as a covariate. ANOVA for repeated measurements was used to analyse the differences between the age groups in total weekly ethanol doses (g/kg), the intoxication levels and the intoxication score/ethanol dose ratios. The differences in BECs between the age groups were analysed by ANCOVA for repeated measurements, with the day of blood sampling as a covariate (V).

Results

1 Blood ethanol concentrations and ethanol tolerence (III, IV, V)

Due to individual differences in ethanol tolerence, the total dose of ethanol during the 12-day ethanol exposure (III) varied slightly from animal to animal. However, there was no difference in the cumulative ethanol doses between the three ethanol-exposed groups (75 ± 1 g/kg in the EtOH group, 74 ± 1 g/kg in the chronically DEX-treated (EtOH + DEX) group, 77 ± 1 g/kg in the EtOH-group with a single dose of DEX (EtOH + SD) group). Neither was there any difference between the ethanol-exposed groups in blood ethanol concentrations. Blood ethanol concentration before the morning dose of ethanol (BEC-0) (mmol/l) was 47 ± 10 in the EtOH group, 34 ± 9 in the EtOH + DEX group and 39 ± 12 in the EtOH + SD group. Blood ethanol concentration 1 h after the morning dose of ethanol (BEC-1h) was 69 ± 9 in the EtOH group, 62 ± 7 in the EtOH + DEX group and 73 ± 12 in the EtOH + SD group.

During the five-week ethanol exposure (IV) ANOVA for repeated measurements revealed no difference between the ethanol-treated groups in the total ethanol doses, but the ethanol doses increased with time during the experiment in both groups (from 34.0 ± 1.6 for week 1 to 40.0 ± 0.8 for week 5 in EtOH group, from 35.8 ± 1.3 for week 1 to 41.8 ± 0.5 for week 5 in EtOH+DEX group). There was no difference between the EtOH and the EtOH+DEX group in the BEC-0 on the first day ($p = 0.65$) or on the second day ($p = 0.94$), or in the BEC-1h on the first day ($p = 0.49$) or on the second day ($p = 0.18$). The development of ethanol tolerance was estimated by calculating the intoxication score/cumulative ethanol dose ratios for each week. ANOVA for repeated measurements revealed no difference between the EtOH group and the EtOH + DEX group in the intoxication/ethanol dose ratio ($p = 0.42$).

ANOVA for repeated measurements revealed that both age and time had an effect on the intoxication score/ethanol dose ratio i.e. on ethanol tolerance, and the development of tolerance with time was slower in the old animals compared with the young ones ($p < 0.01$ for age effect, $p < 0.001$ for time effect, $p = 0.03$ for interaction). The ethanol doses were administered individually according to the intoxication level, slightly smaller doses of ethanol/kg body weight in the old rats compared with the young rats. ANCOVA for repeated measurements revealed no difference between the age groups in blood ethanol concentrations ($p = 0.47$ for age effect). For technical

reasons the blood samples were taken on two consecutive days. There was a difference in the BECs between the two days ($P = 0.000$ for the covariate, i.e. the day of blood sampling). The difference in BECs between the days was due to the different intoxication levels of the animals and thus different ethanol doses given to the animals on each day. However, the intoxication level was higher in the old animals than in the young animals ($p = 0.04$ for age effect). The intoxication level decreased in both age groups with time during the experiment ($p < 0.001$ for time effect, $p = 0.04$ for age x time interaction).

2 Dexmedetomidine in the treatment of ethanol withdrawal symptoms (I, II, IV, V)

The results (I) showed that DEX at doses 10 and 30 µg/kg s. c. diminished the severity of the ethanol withdrawal reaction as measured by the sum score of the three most specific withdrawal signs. The sum score of the three symptoms showed a statistically significant treatment effect [$F(3, 47) = 2.82$, $p = 0.05$], whereas the overall treatment effects on each single symptom did not quite reach statistical significance [rigidity: $F(3, 47) = 2.65$, $p = 0.06$; tremor: $F(3, 47) = 2.25$, $p = 0.09$; irritability: $F(3, 47) = 2.00$, $p = 0.13$].

The dose of 10 µg/kg seemed to be the most effective dose, when the withdrawal period was observed as a whole. During the second and third day of withdrawal (23–58 h after last dose of ethanol) 10 µg/kg of DEX significantly relieved the sum score of the three most specific symptoms compared with the ethanol-treated control group (mean AUC for the sum score was 22.6 in EtOH + DEX10 group and 31.6 in EtOH group, $p = 0.01$), and also relieved tremor compared with the ethanol-treated control group (in tremor rating mean AUC was 6.9 in EtOH + DEX10 group and 9.9 in EtOH group, $p = 0.01$). However, on the first day after ethanol withdrawal the dose of 30 µg/kg seemed to be more effective than 10 µg/kg.Three rats in the EtOH group and three rats in the EtOH + DEX3 group had spontaneous seizures (running fits, tonic-clonic seizures). One rat in the EtOH + DEX30 group had a short-lasting tonic-clonic seizure, 18 h after the last dose of DEX (40 h after ethanol withdrawal). There was no significant difference between the groups in the number of spontaneous seizures ($p = 0.65$).

The effect of DEX on ethanol withdrawal symptoms was compared with that of diazepam and propranolol (II). ANOVA for repeated measurements revealed a significant difference between the groups in tremor [$F(3, 21) =9.03$, $p < 0.001$)], irritability [$F(3, 21) = 10.63$, $p < 0.001$] and the sum score of the three most specific withdrawal signs [$F(3, 21) = 8.50$, $p < 0.001$]. Diazepam relieved the symptoms

significantly compared with the ethanol-treated control group. Diazepam also reduced irritability and tremor. DEX significantly relieved the ethanol withdrawal syndrome compared with the ethanol-treated control group. DEX also relieved tremor compared with the ethanol-treated control group. Propranolol had a relieving effect on tremor only. The differences in the severity of ethanol withdrawal symptoms between the treatment groups are seen in table 3.

During the 5-week intermittent ethanol exposure (IV) DEX significantly relieved the severity of the ethanol withdrawal syndrome compared with the ethanol-treated control group. The severity of the withdrawal symptoms changed with time during the experiment. When comparing the severity of the ethanol withdrawal symptoms in EtOH and EtOH+DEX groups on each week by Bonferroni-corrected t-tests, there was no significant difference between the groups during the first 4 weeks of ethanol exposure. During the last week of ethanol exposure, the severity of the ethanol withdrawal syndrome was significantly relieved in the DEX-treated group, compared with the EtOH group (see Table 4).

Table 3. The severity of the three most specific ethanol withdrawal symptoms (rigidity, tremor, irritability) and the sum score of the three symptoms in the different treatment groups after the four-day ethanol exposure.

	Rigidity	Tremor	Irritability	Sum score
EtOH	17.0 ± 1.8	21.3 ± 1.9	24.0 ± 1.4	62.3 ± 3.5
Propranolol	16.6 ± 1.8	10.6 ± 1.8 **	25.1 ± 2.4	52.3 ± 6.4
Diazepam	12.5 ± 1.1	9.8 ± 1.6 ***	11.5 ± 2.1 ***	33.8 ± 3.4 ***
DEX	14.7 ± 1.4	11.4 ± 1.9 ***	18.3 ± 2.7	44.4 ± 4.2 **

Differences between the groups were analyzed by Bonferroni-corrected *t*-tests,
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with the EtOH group.

Table 4. The ethanol withdrawal symptoms (as the sum of the three most specific symptoms) in the EtOH + DEX group compared with the EtOH group during the 5-week ethanol exposure (IV).

	Week 1	Week 2	Week 3	Week 4	Week 5
EtOH	20 ± 3.6	27 ± 5.1	36 ± 4.9	32 ± 2.5	34 ± 2.7
EtOH + DEX	12 ± 1.8	19 ± 2.8	30 ± 3.4	26 ± 3.2	19 ± 1.4
p =	0.06	0.18	0.28	0.12	< 0.01 **

The values represent AUC (mean + SEM) for the withdrawal symptoms plotted against time. ANOVA for repeated measurements $p = 0.03$ for group effect, $p < 0.01$ for time effect, $p = 0.13$ for group x time interaction. Comparison between the groups was done by Student's *t* test. ** Significantly different from the EtOH group.

3 Morphology of the adrenergic neurons and neuroprotective effects of dexmedetomidine (III, IV)

After a 12-day heavy ethanol exposure (III) the overall level of FIF intensity, reflecting the neuronal concentration of noradrenaline, was found to be higher in the SCG of the EtOH rats compared with the control or EtOH+DEX ganglia. In the group given a single dose of DEX the overall FIF intensity was comparable to, or even higher than in the EtOH SCG. The strongly fluorescent neurons also had strong TH-IR, suggesting a high rate of catecholamine synthesis in these neurons. Also a combination of intense TH-IR with weak or moderate FIF was seen in several neurons of the EtOH and EtOH+SD groups, probably reflecting an extremely high norepinephrine turnover. A large population of neurons in the EtOH group seemed to have lost both TH-IR and FIF, reflecting a loss of catecholamine synthetic activity (see Figs. 1–2 in original publication III).

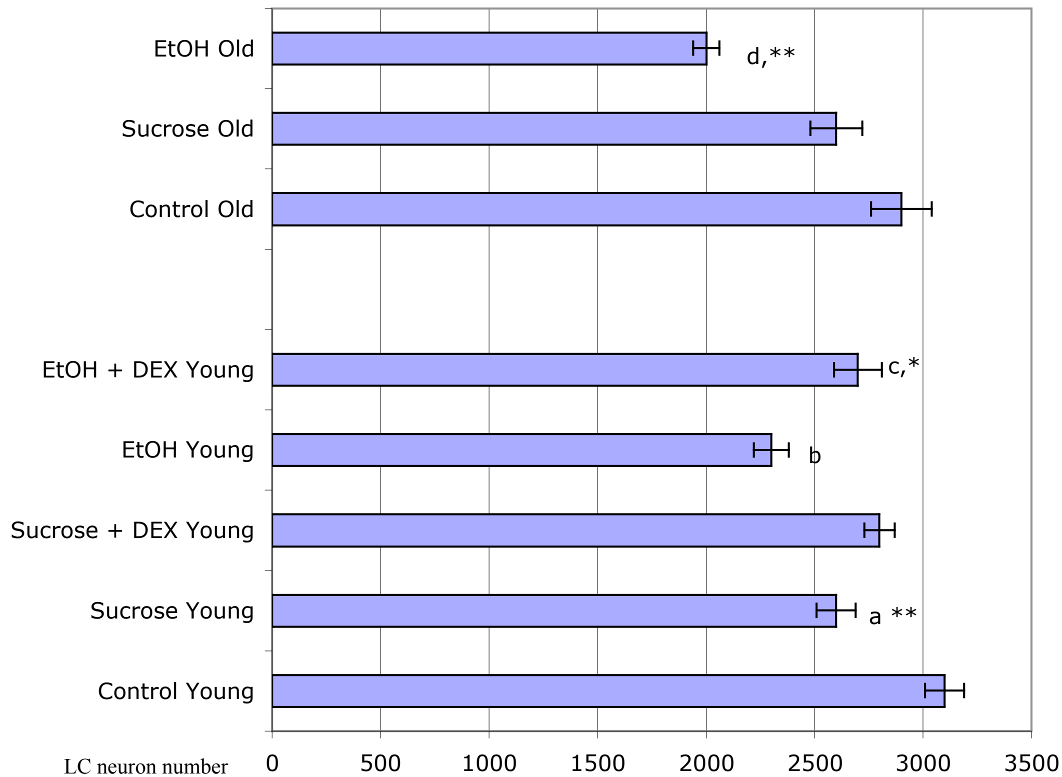
There was a significant difference between the groups in the proportion of TH-negative neurons [$F(4, 55) = 16.45$, $p < 0.001$]. The proportion of TH-negative neurons in the EtOH group (11.3%) was approximately two-fold compared with the sucrose-fed control group (6.4%). EtOH+DEX or EtOH+SD ganglia did not differ from the control ganglia (sucrose) in this respect. However, the proportion of TH-negative neurons was increased in the DEX-treated sucrose-fed group compared with the sucrose-fed control group. There was also a significant difference between the groups in the proportion of vacuolated neurons [$F(4, 55) = 12.39$, $p < 0.001$]. The proportion of vacuolated neurons was approximately seven-fold in the EtOH group compared with the sucrose-fed control group (0.7% in the EtOH group vs. 0.1% in the control group). The proportion of vacuolated neurons was significantly reduced by the chronic DEX treatment (0.2% in the EtOH+DEX group), but a single treatment with DEX had no significant effect on the number of vacuolated neurons (0.4% in the EtOH+SD group).

ANOVA revealed a significant difference between the groups in neuronal size [$F(4, 2995) = 7.80$, $p < 0.001$], as well as in neuronal density [$F(4, 55) = 4.75$, $p < 0.01$]. Neuronal packing density increased by 15% in the EtOH ganglia compared with the sucrose-fed control ganglia, partly due to the decreased neuronal size (11% decrease in neuron cross-sectional area compared with the control group). There was a tendency

towards normalization of the SCG neuronal density by the chronic or the acute treatment with DEX. In the chronically DEX-treated ethanol-fed animals the neuronal size was comparable with the sucrose-fed control ganglia. In the ethanol-treated rats given a single dose of DEX, the SCG neuronal size did not differ from the ganglia of the EtOH rats.

ANOVA showed a significant difference between the groups (IV) in the LC total neuron numbers [$F (4, 23) = 9.68$, $p < 0.01$]. There was a tendency towards lower LC neuron numbers in the EtOH group compared with the control group. However, the number of LC neurons was reduced also in the sucrose-fed control group compared with the non-treated control group, probably reflecting a stress-related loss of LC neurons in the rats subjected to repeated intragastric intubations and a rather strict food restriction (due to the pair-feeding protocol). Dexmedetomidine significantly reduced the LC neuronal loss in the ethanol-treated rats. There was a difference between the groups in the LC volume [$F (4, 23) = 4.04$, $p = 0.01$]. There was a tendency towards a reduced LC volume in the sucrose-fed control group, compared with the non-treated control group (uncorrected $p = 0.04$). However, there was no difference between the EtOH group and the sucrose-fed control group (uncorrected $p = 0.11$), or between the EtOH group and the EtOH+DEX group (uncorrected $p = 0.13$) in the LC volume. ANOVA revealed no difference between the groups in LC neuronal density [$F (4, 23) = 1.96$, $p = 0.13$]. The LC neuron numbers in the different treatment groups are seen in Fig.2.

Pearson's correlation analysis (IV) revealed a tendency towards a negative correlation between the intoxication score and the LC total neuron numbers ($r = -0.51$, $p = 0.09$), a weak positive correlation between the cumulative ethanol dose and the LC neuron numbers ($r = 0.51$, $p = 0.09$), and a negative tendency between the ethanol withdrawal score and the LC neuron numbers ($r = –0.43$, $p = 0.17$). According to analysis of covariance, the total dose of ethanol ($p = 0.34$), the intoxication level ($p = 0.26$) or the ethanol withdrawal score ($p = 0.38$) did not significantly affect the LC total neuron numbers.



Data are expressed as mean ±SEM. Differences between the groups were analyzed by Bonferroni-corrected t-tests.
*p < 0.05, ** p < 0.01, *** p < 0.001
a Significantly different from the young control group
b A tendency towards a reduced neuron number, compared with the young sucrose group
c Significantly different from the young EtOH group
d Significantly different from the old sucrose group

Figure 2. The LC neuron numbers in the different treatment groups after a 5-week intermittent ethanol exposure.

4 Effects of aging on ethanol withdrawal symptoms and ethanol-induced neuronal degeneration (V)

There was no difference between the young (3-4 months) and old (29-30 months) rats in the severity of the ethanol withdrawal symptoms during the 5-week intermittent ethanol exposure (V; ANOVA $p = 0.13$ for age effect). The severity of the symptoms increased with time during the experiment ($p < 0.001$ for time effect, $p = 0.16$ for age x time interaction). ANCOVA showed no difference between the age groups in the severity of the withdrawal syndrome, when the total dose of ethanol ($p = 0.28$ for age difference), or the intoxication level ($p = 0.99$ for age difference) was used as a covariate. However,

the level of intoxication had an influence on the severity of withdrawal symptoms ($p = 0.02$ for the covariate).

Both age and treatments had an effect on the number of LC neurons ($p = 0.02$ for age effect, $p < 0.001$ for treatment effect, $p = 0.31$ for interaction) (see Table 3). Post hoc comparisons showed that the number of LC neurons decreased in the old EtOH group compared with the old sucrose group, but there was only a tendency towards a reduced LC neuron number in the young EtOH group compared with the young sucrose group (uncorrected $p = 0.04$). There was also a tendency towards a reduced neuronal number in the old sucrose group compared with the old control group (uncorrected $p = 0.05$). ANCOVA revealed no age differences in the vulnerability to ethanol-induced neuronal degeneration when ethanol dose (g/kg) ($p = 0.99$ for group difference), intoxication level ($p = 0.88$) or withdrawal symptoms ($p = 0.79$) were used as covariates.

Both age and the treatments had an effect on LC neuronal density ($p = 0.03$ for age effect, $p < 0.001$ for treatment effect, $p = 0.10$ for interaction). The neuronal density decreased in the old EtOH group compared with the old sucrose group, but there was no significant difference between the young EtOH and the young sucrose groups. There was no difference between the groups in LC volume.

Discussion

1 Methodological considerations

1.1 Animal models and ethanol feedings

In human alcoholics several confounding variables, like inaccuracy of ethanol drinking history, abuse of other drugs, poor nutritional status, and possible contributing medical problems, make the evaluation of ethanol-induced neuronal changes difficult (Abel 1984). By using animal models many of these variables can be controlled (Cicero 1980). In addition, the lifetime of the most often used animal model, rat, is short enough to enable the investigation at the effects of ethanol exposure throughout the lifespan. However, the results of animal research cannot be directly generalized to other species, including humans. One difference in rats compared with humans is that the rats do not develop serious liver damage including cirrhosis even with high doses of ethanol (Bosma et al. 1988).

The intragastric feeding method applied in the present series of experiments allows an administration of high and accurate doses of ethanol by the natural, enteral route, and the pair-feeding of the control animals can easily be carried out by isocaloric sucrose intubations (Cicero 1980). The intragastric feeding technique has been previously used in ethanol studies (Ahtee and Svartström-Frazer 1975, Majchrowicz 1975, Jaatinen and Hervonen 1994). The cessation of ethanol feedings, after the shorter 4-day or during the longer 12-day ethanol exposure, produced clear signs of withdrawal, indicating physical dependence on ethanol.

The ethanol feedings consisted of 25% (w/v) ethanol and 5% (w/v) sucrose. Sucrose was added to the ethanol solution to make the solution taste sweet, as well as to avoid the hypoglycemia which may ensure from a severe ethanol intoxication. The addition of sucrose to the solution has been reported to have no influence on the absorption or metabolism of ethanol, and the BEC reached has been shown to be dependent only on the amount of ethanol consumed (Czachowski et al. 1999). Immediately before each ethanol feeding the intoxication of the animals was evaluated by using the standardized six-level intoxication scale (Majchrowicz 1975). To avoid deaths in the course of the experiments, severely intoxicated animals were given reduced doses of ethanol; severely atactic animals received 1–2 g/kg, and rats having lost their righting reflex received 0–1 g/kg.

However, some disadvantages have been reported to be related to the gastric intubations of ethanol. The most severely intoxicated animals did not eat properly, and the organ pathology may thus be partly due to nutritional deficiencies. Althought the pair-feeding regimen was used to ensure that the amount of food eaten was the same in the control and the experimental animals, chronic ethanol exposure may interfere with the absorption of nutrients (Ritzman and Tabakoff 1984), leading to impaired nutrition of the ethanol-exposed animals compared with the control animals. The difference in nutrition was, however, probably negligible during the 4- or 12-day ethanol exposures. During the 5-week intermittent ethanol exposure, the period of poor crow consumption was longer. However, also during that experiment the ethanol exposure period lasted only 4 days continuously, followed by a 3-day withdrawal period. The effect of stress related to intragastric intubations was taken into consideration by including both a sucrose-intubated, pair-fed control group and a nontreated control group without any intragastric intubations in the five-week intermittent exposure. More discussion about stress and stress-induced neuropathology is seen in chapter 4 on page 56.

When the effects of ethanol exposure on the young and the old rats were compared (V), the old rats were given smaller doses of ethanol compared with the young rats during the whole five-week experiment. The ethanol administered is distributed mainly into the body water compartments, including the blood and the cerebrospinal fluid (Harger and Hulpieu 1956). The aging process has been shown to be accompanied by a decrease in the relative body water content and an increase in the relative body fat content (Edelman et al. 1952, Fryer 1962), leading to higher blood ethanol levels and higher intoxication levels in aged subjects compared with younger ones, when the same doses of ethanol (g ethanol /kg body weight) are administered to each age group (Wiberg et al. 1970, Vestal et al. 1977, York 1982). There may also be a difference in the ethanol metabolism between the age groups. Studies have reported a decrease in the rate of ethanol metabolism, e.g. a decrease in the activity of the ethanol- and acetaldehyde-metabolizing enzyme activities in the liver (Fernandez et al. 1988, Rikans et al. 1990, Seitz et al. 1992). Also a reduction of the first pass metabolism and gastric alcohol dehydrogenase activity in the elderly may have a role in the increased vulnerability to the effects of ethanol (Pozzato et al. 1995, Pedrosa et al. 1996). In the present study, the administration of ethanol to the different age groups was successful, because there was no statistical difference in the blood ethanol concentrations between the age groups. However, despite the smaller doses of ethanol in the old rats and the similar blood ethanol concentrations, the intoxication level in the old rats was higher compared with the young rats. In other words, the vulnerability of the CNS to the intoxicating effects of ethanol seemed to increase in the old rats compared with the young rats.

1.2 Measurement of ethanol withdrawal symptoms

The four withdrawal symptoms (rigidity, tremor, irritability, hypoactivity) registered in the present study have been shown to be specific and valid markers of the non-convulsive component of the withdrawal reaction in the rat (Clemmesen and Hemmingsen 1984). Tremor and irritability also reflect the increased sympathetic activity during ethanol withdrawal (Linnoila 1987, Nutt and Glue 1990). When the effects of DEX and diazepam on the severity of the ethanol withdrawal reaction were analyzed (I, II, IV), the sum score of rigidity, tremor and irritability was used. Both DEX (Aho et al. 1992, Scheinin et al. 1993) and diazepam (Baldessarini 1985, Litten and Allen 1991) have been shown to have sedative effects, which diminishes the specificity of hypoactivity in the measurement of the severity of withdrawal reaction. The sum score of the three most specific symptoms has been shown to represent a reliable measure of the withdrawal reaction (Clemmesen et al. 1988). The rats were also observed for spontaneous or handling-induced epileptiformic seizures (tonic-clonic seizures or running fits).

The aim of the study was to measure the behavioral component of ethanol withdrawal reaction and the morphological alterations of adrenergic SCG and LC neurons. Blood pressure and heart rate and hyperthermia might have been other interesting withdrawal symptoms to measure, reflecting also overactivity of the sympathetic nervous system. However, in this study these parametres were not measured, because the measurements of blood pressure and pulse from the rats suffering from ethanol withdrawal symptoms would have caused additional stress to the animals. On the other hand, increase in blood pressure during the ethanol withdrawal phase has been widely documented previously, and according to previous studies DEX has been shown to have hemodynamic stabilizing properties. Thus, measurement of blood pressure would probably not have brought any important new information in the present experiments.

1.3 Morphometric methods

The study on the peripheral adrenergic neurons (III) was based on a previously used fluorescence microscopic morphometric method (Jaatinen et al. 1993, Jaatinen and Hervonen 1994). The areal density of SCG neurons was measured with the aid of an image analysis equipment. The total number of SCG neurons was not measured, as it was improbable that any significant neuronal loss would have taken place during the 12-day ethanol exposure. Because the neuron counts were not corrected according to

the neuronal size in this study, the proportion of large neurons (e.g. vacuolated neurons) may be slightly overestimated. The mean size of the vacuolated neurons, however, was equal in all groups. Thus the increase in the proportion of vacuolated neurons in the ethanol-exposed rats seems to reflect a true increase in the number, not an increase in their relative size. The size distribution of TH-positive and TH-negative neurons was also equal in all groups, and the differences in the proportions of TH-negative and TH–positive neurons can be considered to be real.

In the later morphometric studies (IV, V), unbiased stereological methods were used to analyse the locus coeruleus after the five-week intermittent ethanol exposure. The disector method was developed to eliminate the potential sources of systematic errors inherent in previous techniques (Sterio 1984, Gundersen and Jensen 1987, Gundersen et al. 1988a, 1988b; West and Gundersen 1990). In previously used techniques, already during the sectioning of the tissue for microscopy, there is a bias introduced into the selection of the neurons that appear in the sections. What one observes in the sections are not entire neurons, but profiles of neurons. Profile number depends not only on the actual number of particles, but also on the size, shape, orientation and many other characteristics of the particles being counted (Coggeshall 1992, West 1993). For instance, the profiles of large objects have a higher probability of appearing on a section than those of smaller objects (West 1994).

In this study the total number of LC neurons was estimated by the disector method (Gundersen et al. 1988a, 1988b; West 1993). In stereological terms the disector is a three-dimensional probe that has a set of rules for counting or sampling objects with equal probability in three-dimensional space. It results in unbiased counts because it provides a means of counting the objects without making any assumptions about the size, shape or orientation of the objects. When this method is used, the investigator must be able to identify the sectional profiles that belong to the same object and there must be at least one section in which it is possible to identify each object. However, lost caps, over projection and varying investigator definitions of the presence or absence of a profile in a section do not affect the counts (West 1993).

2 Dexmedetomidine in the treatment of ethanol withdrawal symptoms

Repeated administration of DEX seemed to be necessary because of the short duration of the drug effects; the effect of DEX on withdrawal symptoms could be seen only 1–4 hours after drug administration. In addition, a decreasing dosage of DEX during the withdrawal period seemed to be most effective in releaving the withdrawal symptoms.

One possible explanation for this may be an alteration of kinetics of α_2-adrenergic receptors during the first few days after prolonged ethanol exposure; the initial subsensitivity of α_2-adrenergic receptors may pass off later in the withdrawal phase, diminishing the optimum dose of α_2-agonists. Another explanation may be that the repeated administration of high doses of DEX would lead to accumulation of the medicine beyond the optimum range of concentration.

In previous studies, i.p. or p.o. administration of DEX has been used to study the acute interactions of ethanol and dex (Idänpään-Heikkilä et al. 1995, Seppälä et al. 1994). Based on the previous data, and due to the simplicity of p.o. administration in connection with the ethanol feedings, p.o. administration was chosen to study the effects of chronic and acute DEX treatment during the 12-day ethanol exposure (III). However, DEX has been shown to have poor bioavailability and a short elimination half-life (Scheinin et al. 1992b). Thus, in later studies (I, II, IV, V) repeated s.c. dosage was used to avoid the first pass metabolism of DEX and to ensure an effective concentration of the drug throughout the withdrawal period.

The results of the present study showed that DEX significantly relieved the ethanol withdrawal syndrome, when measured as the sum score of the three most specific symptoms. Previously other α_2-adrenergic agonists, including clonidine and lofexidine, have been used in the treatment of the ethanol withdrawal syndrome (Manhem et al. 1985, Cushman and Sowers 1989, Adinoff 1994, Fahlke et al. 2000). Alpha$_2$-adrenergic agonists have also been reported to have antiepileptic properties (Papanicolau et al. 1982, Shouse et al. 1996). DEX has previously been reported to attenuate kainic acid-induced convulsions and subsequent neuronal damage in rat hippocampus (Halonen et al. 1995). DEX has also been reported to increase the threshold for cocaine-induced seizures (Whittington et al. 2002). In the present study, none of the rats in the EtOH+DEX10 group and only one of the rats in the EtOH+DEX30 group had an epileptiformic seizure, whereas three rats in the EtOH group and three rats in the EtOH+DEX3 group had convulsions. However, the number of seizures observed was too small and therefore no conclusions can be drawn concerning the effects of DEX on ethanol withdrawal-induced convulsions.

When measured as the sum score of the three most specific symptoms, the effects of DEX seemed to be comparable to the effects of diazepam, the clinically most often used medicine in the treatment of the ethanol withdrawal syndrome. DEX seemed to be the most effective drug during the first 12 h of the withdrawal period, but after the first 12 h, diazepam seemed to be the most effective drug. This may be due to the different half-lives of these drugs. DEX has been shown to have a very short elimination half-life, ranging from 1,5–3 h after i.v. administration to 5,6 h after transdermal dosing in human (Scheinin et al. 1992b, Kivistö et al. 1994, Anttila et al. 2003), whereas

diazepam has been shown to be quite long-acting (half-life about 33 h in humans). The concentration of diazepam seemed to accumulate during the course of the experiment, but no effect of DEX could be seen more than 24 h after the onset of the withdrawal reaction, i.e., 4 h after the last dose of DEX.

The main disadvantage of the use of benzodiazepines in ethanol withdrawal has been the development of drug dependence (Schuster and Humphries 1981, O'brien 2005). In previous studies, α_2-adrenergic agonists have not caused any dependence problems (Manhem et al. 1985), and at the doses used in clinical anesthesiology DEX has been shown to be safe and well-tolerated (Aho et al. 1992, Scheinin et al. 1993). DEX has been shown to provide sedation, analgesia and anxiolysis during anesthesia (Aho et al. 1992, Belleville et al. 1992, Bloor et al. 1992). DEX has also been shown to improve hemodynamic stability during surgery and to reduce anesthetic requirements (Bloor et al. 1992, Scheinin et al. 1993).

During the 5-week intermittent ethanol exposure DEX significantly relieved the severity of the ethanol withdrawal reaction. The effect of DEX on ethanol withdrawal symptoms seemed to remain unchanged or even improve during repeated withdrawal periods. This is an important property for a medicine used in the treatment of ethanol withdrawal, because human alcoholics often undergo repeated withdrawal periods, and the severity of the withdrawal reaction has been reported to get worse during repeated withdrawal periods. In conclusion, DEX is a new interesting agent for in-patient withdrawal treatment because of its hemodynamic-stabilizing and sedative properties, and especially for the lack of dependence problems.

3 Alterations of adrenergic neurons induced by ethanol and intragastric intubations

In the study on peripheral sympathetic neurons (III), the neuronal density of the SCG was significantly increased in the rats exposed to ethanol for 12 days compared with the control rats, which was mainly due to a significant shrinkage of neurons in the EtOH group. The overall level of SCG FIF-intensity was higher in the ethanol-exposed animals compared with the control animals, reflecting higher catecholamine concentration. Also the proportion of vacuolated neurons and TH-negative neurons was increased in the SCG of the ethanol-exposed rats.

Vacuolated neurons have been reported to occur normally in several parts of rat peripheral nervous system, including the superior cervical, coeliac, and pelvic sympathetic ganglia (Nouhouayi et al. 1979, Partanen et al. 1979). The appearance of vacuolated neurons in sympathetic ganglia has been regarded as a sign of

neurosecretion or degeneration (Pawlikowski 1962, Partanen et al. 1979). Previously, repeated immobilization stress, which increases the activity of the sympathetic nervous system, has been shown to induce extensive vacuolation in rat SCG (Mikulajova et al. 1989). In this study, the proportion of vacuolated neurons in the EtOH group was almost seven-fold compared with the sucrose-fed control group. Previous studies have shown that most of the vacuolated neurons disappear during the first few days after the ethanol exposure has been finished (Jaatinen et al. 1993). However, it is not clear which proportion of the vacuolated neurons degenerate and die, and which of them are capable of retaining normal function and morphology.

The total number of locus coeruleus neurons decreased by 11% in rats exposed to ethanol intermittently for 5 weeks compared with the sucrose-fed control rats, probably reflecting an irreversible neuron loss (IV). This finding is consistent with previous studies reporting a loss of LC neurons in rats after lifelong ethanol exposure (Wei et al. 1997, Rintala et al. 1998), as well as in human alcoholics (Arango et al. 1994, 1996). However, some other studies found no change in the number of LC neurons between chronic alcoholics and age-matched controls (Halliday et al. 1992, Baker et al. 1994). In addition to the uncontrolled life history variables inherent in human studies, the different morphometric methods used in these studies may have contributed to the discrepant results.

Interestingly, in the present study the LC neuron numbers were found to decrease also in the sucrose-fed rats compared with the non-treated control rats (IV, V). One explanation for this may be the stress caused by the repeated intragastric intubations and by the dietary restriction. The LC has a central role in mediating the stress response (Stanford 1995, McEwen and Magariños 1997, Kawahara et al. 2000). Many kinds of stressors, including sensory and somatosensory stimuli, interoceptive cues like hypotension and hypoglycemia, and complex environmental stimuli have been shown to increase the firing rate of the noradrenergic neurons in the LC (Stanford 1995). Repeated psychosocial stress has been reported to cause atrophy of the apical dendrites in pyramidal neurons of the hippocampus, accompanied by specific cognitive deficits in spatial learning and memory (Luine et al. 1996, McEwen and Magariños 1997, Sousa et al. 2000). However, no neuronal loss in the CNS related to chronic stress has been reported in the literature. Nutritional deficiencies may have contributed to the decreased neuronal numbers in the sucrose-treated animals, because their intake of chow was about half of the amount eaten by the non-treated control animals.

Due to the pair-feeding regimen (IV), there was no difference between the sucrose- and ethanol-treated animals in the amount of food eaten, but the amount of food consumption was about two-fold in the non-treated control animals compared with the sucrose- and ethanol-treated animals. There was also a larger weight increase in the

non-treated control group (+63 ± 3 g) compared with the sucrose-fed control group (+6 ± 10 g). In the ethanol-treated group there was a weight decrease (-28 ± 8 g). The caloric restriction itself has been shown to delay both aging and the number of life-shortening diseases in laboratory animals, by reducing the age-related accumulation of irreversible molecular damage including reduction of oxidative damage to proteins, lipids and DNA (Gredilla and Barja 2005, Sharma and Kaur 2005, Spindler 2005). To our knowledge, caloric restriction has not been shown to induce neuron loss. Crews et al. (2001) even reported that a nutritionally complete diet could increase ethanol-induced brain damage during binge ethanol treatment.

4 Neuroprotective effects of dexmedetomidine

The results of the present study showed that DEX has neuroprotective effects against ethanol-induced neuronal degeneration in both the peripheral and the central adrenergic neurons. Previously some attemps have been made to find pharmacological agents against ethanol-induced neuronal degeneration. Gangliosides have been shown to have neuroprotective effects against a variety of neuronal insults, like mechanical injury, drug toxicity or hypoxia (Hungund and Mahadik 1993) and cerebral gangliosides have been shown to improve the sensory deficits in the alcoholic neuropathy (Mamoli et al. 1980). Membrane lipid changes are associated with these injuries and gangliosides have been shown to restore the membrane fatty acids and facilitate the recovery process. Gangliosides also have an important role in neuronal differentation and development (Hungund and Mahadik 1993). Vitamin E supplementation has been shown to decrease brain lipofuscin content after a 5-month ethanol feeding in mice, but it failed to prevent the ethanol-induced learning deficit (Freund 1979). A recent study by Lamarche et al. (2004) reported that vitamin E could protect neurons against ethanol-induced oxidative stress, notably by contributing to maintaining the intracellular glutathione levels.

The nonpsychoactive cannabinoid cannabidiol has been shown to protect against hippocampal and entorhinal cortical neurodegeneration in a rat binge ethanol model, which was also attributed to reduced oxidative stress (Hamelink et al. 2005). In vitro studies suggest that the excitotoxicity produced by ethanol can be blocked by acamprosate (De Witte et al. 2005), but to our knowledge no neuroprotective effect of acamprosate against ethanol-induced degeneration in vivo has been reported. The diuretic furosemide has been reported to have neuroprotective effects against ethanol-induced cerebrocortical damage in rats by preventing brain edema and electrolyte disturbances in vivo (Collins et al. 1998).

In the peripheral sympathetic neurons of rat, the ethanol-induced overactivity and morpho-functional degeneration (loss of TH-IR, vacuolation, shrinkage) was prevented by chronic DEX-treatment. The EtOH+DEX ganglia, which exhibited normal levels of the catecholamine markers, also showed neuronal vacuolation comparable to the sucrose-fed control ganglia. This may indicate that increased sympathetic activity during chronic ethanol exposure has a central role in the generation of vacuolated neurons. The fact that a single dose of DEX also normalized the proportion of TH-negative neurons may indicate that the loss of TH-IR represents a reversible alteration of the functional state of neurons, not an irreversible damage. Neuronal shrinkage, which was seen in the EtOH-group, was significantly prevented by chronic DEX treatment, but not by a single dose of DEX. In neuronal density, both acute and chronic DEX treatment caused a tendency towards normalization compared with the EtOH group. The difference in neuronal density between the EtOH and the DEX-treated groups may reflect changes in the nerve fibers and/or interstitial elements. A better water balance in the DEX-treated groups compared with the EtOH group may also contribute to this difference.

DEX significantly reduced ethanol-induced neuronal loss in the rat LC during the five-week intermittent ethanol exposure. Previously DEX has been shown to improve neurologic outcome from incomplete forebrain ischemia in the rat (Hoffman et al. 1991). At a dose that reduces the requirements of other anesthetics by 50%, DEX has been reported to be effective in protecting against focal cerebral ischemia in rabbits (Maier et al.1993). DEX has also been shown to have neuroprotective effects against delayed neuronal damage caused by transient global ischemia in gerbils (Kuhmonen et al. 1997). A study by Jolkkonen et al. 1999 showed that DEX significantly reduced the total ischemic volume in rat focal cerebral ischemia. However, DEX did not affect neuronal damage following severe forebrain ischemia in rats (Karlsson et al. 1995). DEX has also been reported to protect against kainic acid induced convulsions and neuronal damage in the rat hippocampus (Halonen et al. 1995).

The pathophysiological mechanisms behind DEX-induced neuroprotective effects are not clear. In this study the neuroprotective effects of DEX seemed not to be due to an altered metabolism of ethanol, as the blood ethanol concentrations were at the same level in all ethanol-exposed groups. Neither have any pharmacokinetic interactions between ethanol and DEX been reported in previous studies (Seppälä et al. 1994, Idänpään-Heikkilä et al. 1995). In addition to the direct neurotoxicity of ethanol, its primary oxidative metabolite, acetaldehyde, has been shown to alter the metabolism of catecholamines and to form potentially neurotoxic compounds like tetra-hydroisoquinolines (Brien and Loomis 1983, Fa and Dryhurst 1991). Ethanol-induced overactivity of the catecholaminergic neurons has been supposed to enhance the auto-

oxidation of catecholamines, producing oxygen free radicals and semiquinones, which may lead to neuronal death (Graham 1978). The reduced noradrenergic activity during DEX-treatment may diminish the formation of these neurotoxic compounds.

The reduced noradrenergic activity during DEX treatment may affect the function of other neurotransmitter systems which are known to contribute to the ethanol withdrawal syndrome and neuronal degeneration. For instance, noradrenergic neurons have been reported to innervate CRF-ergic neurons in rat hypothalamus (Kitazawa et al. 1987). Noradrenaline and CRF have been reported to stimulate each other's release (Al-Damluji et al. 1987, Emoto et al. 1993, Schulz and Lehnert 1996). Overactivity of the hypothalamic-pituitary-adrenal axis has been reported during chronic ethanol exposure and ethanol withdrawal (Spencer and McEwen 1990, Costa et al. 1996, Madeira and Paula-Barbosa 1999, Hundt et al. 2001). DEX-induced reduction of the noradrenergic activity may lead to a reduction in the function of the CRF-ergic neurons, thus relieving the severity of the withdrawal reaction. Increased expression of CRF has been associated with neuronal degeneration in response to an ischemic insult, e.g., in gerbil hippocampus and neocortex (Choi et al. 2001, Park et al. 2002).

Noradrenaline has also been reported to mediate the function of magnesium, which is an endogenous inhibitory modulator of the NMDA function (Whyte et al. 1987). NMDA-receptor -mediated mechanisms have, in turn, been shown to play a central role in the generation of ethanol withdrawal symptoms and excitotoxic neuronal damage. Glutamate has also been shown to stimulate noradrenaline release in several areas of rat brain (Fink et al. 1989, Wang et al. 1992).

Several studies have suggested that withdrawal from chronic ethanol exposure may cause greater loss of CNS neurons than ethanol exposure itself (Phillips and Cragg 1984, Lundqvist et al. 1995, Riikonen et al. 1999a, Obernier et al. 2002). In rat hippocampus, intermittent ethanol exposure has been shown to cause more severe loss of neurons and synapses, and to enhance lipofuscin accumulation compared to continuous ethanol exposure (Lundqvist et al. 1995, Lundqvist 1997). In rat SCG, intermittent ethanol exposure for 5,5 months induced a 28% loss of neurons, whereas no significant neuron loss was seen after a continuous ethanol exposure of the same duration (Riikonen et al. 1999a). In summary, repeated ethanol withdrawal periods seem to be central in the neuropathology of chronic ethanol exposure. Normalization of the increased noradrenergic activity in ethanol withdrawal by DEX relieves the withdrawal symptoms as well as protects the noradrenergic neurons against ethanol withdrawal-induced degeneration.

5 The effects of aging on ethanol tolerance, withdrawal severity and neuronal degeneration

In previous studies, aging has been reported to slow down the development of tolerance to ethanol (Mayfield et al. 1992, York and Chan 1994, Spencer and McEwen 1997). In this study there was no difference between the age groups in the tolerance to ethanol when measured as the BEC/intoxication ratio at the end of the experiment. However, when measured as the intoxication score/ethanol dose ratio, the development of tolerance of the old animals seemed to be slower compared with the young animals, reaching the same tolerance level after 3 weeks of ethanol exposure. Previously, studies about the effect of age on the severity of ethanol withdrawal symptoms have been contradictory (Liskow et al. 1989, Brower et al. 1994, Kasahara et al. 1996, Kraemer et al. 1997, Wojnar et al. 2001). According to the results of this study, there was no significant difference between the age groups in the severity of the ethanol withdrawal reaction, when the doses of ethanol were individually adjusted according to the level of intoxication.

In previous studies, aging has been reported to increase vulnerability to ethanol-induced damage in rat peripheral (Jaatinen and Hervonen 1994) nervous system. The results of the present study showed that ethanol exposure significantly reduced LC neuronal numbers in the old rats, but in the young EtOH group there was only a tendency towards reduced LC neuronal numbers, compared with the respective sucrose fed group. ANCOVA, however, showed no age differences in the sensitivity to ethanol-induced neuronal degeneration when ethanol dose, intoxication score or withdrawal symptoms were used as a covariate, indicating that the difference in the LC neuronal loss between the age groups may be due to the differences in the intoxication levels.

Summary and conclusions

The main findings and conclusions of the study are:

1) Dexmedetomidine significantly relieved the ethanol withdrawal syndrome in the rat, when measured as the sum score of the three most specific symptoms (rigidity, tremor, irritability). The efficiency of DEX in the treatment of ethanol withdrawal symptoms seemed to be comparable to the effect of diazepam, the most often used medication in the treatment of ethanol withdrawal in the clinical setting. During the 5-week intermittent ethanol exposure, the effect of DEX on ethanol withdrawal symptoms seemed to remain unchanged or even improve during repeated withdrawal periods.

2) Dexmedetomidine was shown to have neuroprotective effects against ethanol-induced degeneration in both peripheral and central adrenergic neurons. Chronic treatment with DEX prevented ethanol-induced overactivity and morphological and functional degeneration (shrinkage, loss of TH-IR, vacuolization) of rat peripheral sympathetic neurons. A single dose of DEX offered only marginal neuroprotective effects. During the 5-week intermittent ethanol exposure DEX was found to reduce the ethanol-induced loss of LC neurons.

3) Aging did not significantly affect the severity of the ethanol withdrawal reaction, when the doses of ethanol were individually adjusted according to the level of intoxication. At the beginning of the experiment the old rats were more inebriate than the young rats, but during the 5-week experiment they developed a tolerance equal to the young rats. The LC neurons of the ethanol-exposed rats, especially in the old EtOH group, were smaller than in the control rats and the typical rostro-caudal orientation of neurons was disturbed. There was no difference between the non-treated young and old control groups in the LC total neuron numbers. The LC neuron numbers and LC neuronal density in the old ethanol-exposed animals were significantly reduced compared with the sucrose-fed control animals, but in the young animals, the ethanol-induced neuron loss did not quite reach statistical significance. According to the ANCOVA, the difference in the ethanol-induced LC neuronal loss between the age groups may be due to differences in the level of intoxication.

In conclusion, dexmedetomidine seems to be an interesting new alternative in the treatment of the ethanol withdrawal syndrome, especially with regard to its potential neuroprotective effects. However, clinical studies should be conducted to verify these experimental results of DEX in the treatment of ethanol withdrawal symptoms. Further studies are also needed to illuminate the question whether DEX has neuroprotective effects against ethanol-induced degeneration in other neuronal populations than the noradrenergic ones. Aging does not seem to significantly affect the severity of ethanol withdrawal symptoms or ethanol-induced loss of central adrenergic neurons when a similar level of intoxication is obtained in each age group.

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Dexmedetomidine, Diazepam, and Propranolol in the Treatment of Ethanol Withdrawal Symptoms in the Rat

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In this study, the effects of dexmedetomidine, a selective α_2-adrenoceptor agonist, on ethanol withdrawal symptoms, were compared with those of diazepam and propranolol. The rats were given highly intoxicating doses of ethanol for 4 days. After the intoxication period, rats were divided into four equal groups: a dexmedetomidine-treated group (30 μg/kg, sc), a diazepam-treated group (2 mg/kg, sc), a propranolol-treated group (5 mg/kg, sc), and a control group with no medication. Medication was given in the withdrawal phase—2, 8, 14, and 20 hr after the onset of the withdrawal symptoms. The severity of the ethanol withdrawal symptoms (rigidity, tremor, irritability, and hypoactivity) was observed up to 33 hr after the onset of the ethanol withdrawal symptoms. Both dexmedetomidine and diazepam significantly relieved tremor compared with the control group. Diazepam reduced irritability significantly, compared with the control group. When measured as the sum score of the three most specific withdrawal signs (rigidity, tremor, and irritability), dexmedetomidine and diazepam significantly relieved the ethanol withdrawal reaction. Propranolol attenuated tremor, but was inefficient against other withdrawal symptoms. Dexmedetomidine may thus represent a new effective drug in the treatment of the ethanol withdrawal syndrome.

Key Words: Dexmedetomidine, Diazepam, Ethanol Withdrawal, Propranolol.

ETHANOL WITHDRAWAL symptoms result from disturbances of several neuronal systems, including noradrenergic hyperactivity and GABA/benzodiazepine receptor alteration.[1–4] The withdrawal reaction is characterized by increased anxiety, tremor, muscular tension, sweating, increased blood pressure, and heart rate, and in some cases by epileptiformic seizures.[3,5]

Benzodiazepines, which mainly act on the GABA/benzodiazepine receptors, have been the most widely used agents to alleviate the ethanol withdrawal reaction.[6] Beyond acting on the specific GABA/benzodiazepine receptors, benzodiazepines also act on both the noradrenergic system and the hypothalamic-pituitary-adrenal axis.[6] β-Adrenergic blockers, like atenolol and propranolol, have also been used in the treatment of ethanol withdrawal symptoms.[6–8] During the period of withdrawal, vital signs like blood pressure, heart rate, and body temperature have been shown to return to normal more rapidly with atenolol treatment.[7] α_2-Adrenoceptor agonists, like clonidine and lofexidine, have also been shown to be able to relieve some components of withdrawal syndrome.[6,9–12]

Dexmedetomidine is a highly potent and selective α_2-adrenoceptor agonist,[13,14] with significant sedative, analgesic, and hemodynamic effects.[15–17] We have previously shown[18] a slight suppression of the ethanol withdrawal reaction in rat by perorally administered dexmedetomidine (100 μg/kg 9 and 15 hr after ethanol withdrawal). We have also investigated the dose–response relations of subcutaneously administered dexmedetomidine (3, 10, and 30 μg/kg) in the treatment of ethanol withdrawal symptoms. Dexmedetomidine at doses of 10 and 30 μg/kg significantly diminished the severity of ethanol withdrawal reaction, as measured by the sum score of the three most specific signs (rigidity, tremor, and irritability).[19] In this study, the effects of dexmedetomidine on ethanol withdrawal symptoms were compared with drugs presently used in the treatment of ethanol withdrawal syndrome.

METHODS

Animals and Treatment

Thirty 3- to 4-month-old male Wistar rats were used in the study. Rats were housed individually under standard conditions (+22 ± 1°C, 12-hr light/dark cycle, with lights on between 8 AM and 8 PM). Animals were carefully habituated to handling before the experiment was started.

Rats were kept in a state of continuous ethanol intoxication for 4 days by intragastric feedings of 25% ethanol +5% sucrose every 6 hr for 1 day, then every 8 hr for 3 days. Immediately before each ethanol feeding, the severity of intoxication of each rat was evaluated by using a standardized, six-level intoxication scale.[20–25] The dose of ethanol was individually adjusted according to the level of intoxication.

After the 4-day intoxication period, rats were divided into four groups: a dexmedetomidine-treated group, a diazepam-treated group, a propranolol-treated group, and a control group with placebo medication in the withdrawal phase. The initial weights and the total score of the intoxication ratings were similar in each group. Also, cumulative ethanol doses were similar in each group [34 g/kg (SE: 1 g/kg) in the control group, 35 g/kg (SE: 1 g/kg) both in the propranolol group and the diazepam group, and 36 g/kg (SE: 2 g/kg) in the dexmedetomidine group; $p = 0.80$].

Drugs were given to the treatment groups by subcutaneous injections 2, 8, 14, and 20 hr after the time point, when the first withdrawal signs could be seen. In most of the animals, the first ethanol withdrawal symptoms could be seen 8 to 11 hr after the last dose of ethanol. Correspondingly, the control animals were given subcutaneous injections of the vehicle (Intralipid®). The rating of the withdrawal symptoms was repeated every 3 hr, up to 33 hr after the onset of the first symptoms and was done blind

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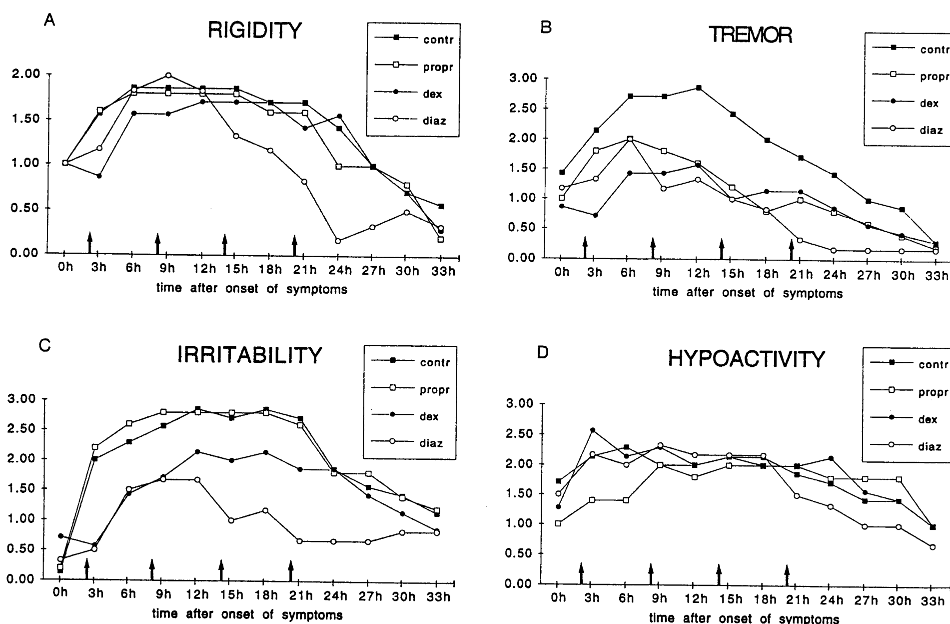

Fig. 1. Time course of ethanol withdrawal reaction in terms of individual symptoms: (A) rigidity, (B) tremor, (C) irritability, and (D) hypoactivity. Propranolol (propr; 5 mg/kg, sc), dexmedetomidine (dex; 30 μg/kg, sc), and diazepam (diaz; 2 mg/kg, sc) were given to the treatment groups 2, 8, 14, and 20 hr after the onset of ethanol withdrawal symptoms. Both dexmedetomidine and diazepam reduced tremor significantly, compared with the control (contr) group (B). Also, propranolol relieved tremor, but was inefficient against other withdrawal symptoms. Diazepam reduced irritability significantly, compared with the control group (C).

to the treatments. The withdrawal reaction was assessed by using a standardized four-item scale.[20–24,26] The four items used (rigidity, tremor, irritability, and hypoactivity) have been shown to be the most specific markers of ethanol withdrawal reaction in the rat.[20,22,26] Furthermore, the animals were observed for possible spontaneous motor seizures.

In the previous studies, diazepam at the dose of 5 mg/kg ip, and at doses from 2 to 15 mg/kg im, has been shown to be effective in the treatment of ethanol withdrawal syndrome in the rat.[27,28] Propranolol has been used in the treatment of ethanol withdrawal symptoms in the rat (e.g., at doses of 2 mg/kg, iv and 20 mg/kg, ip.[23,28]

For this study, the optimal dose of propranolol and diazepam was determined in an open dose-finding study (with doses 2, 5, and 10 mg/kg, sc for propranolol; and 1, 2, 3, and 5 mg/kg, sc, for diazepam). The aim was to find for each drug a dose that would efficiently alleviate ethanol withdrawal symptoms, without causing severe sedation or ataxia. The optimum doses by these criteria were 5 mg/kg for propranolol and 2 mg/kg for diazepam. We have also previously studied the dose–response relations of subcutaneously administered dexmedetomidine in the treatment of ethanol withdrawal symptoms.[19] In that study, 30 μg/kg was the most effective dose during the first day of withdrawal and was chosen to the optimal dose.

Statistical Methods

Data are expressed as mean ± SEM. Area under the curve (AUC) of the individual withdrawal symptoms and sum scores, plotted against time, was used as a measure of the overall severity of the withdrawal reaction. One-way ANOVA was used to analyze overall treatment effects (AUC differences). The comparison of each treatment group with the control group was done by Bonferroni-corrected t tests. A probability of <0.05 ($p < 0.0167$, when three parallel t tests were made) was considered statistically significant.

RESULTS

There was no difference between the groups in weight loss during the 4-day intoxication period [38 g (SE: 3 g) in the control group, 38 g (SE: 3 g) in the propranolol group, 41 g (SE: 2 g) in the diazepam group, and 34 g (SE: 3 g) in the dexmedetomidine group; $p = 0.56$]. During the 2-day withdrawal period, there was a significant difference in weight loss between the groups ($p = 0.002$). The weight loss was 19 g (SE: 3 g) in the control group, 17 g (SE: 5 g) in the propranolol group, and 17 g (SE: 2 g) in the dexmedetomidine group, whereas there was a weight increase of 2 g (SE: 4 g) in the diazepam group.

There was no difference between the groups in the amount of food eaten during the 4-day intoxication period [36 g (SE: 3 g) in the control group, 37 g (SE: 2 g) in the propranolol group, 39 g (SE: 1 g) in the diazepam group, and 40 g (SE: 2 g) in the dexmedetomidine group; $p = 0.37$]. During the 2-day withdrawal period, the rats in the diazepam-treated group ate somewhat more than the rats in the other groups [2 g (SE: 1 g) in the control group, 2 g (SE: 2 g) in the propranolol group, 6 g (SE: 2 g) in the diazepam group, and 3 g (SE: 1 g) in the dexmedetomidine group; $p = 0.097$].

Four rats died during the 4-day ethanol exposure and, in each case, the apparent cause of death was ethanol intoxication. One rat in the propranolol-treated group died during the withdrawal phase. None of the rats died in the other groups. No gross pathology was found in the autopsy. Thus, 25 rats could be used for statistical analysis, 7 rats in the control group, 5 rats in the propranolol group, 6 rats in the diazepam group, and 7 rats in the dexmedetomidine group.

After the 4-day intoxication period, rats exhibited clear symptoms of ethanol withdrawal: rigidity, tremor, irritability, and hypoactivity. The time course of the ethanol withdrawal reaction, in terms of individual symptoms, as well as the sum score, is shown in Figs. 1 and 2. One-way ANOVA revealed no statistically significant difference between the groups in rigidity [$F(3,21) = 1.91, p = 0.158$]. Neither was there any significant difference between the groups in hypoactivity [$F(3,21) = 0.32, p = 0.808$] (i.e., the drugs did not

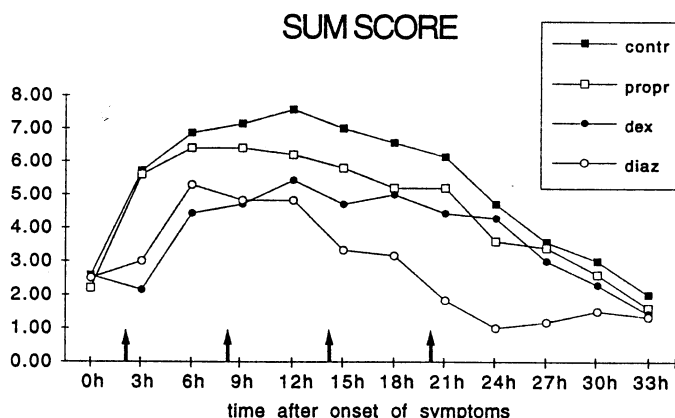

Fig. 2. Time course of ethanol withdrawal reaction in terms of the sum score of the three most specific symptoms (rigidity, tremor, and irritability). Both dexmedetomidine and diazepam significantly relieved the sum score, compared with the control group. Dexmedetomidine was the most effective drug during the first 12 hr; after the first 12 hr, diazepam seemed to be more effective.

cause any measurable sedation of the rats). There was a statistically significant difference between the groups in tremor [$F(3,21) = 9.03$, $p = 0.0005$]. Compared with the control group, tremor was significantly reduced in the diazepam-treated group (mean grade of tremor rating was 9.8 in the diazepam group and 21.3 in the control group, $p = 0.0001$) and in the dexmedetomidine-treated group (mean grade was 11.4 in the dexmedetomidine group, $p = 0.0004$). Also, propranolol reduced tremor compared with the control group (mean grade was 10.6 in the propranolol group, $p = 0.005$).

ANOVA revealed a statistically significant difference between the groups in irritability [$F(3,21) = 10.63$, $p = 0.0002$]. Diazepam reduced irritability significantly, compared with the control group (mean grade of irritability rating was 11.5 in the diazepam group and 24.0 in the control group; $p = 0.0001$). The sum score of the three most specific withdrawal signs (Fig. 2) showed a statistically significant treatment effect [$F(3,21) = 8.50$, $p = 0.0007$]. Diazepam relieved these signs significantly, compared with the control group (mean grade was 33.8 in the diazepam group and 62.3 in the control group; $p = 0.0001$). Also, dexmedetomidine significantly relieved the sum score of the three most specific signs (mean grade was 44.4 in the dexmedetomidine group, $p = 0.0047$). The sum score of the three items (rigidity, tremor, and irritability) has been found to represent a reliable one-dimensional measure of the degree of severity of the nonconvulsive component of withdrawal reaction.[22,26] Both diazepam[6] and dexmedetomidine[16,29,30] have also been shown to have sedative effects, which diminishes the specificity of hypoactivity in the measurement of the severity of withdrawal reaction.

We also observed the animals for possible spontaneous epileptiformic seizures in the withdrawal phase. None of the rats exhibited typical epileptiformic (tonic-clonic) seizure, but a brief clonic seizure was observed in a control rat 6 hr after onset of the withdrawal reaction, and a short-lasting "running-fit" in a dexmedetomidine-treated rat 4 hr after the last drug administration.

DISCUSSION

The overactivity of the noradrenergic nervous system is a central mechanism in the pathophysiology of ethanol withdrawal symptoms.[1–4] The increase in noradrenergic activity may result, at least in part, from a reduced α_2-adrenoceptor activity.[31] Dexmedetomidine is a highly selective, specific, and potent α_2-adrenoceptor agonist.[13,14] It is the dextroenantiomer and pharmacologically active component of medetomidine, which has been used for many years in veterinary practice for its hypnotic, sedative, and analgesic effects.[13,32,33] Recently, dexmedetomidine has been introduced into the clinical practice of anesthesiology. In the perioperative period, dexmedetomidine has shown to provide sedation, analgesia, and anxiolysis.[16,17,29,30,34,35] Dexmedetomidine has also been shown to improve hemodynamic stability during surgery and reduce other anesthetic requirements.[15,16,36,37]

We have previously shown that dexmedetomidine administered at the withdrawal phase at doses 10 and 30 μg/kg significantly diminishes the severity of ethanol withdrawal reaction in rat.[19] We have also shown that chronic dexmedetomidine treatment prevented ethanol-induced overactivity and degeneration of catecholaminergic neurons in rat peripheral nervous system. The single dose of dexmedetomidine offered only marginal protection against the ethanol-induced alterations.[18] Acutely administered dexmedetomidine and ethanol have been shown to enhance each other's effects on rectal temperature and motor actions (e.g., motility and rotarod performance in mice).[38] At the dose of 300 μg/kg ip, dexmedetomidine was found to enhance ethanol-induced sedation and motor impairment of mice.[39]

The results of the present study show that both dexmedetomidine and diazepam significantly relieve the ethanol withdrawal reaction when measured as the sum score of the three most specific withdrawal signs. The dose of dexmedetomidine was chosen according to a previous dose–response study,[19] where 30 μg/kg was shown to be the most effective dose in the first day of ethanol withdrawal. In the present study, the effect of dexmedetomidine seemed to diminish in the course of the withdrawal period. A dose of 30 μg/kg seemed to be very effective during the first 12 hr of the withdrawal period; however, during the next 12 hr, the effectiveness of that dose diminished gradually. This may indicate that the optimum dose of dexmedetomidine alters during the withdrawal period, in line with the findings of our previous experiments.[19]

Dexmedetomidine seemed to be the most effective drug during the first 12 hr of the withdrawal period. After the first 12 hr, diazepam seemed to be the more effective drug. For practical reasons, all drugs were administered every 6 hr, despite the different half-lives of these drugs. Dexme-

detomidine has been shown to have poor bioavailability and a very short elimination half-time,[40,41] whereas diazepam has been shown to be quite long-acting (half-time ~33 hr in humans).[6] This might have led to the accumulation of the concentration of diazepam during the course of the withdrawal period. In the present study, no effect of dexmedetomidine could be seen 24 to 33 hr after the onset of the withdrawal reaction (i.e., >4 hr after the last dose of dexmedetomidine was administered). This was rather an expected result considering the short half-life of dexmedetomidine.

Thus far, benzodiazepines have been the most popular agents to alleviate the ethanol withdrawal reaction.[6] However, addiction to benzodiazepines is a major problem in human alcoholics.[6] β-Adrenergic blockers have also been shown to be useful agents in the treatment of ethanol withdrawal syndrome.[6–8] During the period of withdrawal, vital signs like blood pressure, heart rate, and body temperature have been shown to return to normal more rapidly with atenolol treatment.[7] A troubling issue with β-adrenergic blockers is their possible association with psychotic reactions. Low doses of propranolol may induce a psychotic confusional state, delirium, and hallucinations in alcoholics during the acute ethanol withdrawal period.[42] Consistently, α_2-adrenoceptor agonists, mainly clonidine and lofexidine, have been shown to relieve some withdrawal symptoms, like tremor, tachycardia, and hypertension.[6,9–12] However, these drugs seem less effective in alleviating other withdrawal symptoms, including restlessness, diaphoresis, and insomnia.[33] Because of the many adverse side effects of the drugs used thus far in the treatment of ethanol withdrawal syndrome, alternative forms of treatment and new effective drugs are searched for.

Previous studies show that α_2-adrenoceptor agonists, like clonidine, may also have antiepileptogenic properties.[43–45] Dexmedetomidine (5 μg/kg, sc) was shown to attenuate kainic acid-induced convulsions and subsequent neuronal damage in rat hippocampus.[46] In our previous study, we showed a possible suppression of withdrawal seizure activity by dexmedetomidine at doses 10 to 30 μg/kg sc.[19] In the present study, none of the rats exhibited typical epileptiformic seizures, so we could not get further information on this effect. In previous studies, α_2-agonists have not caused any dependence problems unlike benzodiazepines[10,11]; but, after abrupt discontinuation of high-dose clonidine, a withdrawal reaction may develop.[43] At the doses studied thus far in clinical anesthesiology, dexmedetomidine has been shown to be safe and well-tolerated.[15,16,29,30] Dexmedetomidine might have clinical use in withdrawal treatment, especially in regard to its potential neuroprotective effects.[18] A more frequent administration and different dosage of dexmedetomidine at different stages of the withdrawal period might increase its efficacy against ethanol withdrawal symptoms. In the future, clinical studies should be conducted to verify these experimental results of dexmedetomidine in the treatment of ethanol withdrawal symptoms.

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Effects of Dexmedetomidine on Rat Locus Coeruleus and Ethanol Withdrawal Symptoms During Intermittent Ethanol Exposure

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In the present study, the neuroprotective effects of dexmedetomidine on rat locus coeruleus were studied during a 5-week intermittent ethanol exposure. Male Wistar rats (3 to 4 months old) were given ethanol or isocaloric sucrose by intragastric intubations three times a day for 4 days, which was followed by a 3-day withdrawal period. This 7-day cycle of ethanol exposure and withdrawal was repeated five times. Dexmedetomidine (at a dose decreasing from 30 μg/kg to 10 μg/kg, sc) was given to the treatment group during the withdrawal phase. The results showed that, during the 5-week experiment, dexmedetomidine significantly relieved the ethanol withdrawal syndrome, measured as the sum of the three most specific symptoms (rigidity, tremor, and irritability). The total neuron number of locus coeruleus (LC) decreased in the ethanol-treated group by 24%, compared with the nontreated control group and by 11%, compared with the sucrose-treated control group. Interestingly, the LC neuron numbers were found to decrease in the sucrose-intubated rats as well, compared with the nontreated control group. Dexmedetomidine was found to relieve ethanol-induced neuronal loss in the LC. Dexmedetomidine might be a new interesting alternative in the treatment of ethanol withdrawal syndrome, particularly due to its possible neuroprotective effects in the central nervous system.

Key Words: Ethanol, Intermittent, Locus Coeruleus, Rat, Withdrawal.

OVERACTIVITY OF the noradrenergic nervous system has been reported during chronic ethanol exposure and also during the ethanol withdrawal phase (Nutt and Glue, 1990). The ethanol withdrawal syndrome is characterized by typical symptoms, like anxiety, tremor, increased blood pressure, and heart rate and even epileptiformic seizures, most of which are also classic symptoms of sympathetic overactivity (Nutt and Glue, 1990; Linnoila, 1987). In the withdrawal phase, the increase in noradrenergic activity may result, at least in part, from a reduced sensitivity of α_2-adrenergic receptors (Nutt and Glue, 1990; Hawley et al., 1994; Nutt et al., 1988).

Chronic ethanol exposure has been shown to cause noradrenergic overactivity in both the central nervous system (CNS) and the peripheral nervous system (Ahtee et al., 1975; Pohorecky, 1974; Jaatinen and Hervonen, 1994; Sjöquist et al., 1983). The prolonged noradrenergic overactivity may contribute to the morphofunctional degeneration of the sympathetic nervous system, which has been reported in chronic alcoholics (Eisenhofer et al., 1985; Novak and Victor, 1974), as well as in experimental animals chronically exposed to ethanol (Jaatinen and Hervonen, 1994; Jaatinen et al., 1993).

The locus coeruleus (LC) is the main noradrenergic nucleus in the central nervous system, providing most of the noradrenergic innervation to almost every region of the brain (Foote et al., 1983). The LC is thought to be an important center for maintaining homeostasis within the CNS, and it has been linked with a variety of physiological processes, including regulation of sleep, wakefulness, attention, learning, memory, and stress (Aston-Jones et al., 1991). In previous studies, chronic ethanol exposure has been shown to cause neurodegenerative changes of the LC both in human alcoholics (Arango et al., 1993, 1994, 1996) and in animal models (Lu et al., 1997; Rintala et al., 1998).

The pathophysiological mechanisms behind the ethanol-related neuronal damage are still only partially understood. Ethanol withdrawal and the excitotoxic mechanisms related to it may have a central role in the pathogenesis of ethanol-induced neuronal degeneration (Lovinger, 1993). It has been suggested that intermittent ethanol exposure with repeated withdrawal periods may be more harmful to neurons than continuous exposure (Lundqvist et al., 1994, 1995; Jaatinen et al., 1997).

Dexmedetomidine is a highly potent and selective α_2-adrenoceptor agonist (MacDonald et al., 1991; Savola et al., 1991), with significant sedative, analgesic, and hemodynamic effects (Aantaa et al., 1990; Aho et al., 1991; Scheinin et al., 1992). In our previous studies, dexmedetomidine has been shown to be effective in the treatment of ethanol withdrawal symptoms in the rat (Riihioja et al., 1997a,b). We have also shown that chronic dexmedetomidine treatment may prevent ethanol-induced overactivity and degen-

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eration of catecholaminergic neurons in the rat peripheral nervous system (Jaatinen et al., 1995).

This study was designed to investigate the possible neuroprotective effects of dexmedetomidine and its efficiency in the treatment of ethanol withdrawal syndrome during a 5-week intermittent ethanol exposure.

METHODS

Animals and Treatments

Thirty-four adult (3 to 4 months old) male Wistar rats were used in this study. Rats were housed individually under standard conditions (+22 ± 1°C, 13/11 h light/dark cycle with lights on between 8 AM and 9 PM). Animals were carefully habituated to handling before the experiment was started.

Rats were divided into five groups: a nontreated control group (Control, $n = 6$), a sucrose-fed control group (Sucrose, $n = 6$), a sucrose-fed dexmedetomidine-treated control group (Sucrose + Dex, $n = 6$), an ethanol-fed group (EtOH, $n = 8$), and an ethanol-fed dexmedetomidine-treated group (EtOH + Dex, $n = 8$). Treatment groups were given 25% ethanol or isocaloric sucrose by intragastric intubations three times a day for 4 days (Monday to Thursday), which was followed by a 3-day withdrawal period. This 7-day cycle of ethanol exposure and withdrawal was repeated five times. Medication was given in every withdrawal phase 13 h, 17 h, 21 h, 25 h, and 39 h after the last dose of ethanol. Ethanol-fed nondexmedetomidine-treated rats were given vehicle injections (0.9% NaCl, sc), respectively. Nontreated control animals were given food ad libitum, and sucrose-fed control animals (Sucrose, Sucrose+Dex) were given the same amount of food as the ethanol-treated groups consumed.

We have previously studied the dose–response relations of subcutaneously administered dexmedetomidine in the treatment of ethanol withdrawal symptoms (Riihioja et al., 1997a). In that study, 30 μg/kg seemed to be the most effective dose during the first 20 to 23 hr of withdrawal, and 10 μg/kg seemed to be the most effective dose later in the withdrawal phase. Therefore, in the present study, a decreasing dosage of dexmedetomidine during the withdrawal period was used. To find the optimum sceme of medication, the doses of dexmedetomidine were slightly modified during the course of the experiment. In the first week, the dose of dexmedetomidine was 30 μg/kg at the first two time points, 10 μg/kg at the next two time points and 3 μg/kg at the last time point. In the second week, the doses were raised to 30 μg/kg at the first three time points and 10 μg/kg at the last two time points. During the rest of the experiment, the doses were 30 μg/kg for the first two time points and 10 μg/kg for the last three time points.

Immediately before every ethanol feeding, the severity of intoxication of each rat was evaluated by using a standardized, 6-level intoxication scale (Riihioja et al., 1997a,b; Clemmesen et al., 1984, 1985; Hemmingsen et al., 1979, 1984). The dose of ethanol was individually adjusted according the level of intoxication. The aim was to keep the animals in a state of intoxication graded 3 (clearly impaired walking, impaired elevation of abdomen and pelvis) or 4 (slowed righting reflex, no elevation of abdomen and pelvis) in the previously described intoxication scale.

Blood Ethanol Concentrations (BECs)

BECs were determined by gas chromatography (Eriksson, 1973) from capillary blood samples (0.05 ml), which were taken from the tip of the tail during the last 2 days (Wednesday and Thursday) of ethanol exposure on the fourth week of the experiment. Blood samples were taken before (BEC-0) and 1 hr after (BEC-1h) the morning dose of ethanol. For technical reasons, ethanol samples were taken on two consecutive days.

Measurement of Ethanol Withdrawal Symptoms

Withdrawal symptoms were rated every week, after 11, 14, 18, 22, 26, 36, 40, 48, and 62 hr from ethanol withdrawal, blind to the treatments. The onset of the withdrawal rating was timed according to previous studies, where the first withdrawal symptoms were seen 8 to 11 hr after the last dose of ethanol (Riihioja et al., 1997a,b). The withdrawal reaction was assessed by using a standardized 3-item scale, as described in detail previously (Riihioja et al., 1997a,b; Clemmesen et al., 1984, 1985; Hemmingsen et al., 1979, 1984). The sum score of the three items (rigidity, tremor, and irritability) has been found to represent a reliable one-dimensional measure of the severity of the nonconvulsive component of withdrawal reaction (Clemmesen and Hemmingsen, 1984; Clemmesen et al., 1988). Each of the three items was rated 0 to 3, and the weekly sum score of the three items was used in the statistical analysis. Furthermore, animals were observed for possible spontaneous motor seizures.

Tissue Preparation and Histological Procedures

Rats were killed by decapitation under deep sodium pentobarbital anesthesia (60 mg/kg, ip). After decapitation, the brains were quickly removed. The cerebellar peduncules were transected, and the brainstem was separated by a transverse cut rostral to the inferior colliculi. The brainstem was further cut midsagittally in two halves. The tissue was fixed in 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) at 4°C for 24 hr and then incubated in 10%, 20%, and 30% sucrose in phosphate-buffered saline to avoid cryodamage. For the morphometric analysis, the right side of the LC of each brainstem was used. The brainstem was sectioned sagittally at 8 μm in a cryostat. Six serial sections were collected on one slide for cresyl violet staining and the next two sections on another slide for immunohistochemistry. A total of 8 to 12 series of eight sections were obtained from each LC. The noradrenergic nature of the neurons was ensured by immunohistochemical demonstration of tyrosine hydroxylase by the ABC reaction (Hsu et al., 1981).

Morphometric Analyses

Due to technical problems in processing the samples, a total of 28 rats could be used in the morphometric analyses (5 rats in the Control group, 6 rats in the Sucrose group, 5 rats in the Sucrose + Dex group, 7 rats in the EtOH group, and 5 rats in the EtOH + Dex group). The stereological study was performed by an Olympus Vanox-t microscope (Olympus, Inc., Tokyo, Japan), a Hamamatsu ARGUS-10 image processor, and a video monitor. From the Nissl-stained (cresyl violet) sections, the first and the last sections containing LC proper neurons were identified. The reference section on the first slide was chosen at random, and the next section was the look-up section. If the chosen section was damaged, the next section was used. The boundaries of the LC proper were determined from the Nissl-stained sections. The volume of LC proper was estimated by using the point counting method (Gundersen, 1987) and the total number of neurons was estimated by the disector method (Gundersen et al., 1988; West, 1994) on the Nissl-stained sections, as previously described (Lu et al., 1997; Rintala et al., 1998).

Statistical Methods

Data are expressed as means ± SEM. One-way analysis of variance (ANOVA) was used to analyze the overall differences between the treatment groups, and when ANOVA showed a significant treatment effect, the groups were further compared by Bonferroni-corrected t tests. Student's t test was used to measure the differences between the ethanol-treated groups in BECs. ANOVA for repeated measurements was used to analyze group differences in ethanol doses, intoxication score/ethanol dose ratios, and ethanol withdrawal symptoms during the 5-week ethanol exposure. The effect of cumulative ethanol dose (g/kg), intoxication score, and withdrawal symptom score on the morphometric variables was tested with analysis of covariance and Pearson's correlation analysis. A 5% tail probability was considered statistically significant ($p < 0.0125$ when four pairs of means were compared).

Table 1. Time Course of the Total Ethanol Dose (g/kg) and the Intoxication Score/Ethanol Dose Ratio in the EtOH Group (n = 8) and the EtOH + Dex Group (n = 8) during the 5-week Experiment (Means ± SEM)

	Week 1	Week 2	Week 3	Week 4	Week 5	F	p
Ethanol dose (g/kg)							
EtOH	34.0 ± 1.6	36.4 ± 1.2	39.7 ± 0.8	40.0 ± 0.7	40.0 ± 0.8		
EtOH + Dex	35.8 ± 1.3	35.6 ± 0.9	39.3 ± 1.1	40.6 ± 1.0	41.8 ± 0.5		
EtOH vs. EtOH + Dex[a]						0.69	0.42
Time						13.78	<0.01
Interaction						0.65	0.63
Intoxication score/ethanol dose ratio							
EtOH	0.48 ± 0.13	0.48 ± 0.09	0.26 ± 0.04	0.25 ± 0.03	0.30 ± 0.04		
EtOH + Dex	0.36 ± 0.07	0.52 ± 0.06	0.29 ± 0.07	0.23 ± 0.06	0.18 ± 0.03		
EtOH vs. EtOH + Dex[a]						0.69	0.42
Time						5.99	<0.01
Interaction						0.69	0.60

[a] p values represent results of ANOVA for repeated measurements.

RESULTS

There was no difference between the groups in the initial body weights (Control: 339 ± 11 g, Sucrose: 330 ± 8 g, Sucrose + Dex: 332 ± 8 g, EtOH: 330 ± 11 g, and EtOH + Dex: 331 ± 11 g). Due to the pair-feeding regimen, there was no difference between the sucrose- and ethanol-treated animals in the amount of food eaten (Control: 711 ± 4 g, Sucrose: 380 ± 29 g, Sucrose + Dex: 357 ± 22 g, EtOH: 359 ± 22 g, and EtOH + Dex: 369 ± 23 g). Weight loss was slightly more severe in the ethanol-treated groups (Control: +63 ± 3 g, Sucrose: +6 ± 10 g, Sucrose + Dex: −14 ± 8 g, EtOH: −28 ± 8 g, EtOH + Dex: −24 ± 8 g).

ANOVA for repeated measurements revealed no difference between the EtOH group and the EtOH + Dex group in the total ethanol doses during the 5-week experiment, but the ethanol doses increased with time during the experiment in both groups (Table 1).

The development of ethanol tolerance was estimated by calculating the intoxication score/cumulative ethanol dose ratios for each week (Table 1). ANOVA for repeated measurements revealed no difference between the EtOH group and the EtOH + Dex group in the intoxication/ethanol dose ratio.

BEC

ANOVA revealed no difference in the BECs between the ethanol-treated groups. On the first day of BEC measurements, BEC-0 was 43 ± 17 mmol/liter in the EtOH group and 28 ± 28 mmol/liter in the EtOH + Dex group (p = 0.65), and BEC-1 was 73 ± 16 mmol/liter in the EtOH group and 53 ± 18 mmol/liter in the EtOH + Dex group (p = 0.49). On the second day, BEC-0 was 3.5 ± 2 mmol/liter in the EtOH group and 3.7 ± 2 mmol/liter in the EtOH + Dex group (p = 0.94), and BEC-1 was 21 ± 10 mmol/liter in the EtOH group and 38 ± 6 mmol/liter in the EtOH + Dex group (p = 0.18). The difference in BECs between the 2 days was probably due to the different intoxication levels and ethanol doses given to the animals in the evening before blood sampling.

Ethanol Withdrawal Reaction

ANOVA for repeated measurements showed that dexmedetomidine significantly relieved the severity of the ethanol withdrawal syndrome measured as the sum of the three most specific symptoms. The severity of the withdrawal symptoms changed with time during the experiment (Fig. 1). When comparing the severity of ethanol withdrawal symptoms in EtOH and EtOH + Dex groups on each week by Student's t tests, there was no significant difference between the groups during the first 4 weeks of ethanol exposure (p = 0.06 for week 1, p = 0.18 for week 2, p = 0.28 for week 3, and p = 0.12 for week 4). During the last week of ethanol exposure, the severity of the ethanol withdrawal syndrome was significantly relieved in the dexmedetomidine-treated group, compared with the ethanol-treated control group ($p < 0.01$ for week 5).

Morphometric Results

ANOVA showed a significant difference between the groups in the LC total neuron numbers [$F(4,23) = 9.68$, $p < 0.01$]. Bonferroni-corrected t tests revealed a decreased LC neuron number in the sucrose-treated group, compared with the nontreated controls, and further, a tendency toward lower LC neuron numbers in the EtOH group compared with the sucrose-treated control group (Table 2). Dexmedetomidine significantly reduced the LC neuronal loss in the ethanol-treated rats.

Pearson's correlation analysis revealed a weak negative correlation between the intoxication score and the LC total neuron numbers (r = −0.51, p = 0.09), a weak positive correlation between the cumulative ethanol dose and the LC neuron numbers (r = 0.51, p = 0.09), and a negative tendency between the ethanol withdrawal score and the LC neuron numbers (r = −0.43, p = 0.17). According to analysis of covariance, the total ethanol dose (p = 0.34), the intoxication level (p = 0.26), or the ethanol withdrawal score (p = 0.38) did not significantly affect the LC total neuron numbers.

ANOVA showed a significant overall difference between

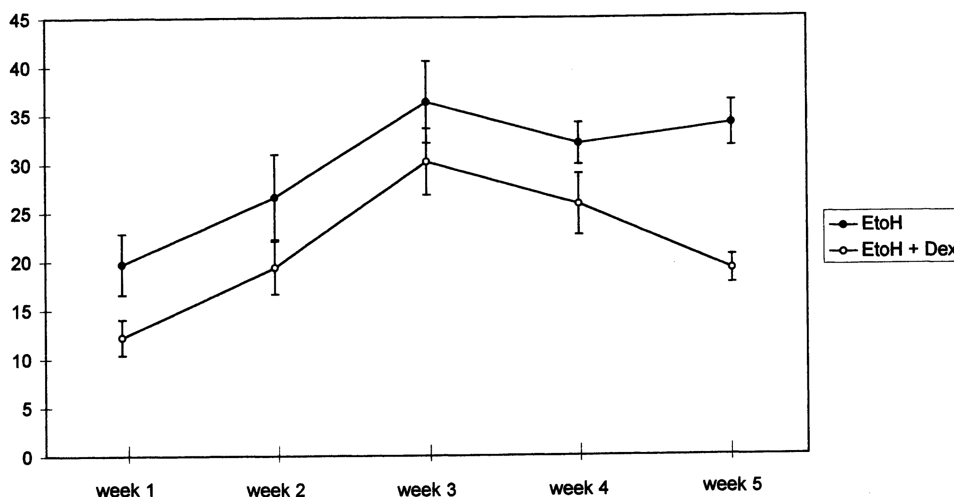

Fig. 1. Time course of the ethanol withdrawal symptoms (as the sum of the three most specific symptoms) during the 5-week experiment (means ± SEM). ANOVA for repeated measurements showed that the severity of the ethanol withdrawal syndrome was significantly relieved in the dexmedetomidine-treated group (n = 8), compared with the ethanol-treated group (n = 8) (p = 0.03 for group effect). Severity of the withdrawal symptoms changed with time during the experiment ($p < 0.01$ for time effect, p = 0.13 for group × time interaction).

Table 2. Total Number of LC Neurons, LC Volume, and LC Neuron Density in Each Treatment Group

	Neuron no.	Volume (μm^3)	Neuron density ($/mm^3$)
Control (n = 5)	3,097 ± 93	44 × 10^6 ± 0.5 × 10^6	70,273 ± 1,590
Sucrose (n = 6)	2,631 ± 89[a,**]	41 × 10^6 ± 1 × 10^6	63,601 ± 1,751
Sucrose + Dex (n = 5)	2,803 ± 72	43 × 10^6 ± 0.9 × 10^6	58,793 ± 6,313
EtOH (n = 7)	2,349 ± 80[b]	40 × 10^6 ± 0.5 × 10^6	59,416 ± 2,375
EtOH + Dex (n = 5)	2,704 ± 110[c,*]	41 × 10^6 ± 1 × 10^6	65,809 ± 3,340

Data are expressed as means ± SEM. Differences between the groups were analyzed by Bonferroni-corrected t tests. * $p < 0.05$; ** $p < 0.01$.

[a] Significantly different from the Control group at the 0.01 level.

[b] A tendency toward a reduced neuron number, compared with the Sucrose group at the 0.10 level.

[c] Significantly different from the EtOH group at the 0.05 level.

the groups in the LC volume [F(4,23) = 4.04, p = 0.01]. Bonferroni-corrected t tests revealed a tendency toward a reduced LC volume in the sucrose-treated group, compared with the nontreated control group (uncorrected p = 0.04). However, there was no significant difference between the EtOH group and the Sucrose group (uncorrected p = 0.11), or between the EtOH group and the EtOH + Dex group (uncorrected p = 0.13) in the LC volume. ANOVA revealed no significant difference between the groups in LC neuronal density [F(4,23) = 1.96, p = 0.13] (Table 2).

DISCUSSION

The results of the present study showed that, during intermittent ethanol exposure, dexmedetomidine significantly relieved the severity of ethanol withdrawal symptoms. These findings are consistent with our previous studies, where dexmedetomidine has been shown to be effective in the treatment of the ethanol withdrawal syndrome in the rat (Riihioja et al., 1997a,b). In the present study, the effect of dexmedetomidine on ethanol withdrawal symptoms seemed to remain unchanged or even improve during the repeated withdrawal periods.

In previous studies, dexmedetomidine has been shown to reduce acute ethanol tolerance (Idänpään-Heikkilä et al., 1995; Seppälä et al., 1994). In the present study, dexmedetomidine seemed not to have effect on the development of ethanol tolerance, when measured as the intoxication score/ethanol dose ratio. This is probably due to the different administration times of ethanol and dexmedetomidine.

The 5-week intermittent ethanol exposure of the present study decreased the total neuron number of rat LC by 24%, compared with the nontreated control group and by 11%, compared with the sucrose-treated group. Previously, lifelong ethanol consumption has been shown to decrease the total neuron number of the LC of AA and ANA rats by 30% in females and 17% in males (Lu et al., 1997; Rintala et al., 1998). In human alcoholics, a 23 to 27% reduction in the total number of pigmented LC neurons has been reported (Arango et al., 1994, 1996). Between ages 30 to 70, alcoholics have been shown to have 12 to 51% fewer LC neurons than controls of the same age (Arango et al., 1996). Interestingly, in the present study, the LC total neuron number was also diminished in the sucrose-treated groups, compared with the nontreated control group. Both the LC volume and neuronal density were slightly, but not statistically significantly, decreased in the ethanol- and sucrose-treated groups compared with the nontreated control group. The tendencies toward decreased LC volumes and neuron densities together resulted in significantly decreased LC total neuron numbers in the ethanol- and sucrose-treated rats.

One explanation for the reduced LC neuron number in the sucrose-treated groups may be the stress related to the intragastric intubations. To our knowledge, neuronal loss in the CNS due to chronic stress has not been reported pre-

viously. Repeated psychosocial stress has been shown to cause atrophy of the apical dendrites in CA3 pyramidal neurons of the hippocampus, accompanied by specific cognitive deficits in spatial learning and memory (McEwen and Magarinos, 1997; Magarinos et al., 1997; Luine et al., 1994). The LC plays an important role in mediating the stress response, and the particular reactivity of the LC system in stressful situations is well documented (McEwen and Magarinos, 1997; Stanford, 1995). Many kinds of stressors, like somatosensory stimuli, interoceptive cues, and complex environmental stimuli, have been shown to increase the firing rate of noradrenergic neurones in the LC, and repeated stressful situations may lead to sensitization to stress (Stanford, 1995). After prolonged periods of stressful situations, region-specific changes have been shown in the activity of brain α_2-adrenergic receptors (Flugge, 1996). In the present study, dexmedetomidine had no significant neuroprotective effect in the sucrose-treated group, which may indicate that other factors than noradrenergic overactivity may have played a central role in the development of neuronal damage in the sucrose-treated rats. Nutritional deficiencies may have contributed to the LC neuron loss in the sucrose-fed animals, as their intake of clow was about half of the amount eaten by the nontreated control group.

The pathophysiological mechanisms by which chronic ethanol exposure produces neuronal damage in the LC have not been established. Ethanol and its toxic metabolites, mainly acetaldehyde, may have direct toxic effects on LC neurons (Lu et al., 1997; Matsubara et al., 1987; Fa and Dryhurst, 1991). Also, ethanol-induced overactivity of catecholaminergic neurons may enhance the auto-oxidation of catecholamines producing oxygen-free radicals and semiquinones, which may lead to neuronal death (Graham, 1978). Chronic ethanol exposure may also reduce the afferent and efferent neuronal connections of the LC (Kjellström et al., 1993). Chronic alcohol abuse has been reported to induce a loss of neurons in the cerebellum, thalamus, hippocampus, and parts of the cerebral cortex (Foote et al., 1983; Mann, 1983), which all have wide efferent and afferent connections with the LC.

The ethanol withdrawal syndrome and excitotoxic mechanisms related to it may have a central role in ethanol-induced neurodegeneration. So far, only a few studies have been performed on the effects of intermittent ethanol exposure in the CNS. In rat hippocampus, intermittent ethanol exposure has been shown to cause more severe neuronal and synaptical loss, and to enhance lipofuscin accumulation compared with continuous ethanol exposure (Lundqvist et al., 1994, 1995; Lundqvist, 1997; Bonthius and West, 1990). In the present study, the results showed a weak negative correlation between the ethanol withdrawal score and the total number of LC neurons. There was also a tendency toward a *positive* correlation between the total dose of ethanol and the LC total neuron number. The explanation for this somewhat unexpected tendency may be that the rats tolerating high doses of ethanol were also resistant to ethanol-induced neuronal degeneration.

The results of the present study show that dexmedetomidine reduced ethanol-induced neuronal loss in the LC. Previously dexmedetomidine has been shown to have neuroprotective effects in the CNS against neuronal damage caused by ischemia (Hoffman et al., 1991; Maier et al., 1993; Kuhmonen et al., 1997) and epileptiformic convulsions (Halonen et al., 1995). We have also shown that dexmedetomidine may protect the peripheral sympathetic nervous system against ethanol-induced neuropathology (Jaatinen et al., 1995). However, during a 5-week *continuous* ethanol exposure, no significant neuroprotection by dexmedetomidine was seen in rat LC (Riihioja et al., 1997c). Thus, the neuroprotective effects of dexmedetomidine may be related to its efficiency in the treatment of the ethanol withdrawal syndrome.

In conclusion, dexmedetomidine was found to alleviate ethanol withdrawal symptoms and to reduce LC neuronal loss caused by intermittent ethanol exposure. Dexmedetomidine may thus prove an interesting alternative in the treatment of the ethanol withdrawal syndrome. Further studies are needed to illuminate the question if dexmedetomidine has neuroprotective effects against ethanol-induced degeneration in other neuronal populations than the noradrenergic ones.

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