Published in final edited form as:

Muscle Nerve. 2013 August ; 48(2): 204–211. doi:10.1002/mus.23744.

EXPERIMENTAL MODEL OF ALCOHOL-RELATED PERIPHERAL NEUROPATHY

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Abstract

Introduction—The aim of this work was to determine the effect of chronic alcohol exposure on peripheral nerves in a nutritionally balanced rat model of alcoholism.

Methods—Three different strains of adult male rats were pair-fed for 8 weeks with isocaloric liquid diets containing 0% or 37% ethanol. Nerve conduction studies (NCS) were performed. Peripheral nerve and muscle were examined histologically with morphometrics.

Results—Ethanol exposure significantly slowed velocity in tibial and fibular nerves, but not in the plantar nerve in all 3 strains. Studies of the sciatic nerve revealed decreased fiber diameters and increased regenerative sprouts in peripheral nerves. There was muscle denervation of ethanol-exposed rats in all 3 strains.

Conclusions—Chronic ethanol exposure caused a polyneuropathy characterized by axonal degeneration despite adequate nutrition. These results suggest that ethanol exposure has direct neurotoxic effects on peripheral nerves. This model may be useful in understanding the underlying mechanism(s) of alcohol-related peripheral neuropathy.

Keywords

alcohol; chronic ethanol exposure; nerve conduction study; neuropathy; rat model

Alcohol-related peripheral neuropathy (ALN) is an underrecognized, chronic, and potentially debilitating disease that lacks effective treatment, because the mechanism of injury is poorly understood.^{1,2} The development of new treatments for ALN depends partly on the understanding of causative mechanisms in a validated rat model of chronic alcoholism. Although alcohol exposure is clearly the critical variable, considerable attention has been paid to thiamine deficiency because of the similarities of the clinical presentation

and electrophysiology of the associated neuropathies. Studies have shown that alcohol impairs thiamine absorption through the gastrointestinal tract,^{3–5} its utilization in tissues,⁶ its hepatic storage,^{7,8} and the phosphorylation of thiamine, thus reducing the availability of the active form, thiamine pyrophosphate.^{9–11} This concept of thiamine as the main etiologic factor causing ALN has lost support, because results from controlled clinical trials and a recent Cochrane Database Review demonstrated that ALN was not abated significantly or reversed by thiamine supplementation.^{12–14} Therefore, additional research is needed to better understand the mechanisms of ALN, particularly with regard to the potential roles of ethanol, nutritional deficiency, and genetic factors that increase risk for developing ALN.

The primary objectives of this study were to determine whether chronic ethanol feeding in a nutritionally balanced rat model of alcoholism would produce a neuropathy consistent with features of ALN. The expectation is that this model can be utilized to understand the underlying mechanism(s) that cause ALN and can help with development of new treatments for this potentially debilitating disease.

METHODS

Chronic Ethanol Exposure Model

Adult male (~200–250 g) Fisher 344 (FS), Long Evans (LE), and Sprague-Dawley (SD) rats (Harlan Sprague-Dawley, Inc., Indianapolis, Indiana) were pair-fed with identical isocaloric liquid diets (BioServ, Frenchtown, New Jersey) containing either 0% ($N=22$) or 37% ($N=28$) ethanol for 8 weeks.¹⁵ These strains were selected because they exhibit different sensitivities to alcohol-induced liver and brain injury. Two weeks prior to the experiment, rats were adapted to the liquid diets by incrementing progressively the ethanol content from 0% to 11.8%, 23.6%, and then 37% of the caloric content. Controls were adapted to ethanol-free liquid diets over the same period. Thereafter, rats were maintained on 0% or 37% ethanol-containing diets throughout the study.¹⁵ Rats were monitored daily to ensure adequate nutritional intake and maintenance of body weight. Blood alcohol levels were measured at 8:00 A.M. using the Analox GM7 apparatus (Analox Instruments USA, Lunenburg, Massachusetts) according to the manufacturer's protocol. Throughout the experiment, rats were housed under humane conditions and kept on a 12-hour light/dark cycle with free access to food. All experiments were performed in accordance with protocols approved by institutional animal care and use committee at the Lifespan–Rhode Island Hospital, conforming to guidelines established by the National Institutes of Health.

Electrophysiology

Nerve conduction studies (NCS) were performed during the seventh week of liquid diet feeding. Under sodium pentobarbital anesthesia (40–50 mg/kg), the rats were placed on a warming pad, and their body temperatures were maintained between 34° and 36°C, monitored with a rectal thermometer. Nerve conduction velocity and amplitude were recorded from the plantar (sensory), tibial (motor), and fibular (motor) nerves with an electromyography system (Viking IV; Nicolet/Carefusion, Madison, Wisconsin) using standard filter settings and subdermal needles (Rhythmlink, Columbia, South Carolina) for motor and sensory NCS. Plantar sensory nerve action potentials (SNAPs) were recorded,

and negative peak latency and peak-to-peak amplitude were measured.¹⁶ Motor nerves were stimulated distally at the ankle and proximally at the knee with recording from the extensor digitorum brevis (fibular nerve) or flexor hallucis longus (tibial nerve). Compound muscle action potentials (CMAPs) were measured after delivery of a supra-maximal stimulus to obtain the maximum response. Latencies and peak-to-peak amplitudes for all stimulations were measured, and velocities were calculated by dividing distance by latency, after subtracting the distal motor latency.¹⁷ At the end of the study, the rats were euthanized by isoflurane inhalation, and segments of peripheral nerve and skeletal muscle (contralateral to the NCS site to avoid artifacts) were immersion-fixed in Histochoice (Amresco, Solon, Ohio) and embedded in paraffin.

Histology and Morphometric Analysis

Histological sections (5 μ m thick) of sciatic nerve were stained with Luxol Fast Blue/hematoxylin and eosin (LHE), and sections from the belly of the gastrocnemius muscle were stained with hematoxylin and eosin (H&E). No tension was placed on peripheral nerves during sectioning. Peripheral nerve samples previously fixed with Histochoice, were rinsed in 0.15 M sodium cacodylate buffer and placed in 2.5% glutaraldehyde in 0.15 M sodium cacodylate buffer for 1 hour. After 3 rinses in cacodylate buffer, samples were postfixed with buffered 1% osmium tetroxide for 1 hour at 4°C, then again rinsed in buffer, dehydrated through a graded acetone series, and infiltrated with Spurr epoxy resin. After overnight polymerization at 70°C, 1- μ m-thick sections were cut using an ultra-microtome (Ultracut S; Reichert, Depew, New York). The sections were stained with methylene blue–azure II for light microscopy and morphometric analysis. Nerve cross-sections were analyzed under 600 $\times$ oil magnification using the dissector, point counting, and nucleator probes of the Stereologer program to determine density and diameter of fibers. H&E-stained cross-sections of skeletal muscle were analyzed at 200 $\times$ magnification using the nucleator probe to measure fiber diameters. All analyses were performed on coded samples.

Statistical Analysis

Data depicted in boxplots reflect group median, 95% confidence interval limit, and range (whiskers), whereas tabulated data reflect mean $\pm$ standard error of the mean (SEM) for each group. Intergroup comparisons were made using 2-way analysis of variance (ANOVA) with *post hoc* Bonferroni tests of significance. Data were analyzed using GraphPad Prism (version 5) software (GraphPad Software, Inc., San Diego, California). Significant *P*-values (*P*<0.05) are shown within the graph panels or included in the tables.

RESULTS

Weight Gain Associated with Liquid Diet Feeding

Despite identical ages upon entry into the study, FS rats had the lowest original and final body weights, SD rats were intermediate, and LEs were the largest. Rats in all groups consumed similar quantities of food and, correspondingly, gained weight continuously over the course of the study. In addition, the pair-fed LE and SD rats had similar mean total and percentage increases in weight such that chronic ethanol-feeding did not impair growth relative to corresponding controls (Table 1). In contrast, ethanol-fed FS rats had

significantly lower mean total and percentage increases in body weight relative to pair-fed controls. Despite these intergroup differences, the mean blood alcohol concentrations were similar in ethanol-fed FS, LE, and SD rats (Table 1). Therefore, any effects of chronic ethanol feeding or differences in response to ethanol among the 3 strains could not be attributed to differences in nutritional status or blood alcohol concentration.

Effects of Ethanol on Nerve Conduction Studies

Nerve conduction studies were performed on the tibial, fibular, and plantar nerves in all 3 rat strains. Mean conduction velocities were significantly reduced in both tibial and fibular nerves of ethanol-exposed relative to control rats in all 3 strains ($P<0.0001$; Fig. 1A and B). The effect of alcohol feeding on the distal latencies and amplitudes in the tibial and fibular nerves was variable among the 3 strains.

In the Fisher 344 strain, NCS of the tibial nerve showed that the mean distal latencies were prolonged in ethanol-fed rats, and the difference from controls reached statistical significance for recordings made from the knee ($P=0.032$). In addition, the CMAP amplitudes were reduced significantly in the tibial nerves of ethanol-exposed rats whether stimulated proximally at the knee ($P=0.044$) or distally at the ankle ($P=0.026$). Recordings from the fibular nerve demonstrated no significant differences in the mean distal latency or CMAP amplitude between control and ethanol-fed rats (Table 2A).

On the other hand, NCS in the Sprague-Dawley strain showed that the mean distal latencies were prolonged significantly in both the tibial and fibular nerves when stimulating distally (from the ankle; $P=0.016$ and $P=0.0057$, respectively), but not proximally at the knee in ethanol-exposed relative to control rats. However, there were no significant effects of chronic ethanol feeding on the CMAP amplitude in the tibial or fibular nerves (Table 2B).

NCS in the Long Evans strain also showed mixed results. With respect to the tibial nerve, the mean distal latency was significantly prolonged when stimulating proximally (from the knee; $P=0.0021$), but not distally in ethanol-exposed relative to control rats. With regard to the fibular nerve, there was no significant effect of chronic ethanol feeding on distal latency. In both tibial and fibular nerves, mean CMAP amplitudes were similar in control and ethanol-fed rats (Table 2C).

Mean latencies, amplitudes, and velocities of nerve conduction in the plantar nerve were similar in control and ethanol-fed rats (Tables 2A and Fig. 1C).

Histopathology of Alcohol-Related Polyneuropathy

The 1- μm -thick, Epon-embedded methylene blue-stained sections of the sciatic nerve demonstrated relatively uniform populations of medium- and large-diameter myelinated axons in control samples, but subtle irregularities of axons were characterized by increased variation in axon size with conspicuous clusters of small, thinly-myelinated axon sprouts in ethanol-exposed relative to control nerves. Axon profiles for all 3 strains showed skewing of nerve fiber diameters to the left, indicating smaller diameters, in ethanol-fed rats (Fig. 2); in addition, average axon diameters were significantly smaller ($P<0.0001$) across all 3 strains in ethanol-fed rats (Fig. 3). These findings were similar to those observed and expected in

the axonal alcohol-related peripheral neuropathy in humans.² The smaller axon diameters were unlikely to completely account for the slowing of the nerve conduction velocities. A potentially noteworthy observation was that the LHE-stained histological sections of sciatic nerves suggested patchy demyelination in ethanol-fed rats among all 3 strains, but objective studies were not performed to substantiate this observation.

Denervation of Muscle in Ethanol-Fed Rats

To determine whether the ethanol-associated functional and structural abnormalities in peripheral nerves were accompanied by denervation of muscle, we examined H&E-stained histological sections of gastrocnemius muscle. Control samples exhibited mainly uniform polygonal myofibers with delicate endomesial connective tissue and no evidence of myofiber degeneration, necrosis, or inflammation. In contrast, in ethanol-fed rats, irrespective of strain, the gastrocnemius muscle samples exhibited increased variation in myofiber size due to the presence of individual and small groups of angulated, atrophic myofibers. There was no accompanying myofiber necrosis, inflammation, hypertrophy, or endomysial fibrosis. These abnormalities are consistent with muscle denervation (Fig. 4).

We used morphometric analysis to measure myofiber cross-sectional area and diameter in at least 100 fibers per sample. Mean myofiber diameter and cross-sectional area differed with respect to rat strain and corresponded with body weight such that myofibers were largest in LE rats, followed by SD rats, and were smallest in FS rats, which had the lowest mean body weights (see Table 1). The mean cross-sectional area and fiber diameter were smaller in ethanol-fed relative to control FS rats; however, due to the marked variation in myofiber diameter, the intergroup difference was only significant with respect to myofiber cross-sectional area (Table 3). Similarly, among SD rats, mean myofiber area and diameter were smaller in the ethanol-fed group, but the intergroup differences did not reach statistical significance due to marked variability in myofiber size in both groups. Among LE rats, however, chronic ethanol feeding resulted in markedly reduced mean myofiber area and diameter, and for both the differences relative to control were highly statistically significant ($P=0.0005$ for area; $P=0.0001$ for diameter) (Table 3).

DISCUSSION

Our experimental models of chronic ethanol feeding showed that a peripheral neuropathy developed in all 3 rat strains in the absence of nutritional deficiency. The control and ethanol-containing liquid diets were isocaloric, and the mean weight gain among pair-fed LE and SD rat strains was similar. Although ethanol-fed FS rats gained significantly less weight than control FS rats, their mean percentage weight gain was similar to that in ethanol-fed LE and SD rats. Moreover, the mean blood alcohol concentrations were similar among the 3 strains, indicating that the ethanol exposures were comparable and that any strain-related differences in the nature or severity of ALN would not likely be attributable to differences in nutritional status or ethanol exposure.

As in humans with ALN, we detected evidence of an axonal neuropathy manifested by significantly reduced CMAP amplitudes in the tibial nerve in FS rats, and significantly reduced mean nerve fiber diameters in all 3 strains after chronic ethanol exposure.

Correspondingly, we observed denervation of muscle characterized by individual and small groups of angulated atrophic fibers in ethanol-fed FS, LE, and SD rats. The manifestations of axonal injury varied in both nerves and muscles among the 3 strains, as would be expected in an acquired toxic peripheral neuropathy and in those that may have differing genetic susceptibilities. The more severe degrees of myofiber atrophy in ethanol-fed LE compared with ethanol-fed FS or SD rats suggests that LE rats are more susceptible to ethanol-induced axonal degeneration and that alcohol-mediated axonal neuropathy may not be entirely due to direct toxic effects of ethanol. Other factors may contribute to the development and propagation of alcohol-related peripheral neuropathy such as insulin/insulin-like growth factor resistance, oxidative stress, and genetic variation, similar to that observed in alcohol-related liver and brain degeneration.¹⁸ Correlation testing with electromyography was not performed, as it is a difficult test to perform and interpret in this animal model.

Chronic ethanol feeding also caused ALN associated with significantly slowed velocities in motor, but not sensory nerves in all 3 strains. The slowed nerve conduction velocities cannot be explained entirely by axonal changes. We observed subjectively that there may be patchy demyelination in the peripheral nerves of the ethanol-exposed strains, although quantitative confirmation of this observation awaits results of further studies, which are in progress. Such demyelination, if confirmed, would be a major factor in slowing nerve conduction. Demyelination has also been shown to contribute to alcoholic brain disease.¹⁸

These results indicate that we have successfully generated models of ALN in the absence of nutritional deficiency, thus nutritional deficiency is not a required pathogenic component of ALN. The results implicate direct toxic effects of ethanol as a mediator of ALN, because the similarly high blood alcohol levels among the different rat strains produced a peripheral neuropathy. As in humans with acquired toxic neuropathies, the effect of chronic alcohol exposure was variable in its effects on different nerves/muscles and in rats with differing genetic susceptibilities. This variation implicates a modulating effect of other factors, including, but not limited to, genetics, nutrition, and dose effect as a predisposing factor, *vis-a-vis* chronic high-level ethanol misuse.

The failure to detect any evidence of sensory nerve abnormalities despite well-documented symptoms of sensory neuropathy in humans² may be related to technical limitations of NCS in the rat model. It is also possible that the electrophysiological abnormalities of a more profound axonal predominant neuropathy seen in humans is a late manifestation of ALN, as many patients present months to years earlier with complaints of burning and tingling, which are more suggestive of small fiber involvement. The duration of alcohol exposure in the rat model may not have been long enough to produce a sensory neuropathy as is typically observed in humans. Demyelination has been observed in human pathological studies.²⁰ Our findings are similar to those observed in other animal models,^{21,22} and this implies there may be different structural targets of ethanol, such as axons and possibly myelin. More studies are needed to determine the role alcohol plays in the pathogenesis of ALN. This refined understanding may contribute to development of effective therapies for the treatment of this potentially debilitating condition.

Acknowledgments

This study was supported by the National Institutes of Health (AA-11431, AA-12908, and K24-AA-16126) and by a special fund from Rhode Island Hospital.

Abbreviations

ALN	alcohol-related peripheral neuropathy
ANOVA	analysis of variance
CMAP	compound muscle action potential
FS	Fisher strain
LE	Long Evans
LHE	Luxol Fast Blue/hematoxylin & eosin
NCS	nerve conduction studies
SD	Sprague-Dawley
SNAP	sensory nerve action potential

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FIGURE 1.

Conduction velocities. Adult Fisher 344 (FS), Sprague-Dawley (SD), and Long Evans (LE) male rats were fed chronically with isocaloric liquid diets containing 0% (control; C) or 37% ethanol (ET). After 7 weeks on the diets, NCS were performed on the (A) plantar, (B) tibial, and (C) fibular nerves. (A–C) Graphs depict nerve conduction velocity data from the 3 paired groups. There is a statistically significant difference in velocity between controls and alcohol-fed rats in the tibial and fibular nerves but not in the plantar nerve.

Representative Axonal diameter distributions

FIGURE 2.

Representative histograms of sciatic axonal diameter distributions for all 3 strains. Morphometric analysis of the 1- μ m-thick sections demonstrates significant leftward skewing of the histograms, reflecting increased percentages of small-diameter fibers in ethanol-fed relative to control rats in all 3 strains. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FIGURE 3.

Mean nerve fiber diameters were significantly smaller in ethanol-fed rats as compared with controls across all 3 strains.

Alcohol Related Myopathy

FIGURE 4.

Histological sections of gastrocnemius muscle in rats with alcohol-related myopathy. Adult male rats were fed chronically with isocaloric liquid diets containing 0% or 37% ethanol for 8 weeks. Gastrocnemius muscle tissue was fixed in Histofix, embedded in paraffin, and 5- μ m sections were stained with H&E. Images show representative areas of muscle from Long Evans (A) control or (B) ethanol-fed rats. Note relatively uniform myofiber size in the controls. There is wide variation in myofiber size, including groups of angulated atrophic fibers (reflecting denervation-marked with line borders), as well as hypertrophied and split fibers (myopathic, arrows). Original magnification: $\times 200$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table 1

Chronic ethanol exposure models; body weight gain (growth) and blood alcohol concentrations

	FS-control	FS-EtOH	SD-control	SD-EtOH	LE-control	LE-EtOH
Number of rats/group	12	14	12	13	12	13
Final body weight	357.70±8.01	339.50±5.89	413.7±9.7	397.10±12.97	429±16.01	392.8±22.8
Net weight gained	82.68±5.35	55.49±3.83 ^{‡§}	104.4±7.93	90.88±6.86	75.05±6.4 [¶]	64.18±6.92 [‡]
Weight gained (%)	30.63±1.98	21.16±1.42 ^{*‡}	34.75±2.73	30.02±2.34	21.04±1.77 ^{*‡}	19.59±2.32 ^{*‡}
Blood alcohol level (mg/dl)	1.8±0.38	108.0±25.8 [¶]	1.88±0.58	107.9±18.9 [¶]	2.62±0.87	129.9±12.0 [¶]

Adult Fisher (FS), Sprague-Dawley (SD), and Long Evans (LE) rats were pair-fed with isocaloric control or ethanol-containing (EtOH) liquid diets for 8 weeks, and weighed weekly. Net mean body weight (grams) and mean percentage body weight gained were calculated. Blood alcohol concentrations (mean±SEM) are indicated. Intergroup comparisons were made using the mixed model ANOVA and Bonferroni post hoc tests of significance. The interstrain differences in blood alcohol concentrations were not statistically significant.

* Significantly lower than FS-control (P<0.05).

[‡] Significantly lower than SD-control (P<0.001) and SD-EtOH (P<0.05).

[‡] Significantly lower than FS-control (P<0.05).

[§] Significantly lower than SD-control (P<0.001) and SD-EtOH (P<0.01).

[¶] Significantly increased in ethanol group relative to control.

Table 2A

Peripheral nerve conduction: Fisher 344 strain.

Peripheral nerve	Latency (ms)		Amplitude (mV)	
	Control	Ethanol	Control	Ethanol
Plantar nerve	0.87±0.03	0.85±0.03	34.43±5.71	58.20±29.59
Tibial nerve (ankle)	1.00±0.08	1.23±0.30	6.23±0.72	3.73±0.59
Tibial nerve (knee)	1.51±0.08	1.95±0.26	4.99±0.49	3.40±0.70
Fibular nerve (ankle)	1.14±0.08	1.08±0.13	6.60±0.62	5.18±1.15
Fibular nerve (knee)	1.65±0.06	1.75±0.06	5.99±0.70	5.08±1.35

NS, not statistically significant.

* Statistically significant (P<0.05).

Table 2B

Nerve conduction: Sprague-Dawley strain.

Peripheral nerve	Latency (ms)			Amplitude (mV)		
	Control	Ethanol	P-value	Control	Ethanol	P-value
Plantar nerve	0.84±0.02	0.84±0.02	NS	91.99±11.16	103.20±12.95	NS
Tibial nerve (ankle)	1.19±0.05	0.90±0.13	0.0161*	6.93±0.43	8.11±1.14	NS
Tibial nerve (knee)	1.68±0.06	1.52±0.14	NS	6.21±0.34	6.82±0.69	
Fibular nerve (ankle)	1.08±0.05	0.78±0.10	0.0057*	10.70±0.90	9.06±1.42	NS
Fibular nerve (knee)	1.56±0.04	1.44±0.12	NS	8.11±0.66	8.14±1.24	NS

NS, not statistically significant.

* Statistically significant (P<0.05).

Table 2C

Nerve conduction: Long-Evans strain.

Peripheral nerve	Latency (ms)		Amplitude (mV)	
	Control	Ethanol	Control	Ethanol
Plantar nerve	0.91±0.01	0.96±0.03	77.29±8.92	78.49±11.18
Tibial nerve (ankle)	1.24±0.06	1.18±0.08	4.37±1.28	4.85±0.45
Tibial nerve (knee)	1.69±0.05	2.00±0.07	3.97±1.13	4.58±0.50
Fibular nerve (ankle)	1.23±0.07	1.11±0.08	5.68±0.81	5.76±0.86
Fibular nerve (knee)	1.71±0.09	1.81±0.08	4.94±0.58	5.42±0.83

NS, not statistically significant.

* Statistically significant (P<0.05).

Table 3

Alcohol-induced gastrocnemius muscle atrophy-rat strain differences in susceptibility.

	Control	Alcohol	<i>P</i> -value
Myofiber diameter (μm)			
Fisher 344	22.62 $\pm$ 5.49	19.93 $\pm$ 3.30	0.08
Sprague-Dawley	35.60 $\pm$ 8.94	28.54 $\pm$ 4.00	0.07
Long Evans	45.83 $\pm$ 5.08	25.34 $\pm$ 4.38	0.0001
Myofiber area (μm^2)			
Fisher 344	718.85 $\pm$ 291.48	406.93 $\pm$ 152.61	0.04
Sprague-Dawley	1517.73 $\pm$ 756.02	1003.55 $\pm$ 351.59	NS
Long Evans	2338.65 $\pm$ 643.04	695.08 $\pm$ 163.35	0.0005

Adult male Fisher 344 (FS), Sprague-Dawley (SD), or Long Evans (LE) rats were maintained chronically on isocaloric liquid diets containing 0% (control) or 37% ethanol by caloric content. After 8 weeks, gastrocnemius muscles were harvested, fixed, and embedded in paraffin. H&E-stained histological sections were subjected to image analysis to measure myofiber diameter and cross-sectional area. At least 200 fibers per specimen are indicated. Intergroup comparisons were made using Student t-tests. NS, not statistically significant.