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Ethanol-related Increases in Degenerating Bodies in the Purkinje Neuron Dendrites of Aging Rats

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Abstract

Chronic ethanol consumption in aging rats results in regression of Purkinje neuron (PN) dendritic arbors (Pentney, 1995), loss of synapses (Dlugos and Pentney, 1997), dilation of the smooth endoplasmic reticulum (SER), and the formation of degenerating bodies within PN dendrites (Dlugos 2006 a, b). Dilation of the SER and the formation of degenerating bodies may be a predictor of dendritic regression. Ethanol-induced effects on mitochondria may be involved as mitochondria cooperate with the SER to maintain calcium homeostasis. The purpose of this study was to determine whether degenerating body number and mitochondrial density and structure are altered by chronic ethanol treatment in PN dendrites. Male, Fischer 344 rats, 12 months of age, were fed an ethanol or pair-fed liquid diet, or rat chow for a period of 10, 20, or 40 weeks (15 rats / treatment; 45rats / treatment duration). Ethanol-fed rats received 35% of their calories as ethanol. At the end of treatment, all animals were euthanized, perfused, and tissue prepared for electron microscopy. The densities of degenerating bodies and mitochondria, mitochondrial areas, and the distance between the SER and the mitochondria were measured. Results showed that there was an ethanol-related increase in degenerating bodies compared to controls at 40 weeks. Ethanol-induced alterations to mitochondria were absent. Correlation of the present results with those of previous studies suggest that degenerating bodies may be formed from membrane re-absorption during dendritic regression or from degenerating SER whose function has been compromised by dilation.

Keywords

alcohol; dendrites; aging; mitochondria; myelin figures; degeneration

I. Introduction

Aging increases alcohol sensitivity and effects (Vestal et al., 1977; National Institute of Alcohol Abuse and Alcoholism, 1998; Breslow et al., 2003) both by enhancing the aging process and by predisposing individuals to premature aging (Spencer and Hutchison, 1999). The mechanisms behind age-related enhancement of alcohol effects may include increased rates of the cellular deterioration mechanisms that accompany normal aging (Yu et al, 1996), the pharmacokinetic-induction of higher BAL in older compared to young adults (Vestal et al., 1977), and age-related decreases in the ability to develop alcohol tolerance (Spencer and

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McEwan, 1997). Brain areas, such as the cerebellum are particularly vulnerable to alcohol (Victor et al., 1959; Walker et al., 1981; Sullivan et al., 2000, 2002; Oscar-Berman and Marinkovic, 2003) and may show enhanced alcohol-induced effects as even moderate drinking of alcohol may result in adult brain pathologies (Mukamal et al., 2001). Ethanol-related effects on the cerebellum appear to be permanent as abstinent uncomplicated alcoholics retain discrete motor deficits related to cerebellar functions (York and Biederman, 1991; Davalia et al., 1994; Woodruff-Pak et al., 1996; Desmukh et al., 2002; Sullivan et al., 2000, 2002).

The cerebellar Purkinje neuron (PN), the sole output neuron of the cerebellar cortex, is a major site of ethanol's action on the cerebellum (Victor et al., 1959; Walker et al., 1981). In an aging Fischer 344 rat model, chronic ethanol consumption for a period of 40 weeks results in regression and degeneration of the very extensive PN dendritic arbor (Pentney and Quackenbush, 1990, 1991; Pentney, 1995; Pentney and Dlugos 2000). PN dendritic regression occurs simultaneously with an accompanying decline in the total number of synapses/ PN (Dlugos and Pentney, 1997). The mechanism behind ethanol-related regression of PN dendrites is unknown but ultrastructural observation of ethanol-related alterations of dendritic organelles may provide cues to cellular components and mechanisms behind the ethanol-related dendritic degeneration that precedes regression.

Structures, named degenerating bodies (Dlugos 2006 a,b) have been observed in PN dendrites with electron microscopy. Degenerating bodies do not fit the well described characteristics of SER, mitochondria, or microtubules, the major PN dendritic organelles (Palay and Palay, 1974). Degenerating bodies in PN dendrites consist of whorls of membranes or vesicular structures bound by membranes (Dlugos 2006a). Degenerating bodies were previously observed in two studies in which ethanol-related SER dilation was quantitated in both male (Dlugos 2006 a) and female (Dlugos 2006 b) rats. Degenerating bodies are not unique to PN dendrites as similar structures have been repeatedly observed in other neurons and neuronal processes and have been given a variety of labels including dense bodies (Babel et al., 1970), myelin ovoids (Pavelka and Roth, 2005), vacuoles (Feldman; 1976), membranous inclusions (Feldman, 1976), myelin figures (Morara et al., 2001), and membranes in the stages of destruction (Bogolepov, 1981). Degenerating bodies occur in cortical neurons (Feldman, 1976; Iontov and Shefer, 1991; Peters et al., 1994), PN (Chen and Hillman, 1999), and brainstem neurons (Casey and Feldman, 1985) and are present in pathologies such as hydrocephalus (Kriebel et al., 1993), chronic *tau* expression (Hall et al., 2000), HIV (human immunodeficiency virus) (Michaud et al., 2001), anoxia (Malunova and Samolilov, 1984), and aluminum intoxication (Wakayama et al., 1992).

The smooth endoplasmic reticulum (SER) in PN forms a continuous calcium storage system extending from the cell body to the dendritic spines (Martone, 1993). Significant levels of SER dilation have been demonstrated with electron microscopy in 22 mo old F344 rats following chronic ethanol consumption for 40 weeks (Dlugos and Pentney, 2000, Dlugos, 2006a,b). Dilation of the SER within PN dendritic branches may indicate increases in intracellular calcium (Garthwaite et al., 1993; Henkart, 1975), lead to SER stress (Szczesna-Skorupa et al., 2004), and culminate in dendritic degeneration. Mitochondria may also be altered as mitochondria work with the SER to maintain calcium balances and accept calcium overload (Simpson and Russell, 1998). Mitochondria reside in close cytosolic proximity to the SER (Mannella et al., 1998; Wang et al., 2000) and have been demonstrated to move even closer when increased buffering with intracellular calcium is required (Darios et al., 2005).

Ethanol-induced structural alterations in the mitochondria, themselves, may also accompany chronic ethanol consumption. Recent studies indicate that normal mitochondrial shape and size are indicative of balances in the molecular components that regulate mitochondrial fusion and fission, and, as such, control mitochondrial function (Chan, 2006). Abnormal mitochondrial

shapes have been observed in PN soma of young rats following 3 or 6 months of ethanol consumption (Tavares and Paula-Barbosa, 1983). Analyses of ethanol-related alterations in mitochondrial size using *in vivo* models, therefore, might be a direct measure of analyzing ethanol-related alterations on mitochondrial fusion and fission pathways. For example, it is now recognized that mitochondrial dysfunctions appear to be at the root of many neuropathologies (Cassarino et al, 1999). Disruption of mitochondrial fusion machinery results in the fragmented mitochondria found in neurodegenerative diseases (Chan, 2006) and may be responsible for the mitochondrial enlargement (Koch et al., 1978, 2004; Tabouy et al., 1998) and altered mitochondrial function (Cahill et al., 2005) that are recognized effects of alcohol in the liver.

There is some precedence for ethanol-related alterations in cerebellar mitochondria as ethanol-induced alteration in volume density (Oudea et al., 1989) and function (Cahill et al., 2005) have been noted in hepatocytes. Reports of ethanol-induced alterations of mitochondrial volume in PN soma are conflicting as ethanol-induced increases have been shown in one study but not in another. In the first study, 3 and 6 months of chronic ethanol treatment in young, adult rats (Tavares and Paula-Barbosa, 1983 b) resulted in a significant increase in PN somal mitochondrial density. In another study, intragastric administration of ethanol of young rats for a 3 month period did not alter the mitochondrial density within PN soma (Lewandowska et al., 1994).

The present study sought to determine whether, chronic ethanol consumption in aging rats results in ultrastructural alterations in any other components of the PN dendritic arbor in addition to the SER dilation previously demonstrated (Dlugos and Pentney, 2000; Dlugos 2006 a, b) that would provide valuable cues to the mechanisms behind ethanol-related dendritic regression. First, the density of degenerating bodies within PN dendrites was evaluated during a time course of 10, 20, and 40 weeks of treatment to determine whether ethanol perturbs age-related degeneration. Second, ultrastructural alterations in the density, size, and distance between mitochondria and the SER in PN dendrites were examined. An ethanol-related increase in the number of degenerating bodies in PN dendrites and mitochondrial involvement in ethanol-induced dendritic degeneration would provide further knowledge about the events surrounding previously reported ethanol-induced PN dendritic regression (Pentney and Quackenbush, 1990, 1991; Pentney, 1995; Pentney and Dlugos, 2000).

2. Results

Brain and body weight

Two-way ANOVA with treatment and duration of treatment as factors showed that ethanol did not alter brain weight [$F(8,135) = 1.75$, $P = .185$]. With longer duration of treatment and increased age, brain weight did increase. Mean brain weight in the 10 week treatment group were significantly smaller than in the 20 or 40 week treatment group [$F(8,135) = 1.75$, $P = .185$], values being 2.02, 2.23, and 2.08 g, respectively. There was a main effect of treatment [$F(8,135) = 20.824$] and duration of treatment [$F(8,135) = 28.702$] on body weight. Rats receiving ethanol and pair-fed treatments weighed significantly more than chow-control rats whereas, all rats treated for 10 weeks, weighed significantly less than those treated for 20 and 40 weeks.

Morphology

Similar to the results of previous studies, chronic ethanol consumption did not result in marked alteration of the general morphology of the molecular layer of the cerebellum (Dlugos and Pentney, 1997; Dlugos 2006 a, b). The molecular layer began superior to the large PN soma at the level of the primary PN dendrite (Fig 1A). PN dendrites, spines, and parallel fibers, and

synapses were easily visualized in all fields of the molecular layer to the pial surface. Many orders of dendritic branches arise from the primary dendrite. Proximal dendritic branches with few dendritic spines and distal dendrites, heavily laden with dendritic spines, were readily observed. In proximal (Fig 1 C) and distal dendritic profiles (Fig 1 D, E), numerous mitochondrial and SER profiles were apparent. Numerous microtubules were observed in the larger dendritic shafts (Fig 1C). Mitochondria were closely opposed to the SER in many of the branches (Fig 1 C, D, E). In the rats treated with ethanol for 20 to 40 weeks, the SER profiles appeared swollen or dilated, a finding that has been confirmed quantitatively (Dlugos and Pentney 2002; Dlugos 2006 a; Dlugos, 2006 b). In some of the PN dendritic shafts and branchlets, degenerating bodies were observed similar to that which has been shown in a host of various diseases and experimental conditions (Feldman, 1976; Iontov and Shefer, 1981; Malunova and Samoilov, 1984; Kriebel et al., 1993; Peters et al., 1994; Michaud et al., 2001; Morora et al., 2001; Dlugos 2006 a, b). Degenerating bodies appeared as whorls of membrane, termed myelin figures (Morora et al., 2001) (Fig 2 C,D, E) or as membrane bound structures with a center core of smaller membrane-bound vesicles (Fig 2 A, B, F). Mitochondria within the PN dendrites did not appear swollen and abnormalities in mitochondrial shape were uncommon and equally distributed among the three treatment groups. In some of the larger dendrites of the ethanol-fed rats, microtubules and other cytoskeletal components were absent in the proximity of dilated SER and/ degenerating bodies (Fig 2F).

Density of degenerating bodies

Two way-ANOVA showed significant effects of treatment [$F(2,126)=9.583$, $P<.001$] (Fig 3) and treatment duration [$F(2,126)=3.185$, $P=.045$] on the density of degenerating bodies within dendrites. There was also a significant interaction between treatment and duration of treatment [$F(4,126)=3.834$, $P=.006$] (Fig 4). The Tukey post-hoc test showed that at 40 weeks, ethanol-fed rats had a significantly higher number of degenerating bodies than pair ($P<.001$) and chow fed rats ($P=.015$). At 20 weeks, the density of degenerating bodies was increased compared to the chow fed rats ($P=.011$) whereas comparison with the pair-fed rats approached ($P=.085$) but did not reach significance. Duration of treatment analysis showed that in addition, to alcohol effects, there was an age-related increase in the number of degenerating bodies at 40 weeks compared to 10 weeks ($P=.045$).

Measurements of mitochondria

There were no effects of dietary treatment ($P=.650$) or duration of dietary treatment ($P=.286$) on the density of mitochondria within PN dendritic branches (Fig 5). At 40 weeks, mean mitochondrial area within PN dendrites was not altered ($F(2, 42)=0.586$, $P=.561$) by ethanol treatment. Similarly, at 40 weeks, the distance between mitochondria and the SER at <1 ($P=.967$), <3 ($P<.996$), or <4 ($P<.838$) microns did not change (Fig 6).

3. Discussion

The results of this study confirm that chronic consumption of ethanol during aging results in increased degeneration within PN dendrites that accompanies ethanol-related dendritic regression in the terminal dendritic segments of the PN of aging, ethanol-fed rats (Pentney and Quackenbush, 1990, 1991; Pentney, 1995; Pentney and Dlugos, 2000). The present study also shows that ethanol-related PN dendritic degeneration probably does not involve structural changes to the mitochondria.

Degenerating bodies have been observed in two other studies on PN in this laboratory. In one study, degenerating bodies were observed in close proximity to dilated SER in aging male rats (Dlugos, 2006 a) but were not quantitated. In another study, ethanol-related increases in degenerating body number were analyzed in the anterior lobe of the cerebellar vermis after 40

weeks of chronic ethanol treatment (Dlugos 2006b). These results, taken with the quantitative results of the present study, suggest that the formation of degenerating bodies is not a gender-specific event. Degenerating bodies are not unique to PN dendrites as they are present in dendrites of the cerebral cortex (Feldman, 1976; Iontov and Shefer, 1991; Peters et al., 1994) and brainstem (Casey and Feldman, 1985). Similarly, degenerating body formation is not a unique effect of chronic ethanol consumption as degenerating bodies are present in hydrocephalus (Kriebel et al., 1993) aluminum intoxication (Wakayama et al., 1992), and HIV (human deficiency virus) (Michaud et al., 2001), over-expression of *tau* (Hall et al., 2000), anoxia (Malunova and Samolilov, 1984), and trichloroethylene treatment (Haglid et al., 1981).

Degenerating bodies do not appear to be age specific as they have been observed during development in PN dendrites undergoing remodeling as they form synaptic connections with climbing fibers (Morara et al., 2001). Degenerating bodies do not however, appear to accompany developmental exposure to alcohol as there is no mention of them in one of the few electron microscopic studies available on this topic (Volk, 1984). The presence of dendritic degenerating bodies may distinguish ethanol-related effects and mechanisms on PN in young, developing rats from the effects of chronic ethanol consumption in old rats. In developing rats, ethanol-induced PN death occurs (Volk, 1984) during a specifically timed developmental window of vulnerability (Hamre and West, 1993; Thomas et al., 1998). In old rats, PN death is not a consequence of lengthy periods of chronic ethanol (Dlugos et al., 2005) rather SER dilation (Dlugos and Pentney 2000; Dlugos 2006 a,b), increased number of degenerating bodies shown here, and PN dendritic regression are constants (Pentney, 1995; Pentney and Dlugos, 2000). These data suggest that degenerating bodies may occur in any region where ethanol consumption results in dendritic regression or remodeling rather than neuronal death. PN dendritic regression has been reported in PN of young adult rats (Tavares et al., 1983 a) and in hippocampal pyramidal neurons (McMullen et al., 1981) but these finding cannot be correlated to the present study as ultrastructural analyses were not performed.

Many of the degenerating bodies found in the dendrites of aging ethanol-fed rats (Fig 2) resemble the myelin figures observed in the early years of electron microscopy (Carbonell and Pollack, 1962; Flaks, 1968, Hohl, 1967). The presence of myelin figures was formerly ascribed to artifacts of glutaraldehyde fixation (Curgy, 1968). Myelin figures, however, have been observed in unfixed specimens (Cole and Aldrich, 1971), well fixed tissue (Morara et al., 1990), human diseases (Tani et al., 1971; Park et al., 1999), and a wide range of species including humans (Tani et al., 1971), fungi (Cole and Aldrich, 1971), and zebrafish (Anken et al., 2004). In the present study as in many of the other studies cited above in which myelin figures were detected (Feldman, 1976; Haglid et al., 1981; Casey and Feldman, 1985; Malunova and Samolilov, 1984; Iontov and Shefer, 1991; Morara et al. 1990 ; Wakayama et al., 1992; Kriebel et al., 1993; Peters et al., 1994; Chen and Hillman, 1999; Michaud et al., 2001), the normal appearance of the tissue argues against myelin figures representing fixation artifact. Furthermore, it is unlikely that the present finding of ethanol-related increases in degenerating structures within PN dendrites would have resulted from a fixation artifact.

There are several significances ascribed to the myelin figure form of degenerating bodies in the literature that may be useful in understanding the presence of these structures in the dendrites of ethanol-fed rats. The hypothesis that myelin figures represent regions of heavy membrane turnover during development (Hildebrand et al., 1994; Morara et al., 1990) or in degeneration or pathological conditions (Tanaka et al., 1988; Tani et al., 1971; Higashi, 1995; Park et al., 1999) is pertinent in the aging rat model in which chronic ethanol consumption results in dendritic regression. Dendritic regression may result in heavy membrane turnover as it involves the retraction of dendritic processes and retrieval of dendritic organelles (Chendotal and Sotelo, 1993). In addition to the plasmalemma, some of the membrane within the

degenerating bodies in PN dendrites may represent retrieved membrane from the SER, the continuous membrane storage system within PN dendrites (Martone, 1993). Ethanol-related SER dilation has been reported in aging ethanol-fed rats (Dlugos and Pentney 2000, Dlugos 2006 a, b) and the presence of degenerating bodies within the dendrites may also portend further dendritic degeneration.

The formation of degenerating bodies was associated with the SER long ago (Feldman, 1976), an association that the results of this study also suggest. A relationship between ethanol-related SER dilation (Dlugos and Pentney, 2000; Dlugos 2006 a) and the formation of degenerating bodies is suggested here as both structures show significant increases in the ethanol-treated groups following 40 weeks of treatment. It is not difficult to envision how the extreme ethanol-induced dilation of the SER demonstrated in previous studies (Dlugos 2000, Dlugos 2006 a, b) may rapidly lead to the formation of degenerating bodies in the PN dendrites of the ethanol-fed rats. The development of degenerating bodies within PN dendrites may be visible evidence of SER collapse as a result of ethanol-related SER dilation (Dlugos and Pentney, 2000; Dlugos 2006 a,b) and ethanol-related stress within the SER of PN dendrites. ER (endoplasmic reticulum) stress has been observed in the SER (Szczena-Skorupa et al., 2004), one of the two major subcompartments of the ER (Rolls et al., 2002).

Ethanol-related alterations in mitochondria within PN dendrites were not found in the study. The lack of alterations in areal mitochondrial density within PN dendrites of the old, ethanol-fed F344 rats reported here suggests that chronic ethanol treatment did not alter the distribution or number of mitochondria in the population of PN dendrites unaffected by previously reported PN dendritic regression (Pentney and Quackenbush 1990, 1991; Pentney, 1995; Pentney and Dlugos, 2000). The total number of mitochondria within the PN dendritic arbor would, however, be decreased after 40 weeks of treatment due to an overall loss of mitochondria within the degenerating branches. Should the process of dendritic regression result in reabsorption of mitochondrial membranes, degenerating structures may also contain fragments of mitochondrial membranes. Mitochondrial densities within PN dendrites have not been previously determined although two studies of ethanol-related alterations of volume fraction of mitochondria within PN soma yielded conflicting results. In one study, there was an ethanol-related decrease in mitochondrial volume density within the PN soma (Tavares and Paula-Barbosa, 1983b) whereas in the other study, mitochondrial volume density remained unchanged. (Lewandowska et al., 1994).

After 40 weeks of treatment, the duration simultaneous with reported dendritic regression (Pentney and Quackenbush 1990, 1991; Pentney, 1995; Pentney and Dlugos, 2000), abnormalities in mitochondrial area indicative of possible changes in mitochondrial function (Chan, 2006) were not determined. This finding is dissimilar to findings of megamitochondria in the liver (Koch et al., 1978; Tabouy et al., 1998) although it has been recognized for a long time that brain mitochondria appear less sensitive than liver mitochondria to ethanol (Thayer and Rottenberg, 1992). In the current study, in addition to measuring mitochondrial area, unusual sizes and shapes of mitochondria were recorded within the three groups. Malformed mitochondria were rare, too few to count, and were distributed equally among the three groups. Elongated or circular mitochondria described in the PN of young, ethanol-fed rats (Tavares and Paula-Barbosa, 1983b) were not noted in this study.

It was expected that ethanol-related alterations in the distance between the mitochondria and the SER would occur as mitochondria assist in buffering of intracellular calcium within cells (Simpson and Russell, 1998). Although this effect has been reported, in the case of ceramide-induced cell death in PC12 cells (Darios et al., 2005), it may not occur in PN dendrites as PN death does not accompany chronic ethanol consumption in old rats (Dlugos et al., 2005). Different mechanisms may underlie dendritic regression and pathways leading to cell death.

The fact that similar magnifications of the electron microscope were used in the ceramide study (Darios et al, 2005) and the current study suggests that if ethanol-related changes were present in the distance between SER and mitochondria occur, they would be detected. Furthermore, in the current study, data were sorted over three ranges of distance so that measuring distances over greater ranges (e.g., < 4 versus < 1 microns) would not mask actual differences within the smaller ranges.

In the present study, it was noted that in some of the larger PN dendrites from the ethanol-fed rats, the region directly surrounding the degenerating bodies was devoid of cytoskeleton, a finding suggesting that ethanol-induced changes in microtubules may also occur. Microtubules were not quantitated here although it is clear that ethanol-related alterations in microtubules would severely impact dendritic trafficking. Ethanol-related alterations in microtubules are not without precedence. For example, in PC-12 cells, chronic ethanol treatment increased microtubule content while decreasing the amount of free tubulin (Reiter-Fund and Dohrman, 2005). Pharmacologically relevant concentrations of alcohol have also been shown to increase phosphorylation of MAP (microtubule associated protein) resulting in inhibition of microtubule assembly (Ahluwalia et al., 2000). In vivo studies, in young rats, chronically fed ethanol for 1,3,6,12, and 18 months have demonstrated a decreased microtubule density within PN dendrites (Paula-Barbosa and Tavares, 1985).

In conclusion, the results of this study show that the appearance of degenerating bodies in PN dendrites occurs in an aging, ethanol-fed model in which SER dilation and PN dendritic regression had been previously reported. The presence of degenerating bodies suggests the presence of membrane reabsorption following regression and/or degeneration as a result of SER dilation. Degenerating bodies become quite large and may serve as barriers that impair dendritic transport leading to functional pathologies within the dendrite. In addition, the lack of structural alterations in mitochondria within PN dendrites suggests that ethanol-related dendritic degeneration must involve the other main dendritic components, such as the SER or microtubules.

4. Experimental Procedures

Animal model

One hundred and twenty male Fischer 344 rats were used in this study. Upon arrival at the laboratory animal facility at the State University of New York at Buffalo, rats were divided into three treatment groups for a period of 10, 20, or 40 weeks. These treatment durations were selected as, previous studies have shown that at 20 weeks, degeneration of dendrites can be detected (Dlugos, 2006a; Pentney and Quackenbush, 1990) and at 40 weeks, ethanol-related degeneration of dendrites has occurred (Dlugos, 2006a, b; Pentney and Quackenbush 1990, 1991). Ten weeks of treatment was used as the analysis starting point because ethanol-related alterations to dendritic structure were not found at this early treatment duration (Dlugos 2006 a).

Rat diets included standard rat chow (CF), the AIN-93M liquid ethanol diet (Dyets Inc, Bethlehem, PA) (EF), or the AIN-93M liquid control diet (PF) (Dyets, Inc). PF rats received the same amount of diet that their EF partners consumed on the previous day. Dietary intakes were recorded daily and rats were weighed weekly. Blood alcohol concentrations (BAC) were determined enzymatically in a subset of EF rats following four weeks of treatment. Tails were nipped one hour after the beginning of the 12 hr dark cycle. Mean BAL was $88.7 \pm \text{dl}$. The health of each rat was assessed daily and weights taken weekly. All animal procedures were approved by the Institutional Animal Care and Use Committee of the State University of NY at Buffalo.

Tissue preparation

Tissue preparation is described elsewhere in detail (Dlugos and Pentney, 1997; Dlugos, 2005; Dlugos 2006 a; Dlugos 2006 b). Briefly, at the end of treatment, rats were anesthetized with sodium pentobarbital (100mg/kg) and the brains fixed by perfusion of a bolus of physiological saline followed by 300 ml of 1% glutaraldehyde; 1% paraformaldehyde in 0.1 M phosphate buffer (pH=7.4). The cerebellum was removed and the posterior lobe dissected through separation of the anterior from the posterior lobes at the primary fissure and elimination of lobule X. Lobules from each animal were coded to prevent bias on the behalf of the investigator. Lobules V1a, VI b+c, VII, VIII, and IX (Larsell, 1952) were separated, washed in PBS, postfixed in 1% osmium tetroxide, dehydrated in acetone, and embedded in an EMBED 812 –Araldite resin (Electron Microscopy Sciences, Washington, PA). Two posterior lobule blocks were selected randomly and 65 nm sections of the molecular layer obtained on a Reichert ultramicrotome. Sections were affixed onto 200-mesh hexagonal thin bar copper grids, and stained with uranyl acetate and lead citrate.

Quantitative analysis

Grids from each of the two posterior lobe blocks/rat were viewed with a Jeol-CX-100 transmission electron microscope. Fifteen fields (every fifth field) of the molecular layer, progressing from the pial surface to the supraganglionic plexus of Purkinje neuron collaterals were randomly selected, viewed, and photographed within the section contained within a 0.009 mm² hexagonal opening. All measurements on the negatives were performed using the UTHSCA Image tool program (<http://ddsdx.uthscsa.edu/dig/itdesc.html>). Standard rules of inclusion and exclusion of structures within the counting frame were applied (Gundersen, 1977). Section thickness was determined with the method of Small (1968).

First, the density of degenerating bodies within the PN dendritic profiles within a 494 cm² grid, representing 93.9 um² of the molecular layer area was analyzed by counting the number of the degenerating bodies/ dendrite and the area of the dendrite within the counting frame. Dendrites were not sorted as to proximal or distal position within the dendritic arbor rather results from all dendrites were pooled. Degenerating bodies were identified in dendrites as multi-lamellar structures in which the lumen was filled with membranous whorls or in which the lumen was filled with small vesicles that were distinctly different from the most normal dendritic components including mitochondria and SER (Feldman, 1976; Iontov and Shefer, 1981; Malunova and Samoilov, 1984; Peters et al., 1994; Dlugos 2006a, b). Analyses were carried out within the 10, 20, and 40 week treatment groups.

Next, the density of mitochondria within PN dendritic profiles within the 10, 20, or 40 week group was also determined within the 494 cm² grid area superimposed on each digitized negative by determining the number of mitochondria and the area of each dendritic profile containing the mitochondria. The area of the mitochondria and the mean distance between, mitochondria and smooth endoplasmic reticulum (SER) was determined at < 4, < 2, and < 1 micron distances were determined at the 40 week timepoint when ethanol-related degeneration reached significant levels (Pentney and Quackebush 1990,1991; Dlugos and Pentney, 1997; Dlugos 2006 a and b).

Statistics

Means and standard deviations were determined for mitochondrial density/ dendrite, degenerating body density/ dendrite, mean distance between the smooth endoplasmic reticulum (SER) and mitochondrial areas. Means were tested with ANOVA using the SPSS program. An alpha level of 0.05 was used.

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Fig 1.

Normal Purkinje neuron morphology in F344 rats between 15 and 22 mo of age. A. Purkinje neuron with visible nucleus and nucleolus (arrow). Purkinje neuron soma (asterisk) becomes tapered at origin of primary dendrite (arrowhead). B. Low magnification of Purkinje neuron dendritic shaft (DSH). Several branchpoints (arrows) are visible. Mitochondria are shown as electron dense profiles within the shaft (arrowheads). C. Higher magnification of a large Purkinje neuron dendritic shaft (DSH) close to the primary dendrite containing many longitudinal profiles of microtubules. A mitochondrion (arrowhead) is found next to the smaller of two SER profiles (asterisks). D. A small dendritic branchlet (arrow) with two dendritic spines (asterisks). A mitochondrion (arrowhead) lies next to a small profile of SER. E. A slightly

larger dendritic shaft (DSh) in which a large mitochondrion (arrow) lies close to an SER profile.
F A large dendritic shaft at a branchpoint (arrow) with a dilated SER profile (asterisk) and degenerating bodies (arrowhead).

Fig 2.

Degenerating bodies in dendritic shafts (DSH) of rats fed the pair-fed diet for 20 weeks (A), the ethanol diet for 20 weeks (B,C) and the ethanol diet for 40 weeks (D, E, F). In some cases the degenerating bodies (arrowheads) are filled with rounded vesicles (A, B, F) while in others they are lamellar in structure (C, D, E). Normal appearing mitochondria (arrowheads) are found in dendritic shafts A–E (DSH). In E a dilated SER profile (asterisk) is found in close proximity to the degenerating structures. In F, there degenerating bodies (arrowheads) occupy a part of the dendritic shaft devoid of cytoskeleton.

Fig 3.

Areal density (mm^2) of degenerating bodies in posterior lobe of the cerebellum following 10, 20 and 40 weeks of treatment. ANOVA showed significant effects of treatment [$F(2,126) = 9.583, P < .001$]. Post hoc analysis showed that at 40 weeks of treatment, EF rats had increased numbers of degenerating bodies compared to PF ($P < .001$) and CF ($P = .015, ++$) rats. At 20 weeks EF rats had increased degenerating bodies compared to the CF rats ($P = .011, +$). Comparison of EF to PF rats approached but did not reach significant levels ($P = .085$).

Fig 4.

The significant interaction between treatment and duration of treatment [$F(4,126) = 3.834$, $P = .006$] showed the pattern of the ethanol-related increases in degenerating bodies with increased treatment duration in Purkinje neuron dendrites. Although the numbers of degenerating structures are relatively the same among the treatment groups at 10 weeks, by 20 and 40 weeks, the density of degenerating structures in the EtOH-fed rats escalated at a higher rate than in any other treatment groups. The interaction occurred because the EtOH-fed group, with the smallest amount of degenerating structures at 10 weeks, far exceeded the other groups at 20 and 40 weeks.

Fig 5. Areal density of mitochondria (mm²) within Purkinje neuron dendrites of the posterior lobe of the cerebellum following 10, 20, and 40 weeks of chow-fed, pair-fed, or ethanol-fed dietary treatment. There was no effect of treatment ($P=.650$) or duration of treatment ($P=.286$) on mitochondrial density within PN dendrites.

Fig 6.

Distance (um) between SER and mitochondria in 40 week Chow-fed, pair-fed, and ethanol-fed rats. SER and mitochondrial distances were measured at <1, 3, and 4 microns. There was not effect of treatment at <1 ($P=.967$), <3 ($P=.998$), and <4 ($P=.838$) micron distances.