INTRODUCTION

Chronic ethanol consumption in adult and aging rats results in degeneration in the extensive dendritic arbor of the Purkinje neuron (PN), the major output neuron of the cerebellar cortex (Pentney, 1982, 1995; Tavares et al., 1983; Pentney and Quackenbush, 1990, 1991; Pentney and Dlugos, 2000). This laboratory has observed ethanol-induced alterations in dendritic components such as dilation of the smooth endoplasmic reticulum (SER) (Dlugos and Pentney, 2000; Dlugos, 2006a,b), formation of degenerating bodies (Dlugos, 2008) and decreases in the total number of synapses on the PN dendritic arbor (Dlugos and Pentney, 1997) that portend or enhance dendritic degeneration. The SER, the main intradendritic calcium storage compartment, is sensitive to chronic ethanol treatment (Lewandowska, et al., 1994; Dlugos and Pentney, 2000; Dlugos 2006a,b), hypoxia (Kaur et al., 2005), decreased calcium levels (Garthwaite et al., 1992) and valproate (Sobaniec-Lotowska, 2001).

The SER, the domain of the endoplasmic reticulum (ER) which lacks ribosomes, is most extensive in neurons such as PN that have extensive dendritic arbor. Three-dimensional reconstruction of the PN SER revealed an interconnecting network of hypolemmal cisternae, tubules aligned parallel to the long axis of the dendrite and small SER tubules within dendritic spines. The specific role of the SER in all neurons is to sequester calcium ions, thus lowering internal levels of intracellular free calcium [Ca^{2+}]_i. Calcium is a second messenger in a plethora of cellular events, including neurotransmission, motility, metabolism, protein synthesis, gene expression, cell cycle progression and apoptosis (Michalak et al., 1999; Corbett and Michalak, 2000). The SER is an essential component of the neuron as it prevents toxicity that results from extreme up-regulation of [Ca^{2+}]_i. Reported ethanol-related dilation of the SER (Dlugos and Pentney, 2000; Dlugos 2006a,b) may be due to ER stress as blockage of the unfolded protein response regulator GRP78/BIP results in SER dilation in HEK293 cells (Li et al., 2008). Further evidence that SER dilation and ER stress are related stems from studies in Trypansoma Brucei, in which the induction of ER stress is followed by ER dilation (Goldshmidth and Michaeli, 2011). A trigger for ER stress may be ethanol-induced alterations of some of the essential components of the SER. The sarco/endoplasmic reticulum Ca^{2+} ATPase (adenosine triphosphate) pump (SERCA) is responsible for uptake of calcium into the SER. Situated on the SER membrane, it couples hydrolysis of ATP with active transport of calcium, removing and storing excess [Ca^{2+}]_i from the cytosol following a calcium wave (Lytton et al., 1992; Berridge, 1998). Of the five isoforms of the SERCA pump, SERCA 2b is the predominant form in non-muscle cells (Wu et al., 1995) and PN dendrites (Villa et al., 1991; Takei et al., 1992, Xia et al., 1998). Ethanol-related decreases in cerebellar SERCA 2b mRNA levels have been shown in rats fed a chronic ethanol diet and further perturbed by ischemia (Xia et al., 1998). Other components of the SER may be altered by ethanol as well. For example, ethanol-induced decreases in mRNA levels for the inositol 1,4,5 triphosphate (IP_3) receptor, which sequesters Ca^{2+} into the SER in peripheral PN dendritic shafts and dendritic spines (Satoh et al., 1990; Villa et al., 1991; Martone et al., 1993), also occur in the hippocampus (Xia et al., 1998). Recent investigations are suggestive of some reciprocity between the SER calcium intake and output systems as decreases in the enzyme that phosphorylates inositol 1,4,5 triphosphate, inositol-1,4,5 triphosphate-3-kinase-A (itpkα) result in upregulation of SERCA 2b levels (Windhorst et al., 2012).

Another SER component is calreticulin, ascribed to be a major ER protein with a C-terminal KDEL ER signal for retention in the ER (Corbett and Michalak, 2000). Calreticulin...
also binds Ca\(^{2+}\) with high affinity, regulates the function of other ER proteins and affects calcium transport across the ER membrane (Ramsamooj et al., 1995; Corbett and Michalak, 2000; Michalak et al., 2002). Calreticulin has also been suggested as a modulator of the SERCA 2b pump as an interaction between the asparagine 1036 on the SER luminal tail of the SERCA 2b pump and calreticulin has been repeatedly proposed (John et al., 1998; Baker et al., 2002; Michalak et al., 2002). Calreticulin has also been localized in proximity to markers for the SER in cultured cells (Wang et al., 2000) and in close association with the IP\(_3\) receptor at presumed calcium release sites in the SER component of the oligodendrocyte (Simpson et al., 1997). Nuclear translocation of calreticulin has also been reported (Rojiani et al., 1991; Michalak et al., 1996; Holaska et al., 2001; Labriola et al., 2010). In situ labeling of the PN for calreticulin mRNA has been shown in rats, chickens and mice (Perrin et al., 1991; Abe et al., 1992), along with immunohistochemical staining in the PN (Perrin et al., 1991; Nori et al., 1993).

There is some indication that alterations in normal calreticulin levels within the SER may be associated with cellular changes that result in ER stress and the SER dilation that is present following chronic ethanol consumption for lengthy periods in aging rats (Dlugos and Pentney, 2000; Dlugos, 2006a,b). For example, in the mutant superoxide dismutase (mSOD1) model of amyotrophic lateral sclerosis, reduced levels of calreticulin triggered the ER stress response leading to death of motor neurons (Bernard-Marissal, et al., 2012). Nuclear expression of calreticulin was enhanced in squamous carcinoma cells that were treated with and resistant to ionizing radiation (Ramsamooj et al., 1995). In addition, calreticulin was considered a marker of ER stress when it was up-regulated in hepatocytes following 6 weeks of chronic alcohol consumption (Orlicky et al., 2011). There was, however, no change in calreticulin expression in a study in which spinal nerve ligation produced hyperalgesia (Gernes et al., 2009).

The purpose of the current study was to examine the possibility that chronic ethanol consumption in aging rats results in alterations in the calcium homeostatic systems within the PN dendritic SER that has been shown to be dilated following chronic ethanol consumption. A time course of expression of the SERCA 2b pump was performed and evaluated in PN soma and their dendritic processes. Similarly, a time course of calreticulin expression was undertaken in PN soma, PN nuclei, Bergmann glia and molecular layer nuclei. Behavioral tests, targeted to cerebellar function, were also undertaken to correlate with changes in PN calcium homeostatic components.

**MATERIALS AND METHODS**

**Animal model**

One hundred male Fischer 344 rats obtained from the NIAAA aging colony (Harlan, Inc.) served as subjects in these experiments. Ten baseline control animals (3 months of age) were used to evaluate the effect of age. The ninety 8-month-old rats were sorted for one of three dietary treatments (30 rats/diet) for durations of 10, 20 or 40 weeks (30 rats/treatment duration). The baseline control rats (3 months of age) and the chow-fed control rats (CF) received Teklad Global 18% Protein Rodent Diet (Teklad) and water *ad libitum*. The baseline control rats were compared with the CF rats and were used as a starting point for age effects on calcium homeostatic proteins and behavior. The pair-fed rats (PF) received the same volume of the AIN-93M liquid control diet that their ethanol-fed partners (EF) had consumed on the previous day. EF rats received 90 ml of the AIN-93M liquid ethanol diet in which 35% of the daily caloric intake was ethanol. Mean weekly intakes (±SEM) of the ethanol rats in the 10-, 20- and 40-week groups are shown in Fig. 1. Dietary intakes were recorded daily and animals were weighed weekly. Brain weights were recorded following perfusion. All procedures were performed with the approval of IACUC (Institutional Animal Care and Use Committee) of the State University of NY at Buffalo (Institutional Animal Welfare Assurance A3354-01).

**Fig. 1.** Mean (±SEM) weekly ethanol intakes over 10, 20 or 40 weeks of ethanol-fed rats. Ethanol-fed rats were offered 90 ml/day (n = 10/treatment group). Pair-fed liquid diet controls received the same amounts as their EF partners the previous day.
**Blood alcohol levels**

Blood alcohol levels were determined 1 h into the 12 h dark cycle. Enzymatic analysis with the Analox Alcohol Analyzer (Analox, Instruments, USA, Inc.) was used on a subset of ten EF rats following 4 weeks of treatment. Mean BAL (±SD) was 125.6 ± 44 mg/dl.

**Behavioral testing**

Ethanol-induced changes in balance, a function ascribed to the rat cerebellum (Tupper and Wallace, 1980), were assessed prior to the end of treatment. Rats were placed first on a square balance beam (2.54 cm/side) and then on a round balance beam (2.54 cm in diameter) suspended 40 cm above the floor (Zausinger et al., 2000). Padding was placed below the balance beam to prevent injury to the rat. The number of seconds/minutes that the rat could remain on the rod following placement on the beam was assessed. One training day was followed by three test days. Means of the three testing days were recorded/rat and/dietary and duration of treatment groups. Baseline control rats were tested 1 week after arrival in the laboratory animal facility and 4 days prior to being euthanized. Rats exposed to dietary treatment were tested during their last week of treatment.

**Tissue preparation**

At the end of treatment, all rats were euthanized by an overdose of Fatal Plus® (100 mg/kg) and perfused through the aorta with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The cerebellum was separated from the cerebrum and divided into the vermis and the two cerebellar hemispheres. Following cryoprotection in 30% sucrose in phosphate buffer, the cerebellum in each animal was completely sectioned at 40 µm and the number of sections recorded. All sections were retained for section selection.

A method for section selection was used to ensure adequate sampling of the cerebellum in the absence of bias (Gundersen, 1986). This process was initiated by choosing a number n from 1–10 with a random number table. This number demarcated the first number used for sampling. Subsequently, n + 1, …, n + 5 sections were also selected yielding a sextuplet of adjacent sections. Sixteen sextuplets of adjacent sections were randomly selected at set intervals throughout the cerebellum. All were stained and every other series (8 total) used for counts. Sections n were labeled with monoclonal anti-Serca 2b (1:500) (Sigma-Aldrich: St Louis, MO, USA) and the Vectastain ABC kit (Vector Laboratories, CA, USA) using DAB (3, 3-Diaminobenzidine) as the chromagen. Sections n + 1 were double labeled with monoclonal anti-SERCA 2b (1:500) (Sigma-Aldrich: St Louis, MO, USA) and polyclonal anti-calbindin (1:1000) (Sigma-Aldrich: St Louis, MO, USA) and secondarily labeled for immunofluorescence with secondary antibodies coupled to Alexa 488 and 568 (Invitrogen™, Life Technologies, Grand Island, NY, USA). Sections n + 2 were stained with anti-calreticulin (1:500) (Novus Biologicals), the Vectastain ABC kit and DAB. Sections n + 3 were stained with polyclonal anti-calreticulin (1:500) and monoclonal anti-calbindin (1:1000) and secondarily labeled for immunofluorescence with secondary antibodies coupled to Alexa 488 and 568 (1:100) (Invitrogen™, Life Technologies, Grand Island, NY, USA). Similarly, section n + 4 was stained with polyclonal anti-calreticulin (1:500) and monoclonal anti-glial fibrillary acidic protein (Sigma-Aldrich: St Louis, MO, USA). One of the extra n + 4 sections/rats were used for staining with neuronal specific nuclear protein (NeuN; EMD Millipore, MA, USA) and calreticulin to determine whether the molecular layer nuclei in the n + 2 sections were mainly stellate neurons or glial cells. Sections n + 5 were affixed on slides and stained with 0.5% cresyl violet in a solution of 1% sodium borate for measurements of the volume of the molecular layer.

Immunohistochemical staining was accomplished as follows. Free floating sections were washed in phosphate buffered saline (PBS), blocked with 1% bovine serum antibody for 30 min and exposed to the primary antibody or, in the case of double labeling, a mixture of primary antibodies overnight. Sections n and n + 3 were secondarily labeled with reagents from the anti-mouse or anti-rabbit Vectastain ABC kits (Vector Laboratories, CA, USA) followed by distilled water (2×) and DAB staining for 5 min. Sections were dehydrated on a slide warmer and mounted in Permount (Fisher Scientific: Pittsburgh, PA, USA). Sections n + 1, n + 3, n + 4 and NeuN stained sections were washed three times with PBS and exposed for 60 min to a mixture of Alexa 488 and 568 (1:100) (Invitrogen™, Life Technologies, Grand Island, NY, USA), dried, coverslipped and mounted in Prolong Gold antifade reagent (Invitrogen™, Life Technologies, Grand Island, NY, USA).

**Quantitative analyses**

The density of SERCA 2b + PN dendritic processes was determined on the eight n sections using an Olympus microscope with attached video camera and automatically moving stage, which is controlled by a keypad (Biopoint Keypad, Ludl Electronics Ltd, Hawthorne, NY, USA). The keypad is used to delineate the size of the tissue section and to program the moving stage to move the tissue section in a raster pattern from the top left to the bottom right corner of the section stopping at 0.85 mm intervals for counts. A Merz test system representing 5184 µm² of the molecular layer was used. This test system was selected to prevent bias due to the anisotropy of the PN dendrites within the cerebellar molecular layer (Merz and Schenk, 1970). Ten fields, five proximal to the PN soma and five proximal to the pial surface, were analyzed/ slide. Several n + 1 sections/rat were viewed with a Zeiss LSM-510 Meta NLO laser scanning confocal microscope to confirm SERCA 2b co-localization with calbindin D-28 in PN dendrites, as calbindin D-28 is a marker for PN and outlines the entire dendritic arbor (Arnold and Heintz, 1997).

Sections n + 2 were analyzed for the presence of calreticulin in the PN soma, dendrites, nucleus, Bergman glial processes and molecular layer nuclei. The Biopoint keypad (Ludl Electronics Ltd, Hawthorne, NY, USA) and moving stage was again used to select the five fields viewed on each of the eight slides used for counts. Fields of PN with calreticulin + nuclei, somas and/or dendrites were photographed at 20X. Densities were then determined by particle counting and measurement of the length of the PN line on the digitized images with the UTHSCSA image Tool program (http://compdent.uthscsa.edu/dig/itdesc.html). Calreticulin + PN dendrites were observed too infrequently to be counted. Areal densities of calreticulin + molecular layer nuclei and Bergmann glial fibers...
were determined at 40x. For each field, nuclei and fibers were counted and the area of the molecular layer/viewing field was measured with the UTHSCSA Image Tool. Densities for each rat were determined by mean number of nuclei or fibers/mean field area. The co-localization of calreticulin in calbindin containing PN and calreticulin in GFAP + Bergmann glial processes was confirmed with sections n + 2 and n + 3.

All sixteen n + 5 sections were stained with cresyl violet and used for volume measurements. The entire slide was digitized and area of the molecular layer was determined on each slide using the UTHSCSA Image Tool program (http://compdent.uthscsa.edu/dig/itdesc.html). Values of all slides were averaged to yield a mean molecular area/rat. Three random measurements of section thickness were taken/slide with a DG-3601 microcator (Ohno Sokki, Addison, Il) to determine a mean section thickness/slide. A mean section thickness/animal (t) was determined by averaging all values/slide. These values and total number of sections/cerebellum were averaged to yield a mean molecular area/rat. Three random measurements of section thickness were taken/slide with a DG-3601 microcator (Ohno Sokki, Addison, Il) to determine a mean section thickness/slide. A mean section thickness/animal (t) was determined by averaging all values/slide. These values and total number of sections/cerebellum were used to determine the reference volume $V_{ref}$ of the molecular layer with a previously reported modification of Cavalieri’s principle ($V_{ref} = P \cdot a \cdot t \cdot k$) (Sterio, 1984; Dlugos and Pentney, 2001). In this modification, molecular layer area (MLA) is determined with the UTHSCSA Image Tool program instead of point counting ($P \cdot a \cdot t \cdot k$) on each slice.

Statistical analyses

Means ($\pm$SEM) for all variables were determined within each rat, treatment group and duration of treatment. Two-way ANOVA analysis was used to compare dietary treatment and duration of treatment in the 90 CF, PF and EF rats treated for 10, 20 or 40 weeks. One-way ANOVA with the post hoc Tukey test was also used to localize differences between dietary treatment groups. One-way ANOVA was also used to evaluate the effects of age. Age comparisons were made between the baseline controls and the CF rats from the 10-, 20- and 40-week treatment durations. An alpha value of 0.05 was considered as significant.

RESULTS

Body and brain weights

There was an effect of dietary treatment [$F_{diet}(2,81) = 5.470, P = 0.006$] and duration of treatment [$F_{duration}(2,81) = 19.58, P < 0.001$] on body weight. When rats from all duration of treatments were combined, the lowest ($\pm$SEM) body weights were in the CF (458 g ± 11.8) and the highest in the PF rats (491 ± 7.4) ($P = 0.005$). Mean ($\pm$SEM) body weight in the EF rats was (468.4 g ± 5.8). After 10 ($P = 0.046$) and 20 weeks of treatment ($P = 0.005$), PF rats were heavier than CF rats. Following 40 weeks of treatment, there were no significant differences in body weights among all the three treatment groups. All the rats gained weight through the treatment, the gain being significant in the PF ($P < 0.001$) and EF ($P < 0.001$) rats between 10 and 40 weeks. Age-related weight increase was demonstrated by the significant gains in weight in the 10-, 20- or 40-week CF rats compared with the 3-month-old baseline controls [$F_{age}(3,36) = 31.083, P < 0.001$].

Dietary treatment [$F_{diet}(2,81) = 14.690, P < 0.001$] had significant effects on brain weight. Combining brain weights of rats from all durations of treatment showed that CF brains weighed more than both PF ($P = 0.006$) and EF ($P < 0.001$) brains. EF rats had lower brain weights than the CF rats after 20 ($P < 0.001$) and 40 weeks of treatment ($P = 0.030$). PF rats had lower brain weights than the CF rats after 40 weeks ($P = 0.041$). Duration of treatment [$F_{duration}(2,81) = 10.762, P = 0.001$] also affected brain weight in the PF rats in which there was an increase in brain weight between 10 and 40 weeks of treatment. In the CF rats, there was a similar increase between 10 and 20 weeks ($P < 0.001$) and 10 and 40 weeks of age ($P < 0.05$). There were no significant increases in brain weight in the EF rats during treatment. Brain weight increased with age at 10, 20 and 40 weeks compared with the 3-month-old baseline controls [$F_{age}(3,36) = 42.048, P < 0.001$].

Behavior

Alcohol consumption decreased balance time on the square rod compared with the CF rats [$F_{diet}(2,81) = 21.897, P < 0.001$] after 20 ($P = 0.006$) and 40 weeks of treatment ($P < 0.001$) (Fig. 2). After 40 weeks of treatment, the performance of the EF rats approached significance compared with the PF rats ($P = 0.093$). There was an overall effect of duration of treatment on square rod performance [$F_{duration}(2,81) = 15.047, P < 0.001$] attributable to the 10- or 20-week EF rats ($P < 0.001$). Differences in square rod behavior between the EF and the control groups were shown by an interaction [$F_{interaction}(4,81) = 2.727, P = 0.035$]. This interaction was produced by the differences in balancing performance in the EF rats compared with the CF and PF rats. The EF rats continually declined in balance at 20- and 40-weeks compared with the 10-week rats. The PF and CF rats, however, did not show a decline in balance until 40 weeks of treatment. CF rats after 40 weeks balanced for shorter times than the baseline control rats [$F_{age}(3,36) = 6.901, P = 0.001$].

Balance on the round rod was much more difficult for the rats than balance on the square rod (Fig. 2). Alcohol consumption decreased balance time on the round rod compared with rats that did not consume alcohol [$F_{diet}(2,81) = 4.366, P = 0.016$]. After 20 weeks, EF rats balanced for significantly shorter periods than the CF group ($P = 0.007$) but not the PF group ($P = 0.058$). After 40 weeks, the EF rats declined in performance relative to the PF ($P = 0.042$) and CF rats ($P < 0.001$). There was also a significant effect of duration of treatment [$F_{duration}(2,81) = 8.132, P < 0.001$] that was due to declines in balancing ability in EF rats between 10 and 40 weeks ($P < 0.001$). There was an interaction between diet and duration of treatment [$F_{interaction}(4,81) = 3.372, P = 0.013$] that was similar to that on the square rod. For example, EF rats showed steady decline in balance from 10 to 40 weeks. PF and the CF rats, however, did not show such declines until 40 weeks. Baseline control rats performed better on the round rod than CF rats of all treatment durations [$F_{age}(3,36) = 18.664, P < 0.001$].

SERCA densities

The molecular, granule and PN layers of the cerebellar cortex of the rats from all diets and durations of treatment appeared morphologically normal and indistinguishable among the treatment groups. As shown in Fig. 3, SERCA 2b staining was most intense in the PN soma and clearly outlined the PN dendrites. Dendritic spines were also SERCA 2b positive. The SERCA 2b antibody stained axons within the granule layer.
SERCA b and calreticulin expression

Fig. 2. Mean (+SEM) balancing duration (sec) of chow-fed (CF), pair-fed (PF) or ethanol-fed (EF) rats. There were significant effects of alcohol consumption on balance on the square \([F_{\text{diet}}(2,81) = 21.897, P < 0.001]\) and on the round rods \([F_{\text{diet}}(2,81) = 4.336, P = 0.016]\). After 40 weeks of treatment, EF rats balanced for shorter times on the round rod compared with the CF and PF groups \((^{*}P < 0.05)\). After 20 weeks, EF rats balanced for shorter times than the CF group \((^{*}P < 0.01)\). On the square rod, the EF rats balanced for shorter times after 20 \((^{*}P < 0.01)\) and 40 weeks \((^{*}P < 0.001)\) compared with the CF rats. There were significant effects of duration of treatment on balance on the square \([F_{\text{duration}}(2,81) = 15.047, P < 0.001]\) and round rod balance \([F_{\text{duration}}(2,81) = 8.132, P < 0.001]\). After 40 weeks the EF rats declined in balance on the square \((^{*}P < 0.001)\) and round rods \((^{*}P < 0.001)\) compared with the 10- and 20-week EF rats. There were significant interactions between diet and duration of treatment on the square \([F_{\text{interaction}}(4,81) = 2.727, P = 0.035]\) and the round rods \([F_{\text{interaction}}(4,81) = 3.372, P = 0.013]\). There were significant age effects on the square rod in the 40-week CF rats compared with the baseline controls \((^{*}P = 0.001)\) and on the round rod in the 10-, 20- and 40-week CF rats compared with the baseline controls \((^{*}P < 0.001)\).

and lightly outlined the bodies of the granule neurons. Neurons of the deep cerebellar nuclei were intensely positive for SERCA 2b. Double immunostaining with anti-SERCA 2b and calbindin D-28, a marker for PN dendrites in the molecular layer, showed co-localization of SERCA 2b and calbindin D-28 in internal dendritic branches and peripheral spiny branchlets (Fig. 3).

Alcohol consumption results in decreased densities of SERCA 2b + PN dendritic processes (Fig. 4) proximate to the PN soma \([F_{\text{diet}}(2,81) = 4.515, P = 0.014]\), the pial surface \([F_{\text{diet}}(2,81) = 5.563, P = 0.006]\) and when measures from both regions were combined \([F_{\text{diet}}(2,81) = 5.119, P = 0.008]\). After 20 weeks of treatment, this decline was shown in the EF compared with the CF rats proximate to the PN soma \((P = 0.021)\), near the PN surface \((P = 0.026)\) and combined regions \((P = 0.023)\). After 40 weeks of treatment, SERCA 2b densities were significantly less in the EF rats compared with the CF and PF groups proximate to the PN soma \((P < 0.001)\), the pial surface \((P < 0.001)\) and when measures from both regions were combined \((P < 0.001)\). Duration of treatment significantly altered the density of SERCA 2b + PN dendrites proximate to the PN soma \([F_{\text{duration}}(2,81) = 5.253, P = 0.007]\), near the pial surface \([F_{\text{duration}}(2,81) = 6.132, P = 0.006]\) and for the combined regions \([F_{\text{duration}}(2,81) = 5.066, P = 0.008]\). This significance was not easily localized due to the interaction between treatment and duration of treatment which occurred proximate to the PN soma \([F_{\text{interaction}}(4, 81) = 3.859, P = 0.006]\), pial surface \([F_{\text{interaction}}(4, 81) = 5.417, P = 0.001]\) and for the combined regions \([F_{\text{interaction}}(4,81) = 4.774, P = 0.002]\). The interaction (Fig. 5) stems from 22% (CF) and 16% (PF) increases in SERCA 2b densities throughout the PN dendritic arbor from 10–40 weeks in the control rats compared with an 11% decrease in the EF rats. Comparison of the CF rats with the baseline controls resulted in decreased SERCA 2b + densities in the 20- and 40-week CF rats proximate to the PN soma \([F_{\text{age}}(3,36) = 5.863, P < 0.01]\), pial surface \([F_{\text{age}}(3,36) = 7.607, P < 0.01]\) and for the combined measures \([F_{\text{age}}(3,36) = 7.165, P < 0.01]\).

**Calreticulin densities**

Calreticulin was present in many but not all PN soma (Fig. 3). Calreticulin nuclear staining was also present in a subset of PN as was staining of Bergmann glial fibers and nuclei within the molecular layer. The presence of calreticulin within Bergmann glia fibers was confirmed with confocal microscopy. The molecular layer nuclei that were stained could not be positively identified as astrocytes as astrocytic processes but not astrocyte nuclei stain with GFAP. In addition, there are many nuclei of stellate neurons, oligodendroglia and microglia within the molecular layer that might stain with calreticulin. However, subsequent double staining of this area with anti-NeuN (EMD Millipore, MA), a nuclear neuronal marker, and anti-calreticulin showed that most of the calreticulin+ nuclei within the molecular layer were glial as they were not
NeuN positive. Nuclei within the molecular layer were completely stained or stained in the perinuclear region.

Alcohol consumption did not alter the density of calreticulin + PN (Fig. 6) compared with rats that did not consume alcohol. There was a significant effect of duration of treatment on the density of calreticulin in the PN soma \( F_{\text{duration}}(2, 81) = 29.277, P < 0.001 \) and nucleus \( F_{\text{duration}}(2, 81) = 8.517, P < 0.001 \). This effect was localized as a significant decline in calreticulin + PN somas in the CF rats \( P < 0.001 \) at 20 and 40 weeks and in the EF rats at 40 weeks \( P < 0.001 \) compared with the 10-week groups. PN

Fig. 3. Representative SERCA 2b and calreticulin immunohistochemistry. (A–C) SERCA 2b staining from a representative slide used in quantitation. In (A), the granule cell layer (GL) and molecular layer (ML) are easily viewed. In (A) and (B), PN cell bodies (arrowheads) and dendrites (arrows) are visible. In (C), dendrites (arrows) are shown within the molecular layer. (D–F) A section through a confocal stack showing SERCA 2b labeling (D), calbindin D-28 labeling (E) and co-localization of SERCA 2b and calbindin D-28 (F) within the PN soma (asterisks), primary dendritic branches (arrows) and smaller spiny branchlets (arrowheads). (G–I) A section through a confocal stack showing SERCA 2b labeling (G), calbindin D-28 labeling (H) and co-localization of SERCA 2b and calbindin D-28 labeling (I) in the regions of the molecular layer closest to the pial surface. PN spiny dendrites (arrows) are visible. Negative images (asterisks) represent stellate or glial cell nuclei. (J–L) Calreticulin staining (J). Molecular layer (ML) and calreticulin staining PN soma (arrows). Inset shows a calreticulin + PN nucleus (arrowhead). (K) PN soma (arrowheads) and PN primary dendrite (large arrow) are calreticulin +. (L) Molecular layer nuclei (arrowheads) and Bergmann glial fibers (arrows) within the molecular layer are calreticulin +.
nuclear calreticulin expression also declined at 40 weeks, compared with the 10-week CF (P = 0.023) and 10- and 20-week PF rats (P < 0.05). Calreticulin densities in PN somas in the 10-week CF rats were less than in baseline controls \[F_{\text{age}}(3,36) = 10.360, P < 0.001\]. Nuclear expression was significantly greater in the baseline controls compared with all of the CF rats \[F_{\text{age}} = (3,36) = 11.535, P < 0.001\]. There was no effect of alcohol consumption on the density of calreticulin + molecular layer nuclei \[F_{\text{diet}}(2,81) = 1.491, P = 0.231\] or Bergmann fibers \[F_{\text{diet}} (2,81) = 2.620, P = 0.079\] (Fig. 7). Duration of treatment did not alter the density of calreticulin + nuclei but had a significant effect on the areal density of Bergmann glial fibers \[F_{\text{duration}}(2,81) = 26.662, P < 0.001\]. In the CF (P < 0.05) and the PF rats (P < 0.05), calreticulin + Bergmann fiber density declined at 20 and 40 weeks relative to the 10-week treatment group. In the EF rats, the density of Bergmann glial fibers similarly declined at 40 weeks compared with the 10-week treatment group (P < 0.001). There was a significant increase in calreticulin expression in molecular layer glial nuclei \[F_{\text{age}}(3,36) = 4.582\] between the baseline controls and 10-month CF rats (P = 0.006).

**Volume of the molecular layer**

Molecular layer volumes (Fig. 8) were not changed by dietary treatment \[F_{\text{diet}}(2,81) = 1.831, P = 0.167\]. There was a significant effect of duration of treatment \[F_{\text{duration}}(2,81) = 3.897, P = 0.025\] which could not be localized. There was no effect of age \[F_{\text{age}}(3,36) = 2.003, P = 0.131\] on molecular layer volume.
Fig. 6. Mean (+SEM) Calreticulin + Purkinje neuron soma and nuclei/mm. There were no effects of alcohol consumption. There was a significant effect of duration of treatment on the density of calreticulin in PN soma ($F_{\text{duration}(2, 81)} = 29.277, P < 0.001$) and nuclei ($F_{\text{duration}(2, 81)} = 8.517, P < 0.001$). There was a significant decline in calreticulin + PN soma in the CF rats at 20 and 40 weeks compared with 10 weeks (*$P < 0.001$) and in the EF rats at 40 weeks compared with 10 weeks. (**$P < 0.001$). Calreticulin + PN nuclear expression declined in CF rats was higher at 10 than 40 weeks ($P < 0.05$). In PF rats ($P < 0.05$), nuclear calreticulin expression was lower at 40 weeks compared with 10 and 20 weeks of treatment. There was an age-related decline in calreticulin expression in the PN soma ($P < 0.001$) between 3 and 10 weeks of age with an accompanying decrease in nuclear calreticulin expression in CF rats at 10, 20 and 40 weeks compared with baseline controls ($P < 0.001$).

Fig. 7. Mean (+SEM) glial nuclei and Bergmann glial fibers/mm$^2$. There was no effect of alcohol on areal density of Bergmann glial fibers or molecular layer nuclei. There was an effect of duration of treatment on Bergmann glial fibers ($F_{\text{duration}(2, 81)} = 26.662, P < 0.001$). In the CF ($P < 0.05$) and the PF rats ($P < 0.05$), calreticulin expression in Bergmann glial fibers was highest in the 10 compared with the 20 and 40-week treatment groups. In the EF, the density of Bergmann glial fibers in the 10-week group was significantly higher than in the 40-week group ($P < 0.001$). After 10 weeks of chow treatment, there was an increase in calreticulin + glial nuclei compared with the baseline controls ($P < 0.01$).
DISCUSSION

The results of this study demonstrate that chronic ethanol consumption for 40 weeks results in decreases in SERCA 2b pump densities in regions of the PN dendritic arbor close to the pial surface and PN soma. In addition, decreases in SERCA 2b levels were robust enough to remain significant when measures from both regions were combined showing that the effect of ethanol on the SERCA 2b pump encompasses every part of the PN dendritic arbor. Ethanol-induced decreases in SERCA 2b is gradual, taking 40 weeks to achieve relative to the two control groups. Behavioral tests of balance showed ethanol-induced deficits during the same duration of treatment as ethanol-induced effects on SERCA 2b pump densities. There were no ethanol-induced alterations in calreticulin densities in PN. In fact, the distribution of calreticulin within PN was not as expected for a major ER calcium-binding protein as it did not extend into the PN dendritic arborization. There were, however, significant age-related changes in calreticulin density within PN. This is the sole report of calreticulin levels within Bergmann glia fibers and glial nuclei of the molecular layer.

Decreases in SERCA 2b densities within PN dendrites are strongly suggestive of ethanol-induced down-regulation of the SERCA 2b pump. A single report of ethanol and ischemia-induced down-regulation of mRNA supports this hypothesis (Xia et al., 1998). SERCA 2b pump down-regulation would impair upon dendritic function be decreasing Ca²⁺ sequestration into the SER. This decline in pump function would result in rise in [Ca²⁺], with increased probability of neuronal toxicity. Another consequence of decreased levels of SERCA 2b would be less calcium within the SER. This would also interfere with normal neuronal activity by affecting the calcium-induced calcium release from the SER which occurs following rises in [Ca²⁺] when membrane calcium channels open (Berridge, 1998). Decreased calcium stores may also result in malfunctions in the SER. For example, depletion of the SER calcium store has been associated with SERCA 2b mutations and ER stress (Wang et al., 2011).

Ethanol-induced decreases in SERCA 2b densities may also be reflective of loss of dendritic branches. As dendritic terminals at branchpoints are the sites of ethanol-induced dendritic regression (Pentney and Quackenbush 1990, 1991; Pentney, 1995; Dlugos and Pentney, 2000), the decreased SERCA 2b densities near the pial surface reported here might simply reflect loss of PN terminal dendrites. Significant ethanol-induced decreases in PN densities, however, also occur closer to the PN soma where the internal, higher order PN branches, described with centripetal Strahler nomenclature, reside (Strahler, 1964; Ito, 1984). Reported ethanol-induced alterations in SERCA 2b densities in the region of the PN soma, in fact, may involve SERCA 2b down-regulation within the internal portion of the dendritic arbor. The effects of down-regulation of the SERCA 2b pump in these internal regions may be enhanced at dendritic branchpoints, sites of calcium release (Manita and Ross, 2009) and regions that calcium waves must traverse (Augustine et al., 2003).

The mechanism behind ethanol-related decreases in the levels of the SERCA 2b pump might be ethanol-induced decreases in expression, trafficking, membrane insertion or degradation of the pump. Alterations in gene expression are a likely cause of SERCA 2b down-regulation as decreased mRNA levels of SERCA 2b occurred in the cerebellum following chronic alcohol consumption (Xia et al., 1998). A mechanism by which changes in SERCA 2b expression may occur is ethanol-induced alterations in epigenetic factors that control gene expression in neural tissue during development (Liu et al., 2009; Zhou et al., 2011) and in human alcoholics (Mayfield et al., 2003). Protein trafficking and membrane insertion have also been shown to be sensitive to ethanol’s effects in some systems. For example, ethanol-induced alterations in trafficking of proteins has been reported (Guppy et al., 1995; Karpyak et al., 2012; Tomas et al., 2012). In addition, ethanol-induced lags in membrane insertion of transferrin and
apolioprotein E have been demonstrated in hepatocytes (Lakshman et al., 2001).

The significant interaction that occurred between treatment and duration of treatment should be noted here as it best demonstrated the opposite effects of alcohol and age on SERCA 2b densities during the 40 weeks of treatment. The ethanol effect is shown by the 10% decrease in SERCA 2b densities in the ethanol rats between 10 and 40 weeks. The age effect is demonstrated by the respective 22 and 16% increase in SERCA 2b levels in combined regions of the dendritic arbor in the CF and PF rats, respectively (Fig. 5) and also by the agerelated increases in SERCA 2b levels in the CF rats between 3 and 18 months of age (Fig. 4). It is hypothesized that age-related up-regulation in SERCA 2b may be a compensatory response to increased levels of \([Ca^{2+}]\), that accompany the aging process (Gibson and Peterson, 1987; Toescu and Verkhratsky, 2000). It appears that age-related increases SERCA 2b density may result in increasing SERCA 2b levels in individual PN branches with age as the volume of the PN dendritic arbor decreases by 18 months of age (Pentney, 1986; Quackenbush et al., 1990).

Behavioral studies (Fig. 2) shows that, after 40 weeks of ethanol treatment, round rod balance was affected in the EF rats, a duration of treatment that correlates well with the ethanol-induced decreases in SERCA 2b densities reported here. The 40-week treatment duration in which behavioral deficits occurred is the same treatment duration in which ethanol-induced SER dilatation (Dlugos and Pentney, 2000; Dlugos, 2006a,b) and dendritic regression (Pentney and Quackenbush, 1990, 1991; Pentney, 1995, Pentney and Dlugos, 2000) were observed in this laboratory. The ethanol-induced effect on balance, if permanent, may also be reflective of studies in recovered alcoholics who demonstrate discrete and permanent deficits in cerebellar functions (York and Biederman, 1991; Davalia et al., 1994; Woodruff-Pak et al., 1996; Sullivan et al., 2000, 2002; Deshmukh et al., 2002; Sullivan and Pfefferbaum, 2005). Figure 2 also shows that, unlike the opposing effects of age and alcohol on SERCA 2b levels, balancing ability on both rods decreased with age. This decrease along with ethanol-induced decreases would enhance balancing deficits. This finding is similar to that shown in human elderly, our most rapidly growing population (Koplan and Fleming, 2000), who are more sensitive to alcohol-induced alterations on motor functions than their younger counterparts (Breslow et al., 2003). The significant interactions shown in both round and square rod studies reinforce ethanol-induced effects on balance. On both behavioral apparatus, there was a steady decline after 10 weeks in the EF rats, whereas in the CF and PF groups, balance did not decline until the 40-week group.

The ascribed functions of calreticulin, as a calcium-binding protein in the SER, a regulator of SER proteins and a modulator of SERCA 2b pump function (John et al., 1998; Corbett and Michalak, 2000; Baker et al., 2002; Michalak et al., 2002), strongly suggest that calreticulin plays an important part in the SER and calcium homeostasis of many neurons. The decreases in SERCA 2b densities, moreover, shown here suggested that, if calreticulin is a modulator of SERCA 2b function, ethanol-induced alterations in SERCA 2b might alter calreticulin expression in the PN. In the present study, however, there were no effects of ethanol on calreticulin expression (Fig. 6). In addition, as calreticulin staining was predominately confined to the PN soma or nucleus in both brightfield and confocal studies, it does not appear that calreticulin expression extends beyond the primary PN dendrite or into the extensive dendritic arbor (Fig. 3). In retrospect, earlier reports of immunocytochemical staining in PN dendrites showed calreticulin staining mainly in the PN soma (Perrin et al., 1991: Nori et al., 1993). It appears, moreover, that calreticulin expression is not strongly detectable in every neuronal type as there is only a weak mRNA signal in the substantia nigra, superior colliculus and cerebral cortex and the absence of that signal in cerebellar granule neurons (Abe et al., 1992). Taken together, these findings suggest that calreticulin is not as important a component of the SER as was assumed in the beginning of the study.

A possible explanation for the assumption that calreticulin would be present in PN dendrites is that the molecular components of the SER within dendrites have never been sufficiently characterized. Early studies identified major components such as inositol 1,4,5-trisphosphate receptor (Satoh et al., 1990) and the SERCA 2b pump (Villa et al., 1991) within PN dendrites. Later studies, such as one in Caenorhabditis elegans, showed that the SER and ER share many proteins (Rolls et al., 2002). Calreticulin, however, originally isolated as an ER protein (MacLennan, 1974), was assumed to function as a calcium chaperone in all ER domains, including the SER. Other calcium-binding proteins, such as calnexin, were identified early on in the Inositol 1,4,5-trisphosphate sensitive Ca2+ store of PN dendrites (Volpe et al., 1990). These findings were largely overlooked mainly because, in the tissues that were surveyed, such as hepatocytes and uterine smooth muscle (Milner et al., 1991), the signal for calreticulin was much stronger than that for calnexin. Calreticulin may play a larger role in non-neuronal cells than in neurons which have specialized dendritic sub-compartments that contain SER and polyribosomes for on-site protein synthesis but little or no RER (Torre and Steward, 1996; Steward and Schuman, 2003; Schuman et al., 2006).

The nuclear expression of calreticulin shown here (Fig. 6) is supported by similar findings of nuclear translocation of calreticulin in a variety of cells and conditions (Rojiani et al., 1991; Michalak et al., 1996; Holaska et al., 2001; Labriola et al., 2010). In neurons, nucleolar calreticulin expression has been described, whereas in glia, expression appears perinuclear or within the nuclear envelope (Opas et al., 1991; Taguchi et al., 2000). A variety of glia express calreticulin including C6 rat glioma cells (Park and Lee, 2010), glia in normal and Alzheimer’s brains (Taguchi et al., 2000) and microglia and astrocytes of the optic nerve (Shimazawa et al., 2012). Calreticulin expression has been shown here in Bergmann glia fibers (Fig. 6) closely aligned with PN dendrites, but not in PN dendrites, themselves. This finding is interesting with respect to the multifunctional role Bergmann glia play with respect to PN dendrites. During development, Bergmann glial processes guide granule neurons, the major input neurons to the PN, to their permanent positions in the granule layer. During adulthood, Bergmann glia surround PN synapses, are responsible for synaptic glutamate uptake, extracellular potassium ion homeostasis and recently have been shown to control the membrane permeability of PN (Wang et al., 2012). In addition, if, as our NeuN findings suggest, calreticulin + nuclei within the molecular layer are mainly glial cells, age-related calreticulin expression in glial nuclei from 3–40 months of age...
occurs concomitantly with decreased PN nuclear expression of calreticulin (Fig. 6). As there was no difference in the volume of the molecular layer between the dietary and duration of treatment groups, there are no concerns that dietary or age changes in molecular layer volumes confound interpretation of the density measurements.

In conclusion, chronic ethanol treatment for long periods in aging, adult rats results in decreased SERCA 2b densities in the PN dendritic arbor. These data, therefore, strongly suggest that ethanol-induced SERCA 2b down-regulation occurs that may contribute to previously reported PN dendritic regression. The significant interaction between alcohol and duration of treatment (age) suggests that aging and alcohol alter SERCA 2b densities by different mechanisms. In addition, there were no ethanol-induced changes related to PN’s expression of calreticulin. In fact, there is little evidence of calreticulin expression outside of the soma and primary PN dendrites. This is the first report of calreticulin expression within Bergmann glial processes and within nuclei of the cerebellar molecular layer.

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