

MORPHOMETRIC EVIDENCE THAT THE TOTAL NUMBER OF SYNAPSES ON PURKINJE NEURONS OF OLD F344 RATS IS REDUCED AFTER LONG-TERM ETHANOL TREATMENT AND RESTORED TO CONTROL LEVELS AFTER RECOVERY

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Abstract — Clinical symptoms of alcohol abuse may be confused with symptoms of age-related neuropathologies in human patients. It is important, therefore, to determine the relationships between alcohol abuse and changes in brain structures in well-controlled studies of ageing subjects. Currently there is little well-documented information of this type available. The purpose of this study was to determine whether long-term ethanol treatment during ageing would lead to reductions in synaptic input to cerebellar Purkinje neurons (PN) of old F344 rats that: (1) were more severe than those attributable to ageing alone; (2) might be responsible for an ethanol-related deletion of dendritic segments of PN in old F344 rats shown previously in this laboratory. The total number of synapses per PN dendritic arbor was determined after ethanol treatment of old F344 rats for 40 weeks between 12 and 22 months of age and in similarly treated rats given a subsequent 20-week period of recovery between 22 and 27 months of age. Groups of age-matched rats fed a chow diet and water and rats pair-fed an isocaloric liquid diet lacking ethanol served as controls. The volume of the molecular layer per PN arbor and the numerical density of synapses in the molecular layer was determined from light microscopic preparations of a fixed volume of the cerebellar cortex. Photographic montages of the ultrastructure of the molecular layer of the cerebellum were also prepared from each rat for measurements of synaptic numerical densities. From the volume of the molecular layer per PN arbor and the numerical density of synapses in the molecular layer, the total number of synapses per PN arbor was estimated for each rat. There was a significant reduction in synapses in the old ethanol-treated rats relative to age-matched chow-fed rats. There were also significant interactions between recovery and treatment prior to recovery. During recovery, synaptic numbers in the old, ethanol-treated rats were restored to pre-recovery control levels whereas synaptic numbers in the old, chow-fed rats were significantly reduced during the same period of time. There were no significant diet- or age-related changes in synaptic numbers in the pair-fed control rats during treatment or recovery. The pattern of reduction in synaptic numbers during ethanol treatment and restoration of synaptic numbers during recovery from treatment paralleled the pattern of ethanol-related segment loss and recovery-related segment regrowth noted earlier in PN arbors of old F344 rats, suggesting that reductions in the numbers of synapses and deletion of terminal dendritic segments were causally related in this strain.

INTRODUCTION

It is well known that long-term alcohol consumption has adverse structural and functional effects on neurons of the cerebellar cortex. Our current understanding of the effects of alcohol on neurons is based largely on studies that have used neonatal or young adult animal subjects. Given the fact that symptoms of overuse of alcohol may be confused with symptoms of age-related neuropathologies in

human patients, it is important to determine the relationships between alcohol abuse, ageing processes, and brain damage in well-controlled experiments. Such information cannot be easily obtained from clinical studies of human patients.

The ongoing goal of this laboratory has been to use the cerebellar cortex of the rat as a model system to analyse the combined and separate effects of alcohol abuse and of ageing on neuronal structures. Cerebellar Purkinje neurons (PN) are remarkable for their exuberant dendritic arbors that extend from the PN monolayer through the overlying molecular layer, composed primarily of

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granule cell axons (parallel fibres), to the pial surface of the cerebellar cortex. The PN are specialized for the processing of sensory information from the spinal cord, brain stem, and neocortex that is relayed by numerous afferents, particularly the mossy fibres, the parallel fibres of granule neurons, and the climbing fibres of inferior olivary neurons. The PN transmit the processed information directly to the deep cerebellar nuclei and/or vestibular nuclei for distribution to other brain stem structures that regulate motor functions. The PN are also known to be affected adversely by long-term alcohol consumption and by ageing (Pentney, 1993).

Previous investigations in this laboratory have assessed a number of quantitative changes in neuronal structures in the cerebella of old F344 rats during normal ageing with and without associated chronic alcohol exposure. These studies demonstrated that, in old F344 rats, chronic ethanol consumption causes the dendritic arbors of the PN to be reduced in size (Pentney, 1982), but the mean length of the terminal segments in these arbors is increased in the ethanol-fed rats (Pentney and Quackenbush, 1990, 1991). Subsequent studies provided evidence that reconciled these two apparently conflicting results. In all dendritic arbors, terminal segments branch from a parent non-terminal segment at a branch point shared with at least one other segment, either another non-terminal segment or another terminal segment. It was shown by a comparison of different theoretical models of dendritic regression that only one model would lead to lengthening of terminal segments in dendritic arbors. In that model, single terminal segments were deleted from branch points shared with other terminal segments that were not deleted. Following such deletions, those branch points ceased to exist and the internal parent segments became continuous with the remaining terminal segments, forming longer terminal segments (Pentney, 1995). Other investigators, using young ethanol-treated Sprague-Dawley rats, have reported that PN had shorter dendritic networks (arbors), reduced dendritic areas, and lower densities of dendritic branching following long-term ethanol treatment (Tavares *et al.*, 1983), but they have not reported increases in the lengths of the terminal segments in those arbors.

Effects of ageing processes, uncomplicated by

ethanol consumption, produced different effects in PN of old F344 rats. Quackenbush *et al.* (1990) demonstrated that terminal segments in the periphery of the dendritic arbors of PN of old ethanol-naïve F344 rats were shorter than those in younger ethanol-naïve F344 rats, but terminal segments located close to the cell body origin of the dendritic arbor lengthened with age. The cause of the lengthening of the terminal segments near the cell body was not apparent, but this effect may represent compensatory growth induced by the 'dying back' of the peripheral terminal segments. The terminal segments of PN in old F344 rats were also partially depleted of dendritic spines (Pentney, 1986).

Other ultrastructural changes within the molecular layer of the rat cerebellar cortex have also been reported following chronic ethanol consumption, e.g. deafferented spines and shrunken profiles of synaptic elements (Tavares *et al.*, 1987). The presence of abnormal and enlarged mitochondria in PN somata and dendrites (Pentney, 1979; Tavares and Paula-Barbosa, 1983) and the dilation of the rough and smooth endoplasmic reticulum of PN dendrites (Lewandowska *et al.*, 1994) have also been noted. Measurements of synaptic densities in the cerebellar molecular layer of ethanol-treated adult Sprague-Dawley rats (Tavares *et al.*, 1987) and of normal old F344 rats (Glick and Bondareff, 1979) showed that ethanol treatment and normal ageing cause similar reductions in the densities of synapses between parallel fibre varicosities and PN dendritic spines. The volume of tissue in which these quantitative changes occurred was not measured, however, leaving open the possibility that changes in overall tissue volumes, rather than specific effects of treatment and ageing, were directly responsible for the decreases in synaptic density. The importance of measurements of relevant tissue volumes has been addressed recently by Coggeshall (1992) and West (1993).

The purpose of the present study was to determine whether long-term ethanol treatment in old F344 rats would lead to severe reductions in synaptic input to dendritic segments of PN that might be responsible for the ethanol-related deletion of dendritic segments noted previously (Pentney, 1995). To examine this question, the total number of synapses per dendritic arbor was quantified in old F344 rats after long-term ethanol intake and in similarly treated rats after a

subsequent lengthy period of recovery. To our knowledge, the total number of synapses per dendritic arbor, a quantitative measure of pre- and postsynaptic structures, has not been reported previously in studies of effects of chronic alcohol consumption in aged rats. The results of these experiments form the subject of the present paper.

METHODS

Animal model

Forty-nine 12-month old, male F344 rats (NIA colony, Harlan Sprague-Dawley, Inc.) were used in this study. Cerebella from four rats that were randomly selected prior to the initiation of treatment were processed to provide measurements for determination of the mean numbers of synapses at the start of treatment. All remaining rats were randomly assigned to one of three dietary treatment groups ($n = 15/\text{group}$). Rats in the ethanol-fed group received a liquid diet (Bio-Serv, Frenchtown, NJ, USA) in which 35% of the dietary calories were derived from ethanol. Rats in the pair-fed control groups received the control liquid diet made isocaloric to the ethanol diet by substituting maltose/dextrins for ethanol. Rats in a group that received a nutritionally complete chow pellet (RMH 1000 diet, Agway, Inc., Syracuse, NY, USA) and water diet *ad libitum* served as controls for any likely effects of the liquid diet that were not dependent upon the presence of ethanol and for effects related to normal ageing. Pair-fed control rats were matched by weight to the ethanol-fed rats. The intake of each pair-fed rat was then determined on a daily basis by the intake of its ethanol-fed partner. The pair-fed control rats, therefore, received the same dietary volumes as the ethanol-fed rats throughout the treatment period.

Procedures for introducing the rats to the ethanol-containing diet were as described previously (Pentney *et al.*, 1989; Pentney, 1995). At the end of 40 weeks of ethanol treatment, 10 ethanol-fed rats, and their pair-fed and chow-fed control partners were euthanized as described below (non-recovered rats). The remaining five ethanol-fed rats and their pair-fed and chow-fed control partners were weaned from the liquid diets over a 9-day period to prevent overt withdrawal reactions, as described previously (Pentney, 1995),

and returned to the chow pellet diet and water administered *ad libitum*. The groups of weaned rats and their continuously chow-fed partners were then maintained on the chow diet for a 20-week recovery period (recovered rats). Throughout treatment and recovery, all rats were weighed weekly to monitor their health.

Blood ethanol concentrations

Blood ethanol levels were monitored in randomly selected subsets of the ethanol-fed rats after 3 days ($n = 8$), after 12 weeks ($n = 3$), and after three weeks ($n = 3$), and after 33 weeks ($n = 3$) of dietary treatment. The blood samples were collected for enzymatic measurements (Sigma Diagnostics Kit #332A) from the tail veins at 0800, i.e. 23 h after fresh diet was placed on the cages. The mean circulating levels of ethanol at these intervals averaged 66 ± 43 mg/dl, 91 ± 26 mg/dl, and 56 ± 43 mg/dl, respectively. The highest measurements recorded were 142 mg/dl, 113 mg/dl, and 98 mg/dl at these respective time points. These measurements showed that ethanol levels judged to be intoxicating in humans (currently 100 mg/dl) were achieved by these rats over many weeks of treatment. Circulating levels of ethanol varied during each 24 h period, with peak levels of 100–200 mg/dl declining to low levels of 0–10 mg/dl between periods of drinking. For this reason, measurements presented here were not considered to be peak circulating levels of ethanol, because the intervening time between each rat's ethanol intake and collection of the blood samples was not known. Measurements in this group of old ethanol-treated rats were consistent with measurements in previous studies in which similar treatment protocols were used (Pentney *et al.*, 1989; Pentney and Quackenbush, 1991).

Tissue preparations

Rats were anaesthetized with sodium pentobarbital (65 mg/kg) and perfused through the aorta with 40 ml of saline containing 2000 units of heparin (50 units/ml) followed by a solution of 1% glutaraldehyde/1% paraformaldehyde in 0.1 M Sorenson's phosphate buffer (pH 7.4). The brains were removed and coded to avoid investigator bias during measurements. Each cerebellum was then separated from the brain stem and weighed. The cerebellar vermis was hemisected sagittally; one half was prepared for light microscopy (LM) and

the other half for transmission electron microscopy (EM). Epon-Araldite embedding was used for LM and EM preparations. The LM preparations were used to determine the mean volume of the molecular layer that was associated with each PN arbor. The EM preparations were used to determine the synaptic density within that volume.

At the end of the dietary treatments, three rats (an ethanol-fed rat, its pair-fed control partner, and a chow control rat) were euthanized and their brains processed at the same time to ensure that treatment of the tissues was identical for all dietary groups. These precautions ensured that any inadvertent technical bias would not pose a serious problem since its effects would be similar in all treatment groups (Mayhew and Reith, 1988; Flood, 1994). Corrections for tissue shrinkage were not made, because: (1) this was a comparative study in which uniform conditions were used for all groups; (2) the dietary treatments did not induce differential tissue shrinkage of the entire cerebellum or its cortical layers in similarly treated cohorts of the rats used here (unpublished data from this laboratory); (3) shrinkage in tissue volume following fixation and Epon embedding has been reported not to exceed 3–5% (Weibel and Knight, 1964; Reitan, 1988).

For LM preparations, the vermal halves were separated into anterior and posterior lobes and lobule X (nodulus). The anterior and posterior lobes were then washed in 0.1 M Sorenson's buffer (pH 7.4), osmicated in 1% osmium tetroxide, dehydrated in acetone, and embedded separately in Epon-Araldite. Each lobe was sectioned parasagittally at 1.75 μm on a JB-4 microtome. Section thickness was measured with the step method of Gundersen *et al.* (1983). After the first complete section of each lobe was obtained, 30 serial sections were collected. The serial sections were stained with 0.5% toluidine blue in 1% sodium borate. Lobules III and IV from the anterior lobe and lobules VII and VIII from the posterior lobe were used for all subsequent measurements.

For EM preparations, lobules III and IV–V were separated from lobules I–II in the anterior lobe, and lobules VI–VII and VIII were separated from lobules IX and X in the posterior lobe. The tissue blocks were then washed in 0.1 M Sorenson's phosphate buffer, osmicated in 1% osmium tetroxide, and embedded in Epon-Araldite in flat

embedding moulds for parasagittal sectioning. The embedded blocks of tissue were sectioned at 65 nm. Section thickness of ultrathin sections was determined by the fold method of Small (1968) (see also DeGroot, 1988). The sections were transferred to single-slot, Formvar-coated copper grids and stained with uranyl acetate and lead citrate. Lobules III and IV from the anterior lobe and lobules VII and VIII from the posterior lobe were used for all subsequent measurements.

LM determination of the mean volumes of the PN arbors

The first step in the quantification procedure was to determine the mean number of PN in sections of lobules III and IV of the anterior lobe and in lobules VII and VIII of the posterior lobe with the disector methodology (Sterio, 1984; Gundersen, 1986). A random systematic method (West, 1993) was used to select four pairs of sections in the anterior lobe and four pairs of sections in the posterior lobe from the 30 serial sections collected from each lobe. Each pair of sections served as a disector pair in which PN with visible nucleoli were counted. One section of the disector pair initially served as the reference section and the other as the look-up section.

In the study reported here, an adaptation of the disector method described by Black *et al.* (1990) was used for the counts of PN. In this adaptation, the entire PN layer within a cerebellar lobule was used. The anterior and posterior boundaries of the lobules were marked on the sections. The reference section was then projected at $\times 150$ through a drawing tube mounted on a Zeiss microscope, and the PN layer was traced on the drawing surface. The profiles of all PN nuclei with visible nucleoli, including cells that touched the posterior boundary and excluding cells that touched the anterior boundary, were outlined on the tracing (the reference drawing). The corresponding image of the PN layer on the look-up section was then superimposed on the reference drawing, and all nuclei with visible nucleoli were added to the drawing in a different colour. PN nuclei were counted only if they were present in the reference section and not in the superimposed image of the look-up section (Gundersen, 1986). A second measurement was then obtained from the same pair of sections by designating the second section in the disector pair as the reference section

and the first section as the look-up section and repeating the above procedure. Eight counts were thus obtained from lobules III and IV in the anterior lobe and lobules VII and VIII in the posterior lobe in each rat (32 measurements/rat). The mean coefficients of error for these counts in all groups were between 6 and 10%. Mean values for the number of PN (Q^-) in specific lobules and in the anterior and posterior lobes were then determined.

The reference volume for the PN counts was the volume of the overlying molecular layer (ML) that contained the dendritic arborizations of the PN (Black *et al.*, 1990). Thus, the second step in this procedure was to determine the area of the ML (A_{ML}) in lobules III, IV, VII and VIII. The sections were projected at $\times 24.6$ with a Bausch & Lomb Trisimplex microprojector and the ML within the projected boundaries were outlined on the drawing surface for measurements on a digitizing tablet. Mean values for A_{ML} in the lobules and in the anterior and posterior lobes were then determined.

From the above measurements, several parameters were derived. The numerical density of PN in the reference volume (N/V) was determined by the number of PN (Q^-) divided by the area of the molecular layer (A_{ML}) times the distance between the two planes of the disector (d) (Mayhew and Gundersen, 1996).

$$\Sigma Q^- \div (A_{ML} \times d) = N/V \quad (1)$$

The A_{ML} was then multiplied by the total thickness of the section series ($30 \times t$) to obtain the volume of the molecular layer (V_{ML}) in each lobule.

$$A_{ML} \times 30 \times t = V_{ML} \quad (2)$$

The total number of PN (N_{PN}) was equal to the numerical density of the PN (N/V) multiplied by the volume of the molecular layer (V_{ML}).

$$N/V \times V_{ML} = N_{PN} \quad (3)$$

The volume of the ML (V_{ML}) divided by the total number of PN (N_{PN}) then yielded the volume of the molecular layer per PN arbor (V_{PN}).

$$V_{ML} \div N_{PN} = V_{PN} \quad (4)$$

Determination of the numbers of synapses on the PN arbors

Montages of the ultrastructure of the molecular

layer were used for these measurements. Each montage represented a parasagittal strip of tissue extending from the pial surface to the PN monolayer of the cerebellar cortex. Two tissue sections from the anterior lobe (one each from lobules III and IV) and two sections from the posterior lobe (one each from lobules VII and VIII) were viewed with a JEOL 100 CX electron microscope and photographed at $\times 14\,000$ for construction of photomontages. The final magnifications of the photomicrographs were $\times 37\,500$, and each constructed montage represented approximately $750 \mu\text{m}^2$ of the molecular layer. A square $16 \text{ cm} \times 16 \text{ cm}$ counting frame (representing $18.27 \mu\text{m}^2$) was superimposed over the montage in a sequential linear pattern from the pial surface to the PN layer. The counting frame could be positioned on each montage, as described, ~ 35 times. Accepted rules for inclusion/exclusion of structures touching the frame boundaries were followed (Gundersen, 1977). The criteria for identifying a synapse were the presence of opposed membranes with a minimum of two associated synaptic vesicles and a postsynaptic thickness or density.

On each montage, the numbers of synapses within sequential counting frame areas were recorded, the mean number of synapses per unit area (N_a) was determined, and the mean length per postsynaptic density (D) was determined from digitized measurements of 15 randomly selected postsynaptic densities. The numerical density of synapses in the molecular layer, (N_s) divided by the sum of the mean length of the postsynaptic densities (D) and the section thickness (t) yielded the numerical density of synapses (N_v) in the molecular layer (Anker and Cragg, 1974; Black *et al.*, 1990).

$$N_a / (D + t) = (N_v) \quad (5)$$

The volume of the PN arbor (calculated in step 4) multiplied by the numerical density of synapses (N_v) was equal to the total number of synapses per PN arbor (N_{PN}) (Black *et al.*, 1990).

$$V_{PN} \times N_v = N_s \quad (6)$$

Mean values for N_s were determined for PN in the anterior and posterior lobes in each animal.

Statistical analyses

The mean values for the total number of synapses in the anterior and posterior lobes of

Table 1. Body and brain weights of rats in different treatment groups

Treatment	Age (months)	Body wt (g)	Brain wt (g)	Ratio
Chow controls	12	410 ± 23	2.096 ± 0.039	196 ± 13
Chow controls	22	440 ± 39	2.139 ± 0.086	206 ± 19
Pair-fed controls	22	489 ± 28*	2.122 ± 0.112	231 ± 10*
Ethanol-fed	22	492 ± 44*	2.096 ± 0.092	234 ± 19*
Chow controls	27	421 ± 66	2.150 ± 0.047	195 ± 29
Pair-fed/recovered	27	437 ± 24	2.126 ± 0.056	206 ± 16
Ethanol-fed/recovered	27	438 ± 31	2.136 ± 0.111	205 ± 16

Mean body weight for male F344 rats at the beginning of treatment (12 months old, $n = 4$), after 40 weeks of treatment (22 months old, $n = 10$ /group) and after 20 weeks of recovery (27 months old, $n = 5$ /group). There were no significant effects of the liquid diets *per se* or of ethanol on brain weights.

Values are means ± SD.

*Significant main effect of diet, $P < 0.05$ (ANOVA, F -values and exact P -values in text).

non-recovered and recovered rats were tested by two-way ANOVA with treatment and lobe being the independent variables (SPSS PC+ program). In tests in which the lobe was an independent variable, a modified repeated measures ANOVA was used to correct for the fact that values for anterior and posterior lobes were from the same animal. An alpha level of 0.05 was accepted as significant. Significance of main effects was localized by a post-hoc Duncan multiple comparison test. Analysis of statistically significant interactions between recovered and non-recovered groups used simple main effect ANOVA followed by a post-hoc Scheffé multiple comparison test for localization of significance within groups.

RESULTS

Effects of diets

The mean body weights, brain weights, and ratios of body weights to brain weights for rats in different treatment groups are listed in Table 1. There was a significant main effect of the liquid diets on body weights ($F_{6,42} = 4.937$, $P = 0.001$) with the 22-month-old pair-fed and ethanol-fed groups of rats being significantly heavier than cohorts that were allowed a 20-week period of recovery after similar dietary treatment and also significantly heavier than all groups of chow-fed control rats. There were no significant differences in brain weights in any of the treatment groups,

but there were significant differences in the body weight to brain weight ratios that reflected the increased body weights in the non-recovered pair-fed and ethanol-fed groups described above ($F_{6,24} = 5.954$, $P = 0.0001$). There were no significant effects of ethanol treatment *per se* on body or brain weights.

Qualitative features of the ultrastructure of the molecular layer

The typical ultrastructural morphology of the parasagittally sectioned ML in rats from all treatment groups is shown in Fig. 1A–D. Major constituents of the ML were the extensive dendritic arbors of the PN and the massive numbers of parallel fibres of the granule neurons. Profiles of longitudinally or obliquely oriented PN dendritic segments were easily identified in the montages. Bundles of parallel fibres, oriented perpendicularly to the plane of the section, were present between the dendritic segments, and many parallel fibre varicosities formed asymmetric synaptic contacts with PN dendritic spines. The synaptic vesicles in the varicosities were round and clear. Synapses of climbing fibres and axonal terminals of interneurons with PN dendritic shafts were also noted. Cell bodies of interneurons and glial cells were also present in small numbers. Despite the generally similar appearance of the neuropil in rats from all treatment groups, there were indications of treatment-related differences in the organization of the ML. In the ethanol-

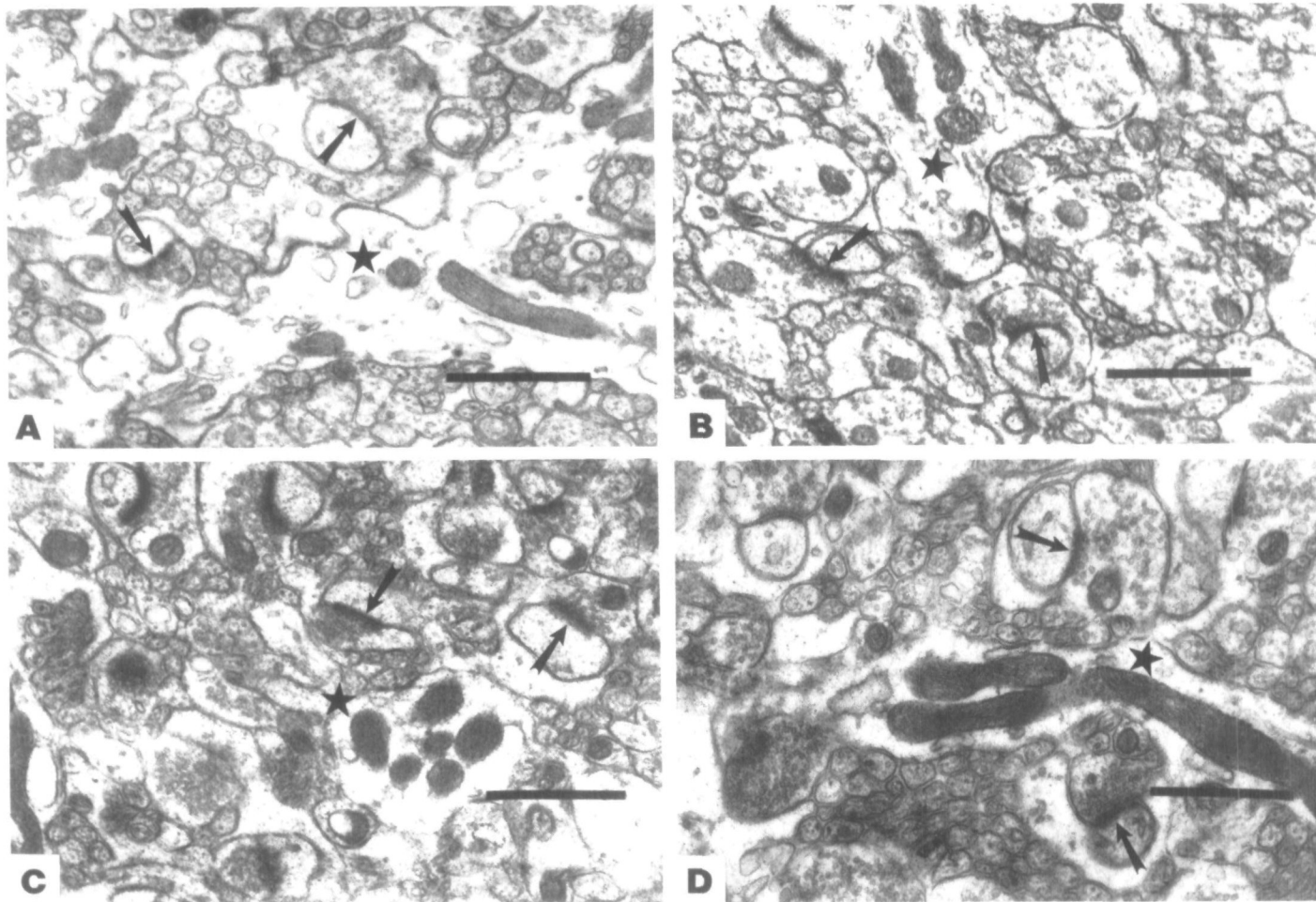


Fig. 1. Photomicrographs of the molecular layer in the posterior lobe of the cerebellum (A) in a 12-month-old chow-fed control F344 rat, (B) in a 22-month-old chow-fed control F344 rat, (C) in a 22-month-old pair-fed control F344 rat, and (D) in a 22-month-old ethanol-fed F344 rat. Stars denote Purkinje neuron (PN) dendritic shafts. Arrows point to synapses between parallel fibre varicosities and PN dendritic spines. Calibration bars = 1 μ m.

Table 2. Volume per Purkinje neuron dendritic arbor (μm^3)

Treatment	Age (months)	Anterior lobe Mean (CV)	Posterior lobe*** Mean (CV)
Chow controls ($n = 4$)	12	111 719 (0.14)	145 394 (0.12)
Chow controls ($n = 10$)	22	138 942 (0.19)	161 449 (0.18)
Pair-fed controls ($n = 10$)	22	122 617 (0.17)	144 330 (0.14)
Ethanol-fed ($n = 10$)	22	126 003 (0.15)	140 387 (0.19)
Chow controls ($n = 5$)	27	108 947 (0.17)***	161 870 (0.27)
Pair-fed/recovered ($n = 5$)	27	126 527 (0.15)	194 468 (0.23)**
Ethanol-fed/recovered ($n = 5$)	27	139 537 (0.18)	201 360 (0.20)**

Mean values are given for the volumes of the cerebellar molecular layer filled by individual PN dendritic arbors in the anterior and posterior lobes of the cerebellar vermis (CV). There was a significant age-induced decrease in the 27-month-old chow controls. Post-recovery volumes were significantly larger than pre-recovery volumes in groups given the liquid diets. Mean volumes in the posterior lobe were significantly larger than those in the anterior lobe.

*Significant main effect of ageing, $P < 0.05$; **significant interaction between treatment and recovery period, $P < 0.05$;

***significant main effect of lobe, $P < 0.05$ (ANOVA, F -values and exact P -values in text).

treated rats, for example, the dendritic shafts and parallel fibre varicosities frequently appeared to be swollen, and the ML was often less compactly organized than in control tissue.

Volumes of Purkinje neuron arbors (V_{PN})

The results in Table 2 show that there was a main effect of ageing in the chow-fed control rats. The mean volume of the molecular layer per PN dendritic arbor increased in chow control rats between 12 and 22 months of age and then decreased between 22 and 27 months of age. In the anterior lobe, the increase in volume between 12 and 22 months of age did not reach significance, whereas the decrease in volume per PN between 22 and 27 months of age, a much shorter period of time, was significant ($F_{2,18} = 3.836$, $P < 0.05$). This effect of ageing was also detected as a significant interaction between treatment and recovery ($F_{2,39} = 3.607$, $P < 0.04$). A simple main effect ANOVA showed this interaction to be a significant effect of ageing ($F_{1,39} = 6.207$, $P < 0.025$), as shown above. An effect of ageing on the volume per PN was not present in the posterior lobe of those rats, and effects of ageing on the volume per PN were not detected in either lobe of rats that were treated with the liquid diets.

There was a consistent main effect related to the anterior or posterior lobe origin of the PN. In all groups of rats, the mean volume of the ML per PN arbor was consistently larger in the posterior lobe than in the anterior lobe (all 22-month-old rats:

$F_{1,27} = 15.80$, $P < 0.01$; all 27-month-old rats: $F_{1,27} = 31.27$, $P < 0.001$; Table 2).

There were also significant main effects of recovery on the volume of the ML per PN arbor, with the volume per PN arbor being significantly larger after recovery from treatment with the control and ethanol-containing liquid diets ($F_{1,39} = 8.579$, $P < 0.01$ and $F_{1,39} = 12.687$, $P < 0.001$, respectively). There were no effects of ethanol treatment *per se* on the mean volume per PN arbor.

Total numbers of synapses on PN arbors (N_s)

Analysis of the results in Table 3 showed that the mean total number of synapses per PN arbor differed significantly in the anterior and posterior lobes. In all treatment groups, the total numbers of synapses on PN arbors were significantly higher in the posterior lobe than in the anterior lobe (non-recovered rats: $F_{1,27} = 9.24$, $P < 0.01$; recovered rats: $F_{1,27} = 19.04$, $P < 0.001$).

There was a significant main effect of ethanol treatment on the numbers of synapses in the 22-month-old, non-recovered rats ($F_{2,27} = 4.36$, $P < 0.025$), an effect present only in the posterior lobe of those rats ($F_{2,27} = 3.71$, $P < 0.05$, Table 3). Post-hoc analysis showed that the mean number of synapses in the ethanol-fed rats was significantly lower than in the age-matched chow rats. There was also a significant interaction in the posterior lobe between recovery and previous dietary treatment ($F_{2,39} = 4.481$, $P = 0.02$). Analysis of

Table 3. Total number of synapses per Purkinje neuron dendritic arbor

Treatment	Age (months)	Anterior lobe Mean (CV)	Posterior lobe*** Mean (CV)
Chow controls (<i>n</i> = 4)	12	60 491 (0.13)	75 754 (0.29)
Chow controls (<i>n</i> = 10)	22	71 236 (0.22)	86 637 (0.27)
Pair-fed controls (<i>n</i> = 10)	22	62 911 (0.27)	73 786 (0.22)
Ethanol-fed (<i>n</i> = 10)	22	56 799 (0.17)	63 259 (0.26)*
Chow controls (<i>n</i> = 5)	27	49 383 (0.28)	65 197 (0.27)**
Pair-fed/recovered (<i>n</i> = 5)	27	62 299 (0.27)	81 845 (0.21)
Ethanol-fed/recovered (<i>n</i> = 5)	27	61 331 (0.19)	85 681 (0.26)**

Mean values are given for the total number of synapses (N_s) per PN dendritic arbor in the anterior and posterior lobes of the cerebellar vermis (CV). There was a significant age-induced decrease in N_s in the 27-month-old chow controls. There was a significant ethanol-related decrease in N_s in the 22-month-old ethanol-fed rats. In rats recovered from the ethanol diet, the post-recovery value for N_s was significantly larger than the pre-recovery value. The mean N_s on PN in the posterior lobe was significantly larger than that in the anterior lobe in all groups.

*Significant main effect of ethanol, $P < 0.05$; **significant interaction between treatment and recovery period, $P < 0.05$;

***significant main effect of lobe, $P < 0.05$ (ANOVA, F -values and exact P -values in text).

the interaction showed that there were significantly fewer synapses per PN in the chow-fed control rats at the end of the recovery period (simple main effect ANOVA, $F_{1,39} = 4.119$, $P = 0.05$), and significantly more synapses per PN in the ethanol-fed rats at the end of the recovery period (simple main effect ANOVA, $F_{1,39} = 4.505$, $P < 0.05$). There was no significant change after recovery in the number of synapses per PN arbor of the pair-fed control rats.

DISCUSSION

The purpose of the study reported here was to determine whether the total number of synapses on PN dendrites in old F344 rats was reduced after long-term ethanol imbibition. In previous studies, we showed that, when 12-month-old F344 rats consumed ethanol for long periods of time (40–48 weeks), the terminal dendrites of PN arbors appeared to lengthen (Pentney and Quigley, 1987; Pentney and Quackenbush, 1990, 1991; Pentney *et al.*, 1989). It was subsequently shown that in at least 30% of the ethanol-exposed PN, the apparent lengthening of dendritic segments was a result of deletion of single terminal segments at branch points (Pentney, 1995). Each segment deletion removed a branch point that marked the end of a non-terminal parent segment and the beginning of a surviving daughter terminal segment. Without the intervening branch point, these latter two segments formed a single longer

terminal segment. In old chow-fed rats, dendritic segments shortened with age, and there was no evidence of deletion of terminal segments at branch points (Quackenbush *et al.*, 1990). An adequate explanation for ethanol-induced deletion of segments in PN arbors has not yet emerged. The best-documented causes of dendritic loss in other types of neurons are: (1) regression resulting from a loss of afferent supply to dendrites; (2) regression following loss of neuronal targets; (3) regression integral to neuronal death (Flood and Coleman, 1993). In seeking to understand the reason for segment deletion in PN of ethanol-treated rats, we focused here on the most fundamental characteristic of synaptic sites on dendritic arbors, i.e. their number. The question we sought to answer was whether there were fewer synapses on dendritic arbors of PN in the old, ethanol-treated rats than in the old controls, in terms of total number per arbor.

The answer that we expected to obtain was that the total number of synapses on PN in the non-recovered ethanol-fed rats was reduced. Earlier investigations had shown that PN dendritic field area, PN dendritic branching density, PN total dendritic length (Tavares *et al.*, 1983) and synaptic density in the molecular layer of the cerebellum (Tavares *et al.*, 1987) were all reduced when Sprague–Dawley rats consumed ethanol chronically from the time they were 2–3 months of age. It had also been shown that regression of terminal dendrites occurred in PN dendritic arbors of old

F344 rats after similar durations of ethanol treatment, begun when the rats were 12 months of age (Pentney, 1982, 1995). Since synaptic input to terminal segments of PN occurs largely through synapses of afferent terminals with dendritic spines (Palay and Chan-Palay, 1974), it was theorized that ethanol-induced reductions in afferent input to potential synaptic sites or ethanol-induced reductions in spine formation might lead to synaptically depleted terminal segments that might subsequently be deleted. A significant main effect of ethanol treatment on the number of synapses, a reduction in the total number of synapses per PN arbor, was shown as expected.

The fact that the reduction in the number of synapses was present only in the posterior lobe was unexpected, however. It was of interest that in all groups of rats, regardless of treatment, the volume of the molecular layer per PN arbor and the number of synapses per arbor were significantly larger for PN in the posterior lobe than for PN in the anterior lobe. We suspect that these differences reflect fundamental differences in synaptic connectivity of neuronal populations in the two lobes. A previous study in this laboratory showed that, in F344 rats, the ratio of granule neurons to PN in lobule VII of the posterior lobe was significantly larger than that in lobule IV of the anterior lobe at all ages (Dlugos and Pentney, 1994). A possible reason for this quantitative difference is that the afferent input relayed to PN terminal segments by granule neurons in the anterior lobe of the cerebellar vermis is primarily from spinocerebellar mossy fibres, whereas that to PN in the posterior lobe is from spinocerebellar mossy fibres and also from pontocerebellar mossy fibres that relay information from higher brain centers (Gilman, 1986). The pontocerebellar input is the largest source of cerebellar mossy fibres and pontocerebellar projection patterns are more complicated than those of the spinocerebellar projections (Ito, 1984). The data reported here are consistent with the current view that PN in the posterior lobe of the cerebellum interact synaptically with a larger number and more varied source of afferents than do PN in the anterior lobe. The restriction of the synaptic loss to the posterior lobe of the cerebellum may be related to those differences.

The most interesting and revealing effects of ageing and of ethanol on the numbers of synapses

on the PN in the ageing F344 rats were those that resulted from significant interactions between the recovery period and treatment prior to recovery. It is important to keep in mind that during the 20-week recovery period following the ethanol treatment, all rats were given the standard chow pellet diet and water *ad libitum*. The standard controls, therefore, continued to receive their normal diet, and the pair-fed controls and the ethanol-fed rats were weaned from the liquid diets and returned to the chow diet. During the recovery period, there was a significant interaction between recovery and previous dietary treatment in the posterior lobe of the cerebellum, i.e. the post-recovery numbers of synapses on PN in particular treatment groups were significantly different from the pre-recovery numbers in the same groups. The post-hoc analyses showed that a significant *decrease* in the mean number of synapses occurred in rats on the chow diet during the 20 weeks of the recovery period and a significant *increase* in the mean number of synapses occurred in recovered rats that had been on the ethanol liquid diet. As noted above, there was no significant change in the number of synapses in PN of rats that had been on the control liquid diet.

In the chow controls, the decrease reflected a normal effect of ageing processes, and the decrease in the number of synapses paralleled a significant decrease in the volume of the molecular layer per PN arbor, suggesting that the age-related loss of synapses may have resulted from a loss of afferents. This change agrees with the significant decrease in the volume of the molecular layer of old F344 rats reported previously (Dlugos and Pentney, 1994).

In the ethanol-treated group, the increase in the number of synapses reflected an effect of recovery from the ethanol treatment with restoration of the number of synapses to pre-recovery control levels. The restoration of synaptic numbers in the recovered ethanol-fed rats was not a direct result of an increased volume of the molecular layer per PN for the following reason. Increases in the volume of the molecular layer per PN were of similar magnitude in the pair-fed and ethanol-fed groups ($+50\,138\text{ mm}^3$ and $+60\,973\text{ mm}^3$, respectively), showing that these increases were effects of recovery from the liquid diets *per se* and not specifically related to ethanol. In contrast, the increase in the number of synapses per PN during

recovery was three times larger in the ethanol-fed group (+22586) than in the pair-fed group (+8059), a difference that must be specifically related to ethanol.

The most telling feature of the changes produced by ethanol treatment and recovery in the old F344 rats used in this study was the pattern of change in the numbers of synapses over time. The loss of synapses during ethanol treatment and the regaining of synapses during recovery paralleled the previously described reversible loss of terminal dendritic segments in old, ethanol-treated and recovered F344 rats (Pentney and Quackenbush, 1990, 1991), suggesting that these separate structural modifications were causally related. The direction of causality is not clear, however. The fact that a loss of afferents is one of the best documented causes of dendritic loss, as noted above (Flood and Coleman, 1993), lends support to the view that ethanol-induced reductions in synaptic input to PN dendrites may have *caused* deletion of affected dendritic segments in PN. The reverse view, that the loss of synapses may have *resulted* from an ethanol-induced deletion of dendritic segments, cannot currently be eliminated, however. It remains to be shown whether the reductions in synaptic input result from prior pre- or postsynaptic events. A study addressing this problem is currently underway.

Remodelling of synapses on PN during long-term ethanol treatment has been previously reported in young adult rats following removal of parallel fibre synaptic terminals (Tavares, 1985) attributed to death of granule neurons (Tavares and Paula-Barbosa, 1982) and compensatory hypertrophy of climbing fibre terminals (Tavares *et al.*, 1986). There was no evidence in the old, ethanol-treated F344 rats that a loss of granule neurons was responsible for the decrease in synaptic numbers on ethanol-exposed PN (Pentney and Dlugos, 1996). Furthermore, the restoration of synapses on PN in the present study occurred during recovery, rather than during ethanol treatment. As encouraging as these results are, it has not yet been possible to determine whether the synaptic component of each arbor was re-established qualitatively as well as quantitatively during recovery. If the quantitative restoration of synapses during recovery reflects a qualitatively different synaptic arrangement on PN, it will be important to define that difference in

order to understand the functional consequences of the ethanol-induced synaptic changes that have been reported here.

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