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The effect of the timing of ethanol exposure during early postnatal life on total number of Purkinje cells in rat cerebellum

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ABSTRACT

We have previously shown that exposing rats to a high dose of ethanol on postnatal d 5 can affect Purkinje cell numbers in the cerebellum whilst similar exposure on d 10 had no such effect. The question arose whether a longer period of ethanol exposure after d 10 could produce loss of Purkinje cells. We have examined this question by exposing young rats to a relatively high dose ($\sim$ 420–430 mg/dl) of ethanol for 6 d periods between the ages of either 4 and 9 d or 10 and 15 d of age. Exposure was carried out by placing the rats in an ethanol vapour chamber for 3 h per day during the exposure period. Groups of ethanol-treated (ET), separation controls (SC) and mother-reared controls (MRC) were anaesthetised and killed when aged 30 d by perfusion with buffered 2.5% glutaraldehyde. Stereological methods were used to determine the numbers of Purkinje cells in the cerebellum of each rat. MRC, SC and rats treated with ethanol between 10–15 d of age each had, on average, about 254–258 thousand cerebellar Purkinje cells; the differences between these various groups were not statistically significant. However, the rats treated with ethanol vapour between 4–9 d of age had an average of only about $128\,000 \pm 20\,000$ Purkinje cells per cerebellum. This value was significantly different from both the MRC and group-matched SC animals. It is concluded that the period between 4 and 9 d of age is an extremely vulnerable period during which the rat cerebellar Purkinje cells are particularly susceptible to the effects of a high dose of ethanol. However, a similar level and duration of ethanol exposure commencing after 10 d of age has no significant effect on Purkinje cell numbers.

Key words: Cell death; stereology; fetal alcohol syndrome.

INTRODUCTION

Alcohol ingestion by mothers during pregnancy can result in the fetal alcohol syndrome (FAS) in their offspring (Jones & Smith, 1973). This is characterised by specific facial features (short palpebral fissures, epicanthic folds, lower nasal bridge, short nose, indistinct philtrum, thin lip and flat midface), pre- and postnatal growth retardation, microcephaly and mental retardation (Jones et al. 1973, 1976; Clarren et al. 1978; Sampson et al. 1997). Damage to the central nervous system (CNS) has emerged as one of the most serious consequences of FAS (Clarren & Smith, 1978; Streissguth et al. 1986). The cerebellum is most consistently affected by prenatal ethanol exposure in man. Children with FAS show various symptoms associated with cerebellar damage including delay in motor development (Streissguth et al. 1980; Little et al. 1989), problems with fine motor tasks (Jones & Smith, 1973; Beattie, 1986; Barr et al. 1990) tremor (Jones & Smith, 1973; Kyllerman et al. 1985; Marcus, 1987), ataxia (Kyllerman et al. 1985; Marcus, 1987) and gait disturbance (Marcus, 1987).

In man the 'brain growth spurt' (Dobbing & Sands, 1973), a period during which the brain is particularly vulnerable to environmental influences, begins at

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about midgestation and peaks around birth. In laboratory animals, such as rats, the brain growth spurt occurs primarily during the first 2 wk after birth, with a peak at about postnatal days (PND) 6–8 (Dobbing & Sands, 1979). The period of maximal vulnerability in the rat is therefore thought to coincide with this postnatal period. Ethanol administration to rats during early postnatal life has been shown to result in behavioural anomalies (Goodlett et al. 1987; Kelly et al. 1987; Meyer et al. 1990; Pauli et al. 1995) and various cerebellar deficits (Phillips & Cragg, 1982; Pierce et al. 1989; Bonthius & West, 1990; Goodett et al. 1990; Hamre & West, 1993; Pauli et al. 1995).

In a recent study we (Pauli et al. 1995) have found that acute exposure of rats to alcohol for a period of just a few hours on PND 5 resulted in a marked deficit in Purkinje cell numbers. However, similar exposure on PND 10 had no significant effect. From this study it remained uncertain whether a more prolonged period of exposure to ethanol after 10 d of age would result in changes in Purkinje cell number or whether these cells were somehow protected from the effects of alcohol after this age. We decided to investigate this question by exposing rats to relatively high doses of ethanol for a few hours a day between the periods of PND 4–9 or PND 10–15. A knowledge of the exact timing and duration of the vulnerable period for Purkinje cell development may help in understanding the mechanisms involved which result in damage to the CNS by exposure to ethanol.

MATERIALS AND METHODS

Animals

Pregnant female Wistar rats were obtained from the University of Queensland Central Animal Breeding House and housed in individual cages in a temperature-controlled room (21.6 ± 0.5 °C) maintained on a 12/12 h light/dark cycle. They were fed a normal rodent diet and given drinking water ad libitum. The rats were checked at 9.00 am each day to determine whether they had given birth. The day of birth for any given litter was designated as PND 0 for those rats. On PND 2 pups from groups of 3 separate litters were removed from their mothers and placed together in a temporary holding cage. The pups from this 'pool' were then randomly assigned back to the lactating mothers such that each mother within a given group received 12 pups. These litters were then randomly assigned either to ethanol treatment (ET), separation control (SC) or to mother-reared control (MRC) groups.

Ethanol treatment

Exposure to ethanol was achieved by a vapour inhalation method similar to that described by Karanian et al. (1986). Specifically, on each day between PND 4 and 9 or between PND 10 and 15 all pups from a given litter assigned to the ET group were removed from their 'mother' between the hours of 10 am to 1.00 pm and placed in a holding cage containing wood shavings. This was located in an enclosed perspex chamber ($600 \times 600 \times 1100$ mm). Ethanol vapour mixed with medical grade air was allowed to flow into this chamber at a rate of 10 l/min. The concentration (mean $\pm$ S.E.M.; $n = 9$) of ethanol in the chamber was controlled to be about 58.7 ± 2.3 mg/l for the PND 4–9 treatment group and 76.3 ± 5.3 mg/l for the PND 10–15 treatment group. Pilot experiments had shown that these concentrations of ethanol produced similar blood alcohol concentrations in the different-aged rats used. After the 3 h treatment period each day the pups were weighed and returned to their 'mothers'. The pups within the SC litters were also removed from their mother for a period of 3 h each day between PND 4–9 or PND 10–15 as appropriate. During the period of separation these pups were placed in a chamber under similar conditions as the ET groups except that ethanol vapour was not added to the air. The pups within the MRC groups were allowed to remain with their mother for the duration of the experiment. All pups were weaned by separation from their mothers at 25 d of age.

Determination of blood alcohol concentration (BAC)

Blood alcohol concentrations (BAC) were measured immediately after ethanol exposure on PND 4, 6, 9, 10, 12 and 15 in the ethanol treated groups using the enzymatic method described by Lundquist (1959). The pups used for this purpose were not those used in the main part of the experiment.

Tissue preparation

At 30 d of age, groups of ET, SC and MRC pups were anaesthetised with a mixture (0.1 ml/kg body weight) of 50:50 (v/v) Ketamine (100 mg/ml; Apex Laboratories, Australia) and Xylazine (20 mg/ml; Cooper Animal Health, Australia), and perfused intracardially with a few millilitres of buffered saline followed by about 350 ml of a fixative containing 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Each group contained 9 pups with no more

than 3 from any given litter. After perfusion the brains were removed and the 'forebrain' (here defined as brain minus cerebellum and brainstem) and cerebellum from each animal weighed.

The Fractionator

The Fractionator method (Gundersen, 1986) was used to estimate the number of Purkinje cells in each cerebellum. This method utilises a number of systematic sampling stages, summarised as follows.

Stage 1. Each cerebellum was cut into 1–2 mm parasagittal slices. It was ensured that the position of the first slice was 'uniform random' (equiprobable) in the interval 0–2 mm. This guaranteed that all regions of the cerebellum had an equal chance of being sampled. All of these slices were then cut into strips about 1–2 mm wide. The strips were arranged in approximate order of size with the biggest strips in the middle. This 'dome' arrangement of the strips reduces interindividual variation (Ogbuihi & Cruz-Orive, 1990; Mayhew, 1991, 1992). A systematic random procedure (Gundersen, 1986) was used to select a known fraction of the strips (Gundersen, 1986; Nairn et al. 1989; Bedi et al. 1992). This was accomplished by selecting a number between 1 and 4 by lottery. If the number 4 was chosen for a given cerebellum, the strips 4, 8, 12, etc. were selected for further analysis. A new random number was chosen for each cerebellum. The probability of selecting any given strip was therefore 1 in 4 ($f1 = 4$) for each cerebellum.

Stage 2. The strips sampled at stage 1 were cut into smaller pieces approximately 1–2 mm^3 in size and arranged as described above. A systematic random procedure was used to select 1 in 5 ($f2 = 5$) of these pieces of tissue.

Stage 3. The tissue sampled in stage 2 for any given cerebellum was processed for routine paraffin wax histology (Drury & Wallington, 1976) and embedded together in the same mould. The tissue blocks were cut subsequently on a Historange microtome at a nominal section thickness of 6 μm until all the tissue had been exhaustively serially sectioned. A sample of 1 in 20 ($f3 = 20$) of these sections was chosen by a systematic random sampling procedure (Bedi et al. 1992; Pauli et al. 1995), mounted on glass slides and stained with 0.1% cresyl violet (Drury & Wallington, 1976).

Stage 4. The sections sampled in stage 3 were examined with a Vickers M17 light microscope fitted with a $\times 25$ magnification objective lens. All Purkinje cell nucleoli visible in these sections were counted (total number, n). The total number (N) of Purkinje cell nucleoli in the entire cerebellum was estimated from the equation (Gundersen, 1986)

$$N = f1 \times f2 \times f3 \times n.$$

It was assumed that each Purkinje cell nucleus contained only 1 nucleolus. If this assumption is correct, then the estimate of nucleolar number must also be equal to the number of Purkinje cells. In this experiment, we found no case in which a profile of a Purkinje cell contained more than 1 nucleolus.

All analyses were carried out without prior knowledge of the experimental group to which any given cerebellum belonged. This was achieved by allocating a code number to each cerebellum, the code not being broken until all the cerebella in a given experiment had been analysed.

Statistics

Group means and standard errors were calculated for all control and experimental groups of animals. Data were analysed by 2-way analysis of variance (ANOVA) procedures. Post hoc tests were carried out where appropriate using the Tukey–Kramer test (Sokal & Rohlf, 1981). All statistics were carried out using Minitab (Version 11) on an IBM-compatible computer.

RESULTS

Blood ethanol concentrations

The mean blood ethanol concentration of rats exposed to ethanol vapour during PND 4–9 was 422 ± 17 mg/dl (mean $\pm$ S.E.M.; $n = 21$) while rats exposed to ethanol vapour during PND 10–15 showed a blood concentration of 432 ± 38 mg/dl ($n = 21$).

Body weights

The mean $\pm$ S.E.M. body weights of PND 4–9 and PND 10–15 groups are shown in Table 1, both at the end of the treatment period (i.e. 10 and 16 d of age respectively) and at 30 d of age when the animals were killed for histological examination. Two-way ANOVA of the body weight data at 10 and 30 d of age from the PND 4–9 groups showed a significant main effect of Groups, Age and a significant Group $\times$ Age interaction (Table 1). Post hoc tests showed that these observations were due to differences between the ET animals on the one hand and both MRC and SC age-matched controls on the other. There were no significant differences between the MRC and SC

Table 1. *Mean ± S.E.M. body weights (g) of rats exposed to ethanol vapour at either PND 4–9 or PND 10–15 and their age-matched controls*

Group	PND 10	PND 16	PND 30	ANOVA results		
PND 4–9						
MRC	27.3 ± 0.7 (9)	—	112.0 ± 3.8 (9)	Group	df 2, 48	$F = 25.33$***
SC	25.3 ± 0.9 (9)	—	111.4 ± 3.6 (9)	Age	df 1, 48	$F = 1434$***
ET	16.6 ± 0.9 (9)	—	89.2 ± 3.4 (9)	Group × Age	df 2, 48	$F = 4$*
PND 10–15						
MRC	—	33.4 ± 0.9 (9)	103.4 ± 3.2 (9)	Group	df 2, 48	$F = 4.88$*
SC	—	34.5 ± 1.1 (9)	106.4 ± 3.2 (9)	Age	df 1, 48	$F = 1327$***
ET	—	31.3 ± 1.0 (9)	95.5 ± 2.9 (9)	Group × Age	df 2, 48	$F = 1.47$

Figures in parenthesis indicate the number of animals examined in each group.
*** $P < 0.001$, * $P < 0.05$. MRC, mother reared control; SC, separation control; ET, ethanol treated animal; PND, postnatal day; ANOVA, analysis of variance; df, degrees of freedom; *F*, *F* value.

Table 2. *Two-way ANOVA of body weight data of 30-d-old rats in the PND 4–9 and PND 10–15 treatment groups*

Group	df 2, 48	$F = 15.0$***
Age at treatment	df 1, 48	$F = 0.79$
Group × Age at treatment	df 2, 48	$F = 2.71$

animals. Similar 2-way ANOVA of the body weight data from the PND 10–15 groups of animals showed significant effects of Groups and Age but no significant interaction (Table 1). Post hoc tests showed once again, that the main effects were due to differences between the ET treated animals and their respective MRC and SC controls with no significant differences between the 2 control groups. The body weight data at 30 d of age from the 2 treatment groups (i.e. PND 4–9 and PND 10–15) was further analysed by a 2-way ANOVA with the Treatment Age and Groups as the 2 factors. This analysis showed a significant main effect of Groups, no significant effect of Treatment Age or interaction (Table 2). However, post hoc tests showed the deficit in body weight at 30 d of age of the PND 4–9 ethanol-treated rats was significant compared with their controls whilst that for the PND 10–15 was not.

Brain weights

Table 3 shows the means ± S.E.M. for 'forebrain' and cerebellar weights of rats exposed to ethanol vapour during PND 4–9 or PND 10–15 and their respective MRC and SC controls. Two-way ANOVA revealed a significant main effect of Group and Group × Age interaction (Table 3) for both the forebrain and cerebellum. These differences were reflected in post hoc tests which showed significant deficits in forebrain and cerebellar weights between ET animals and their MRC or SC controls. There were no statistically significant differences in these measures between MRC and SC animals within any given treatment group (Table 3).

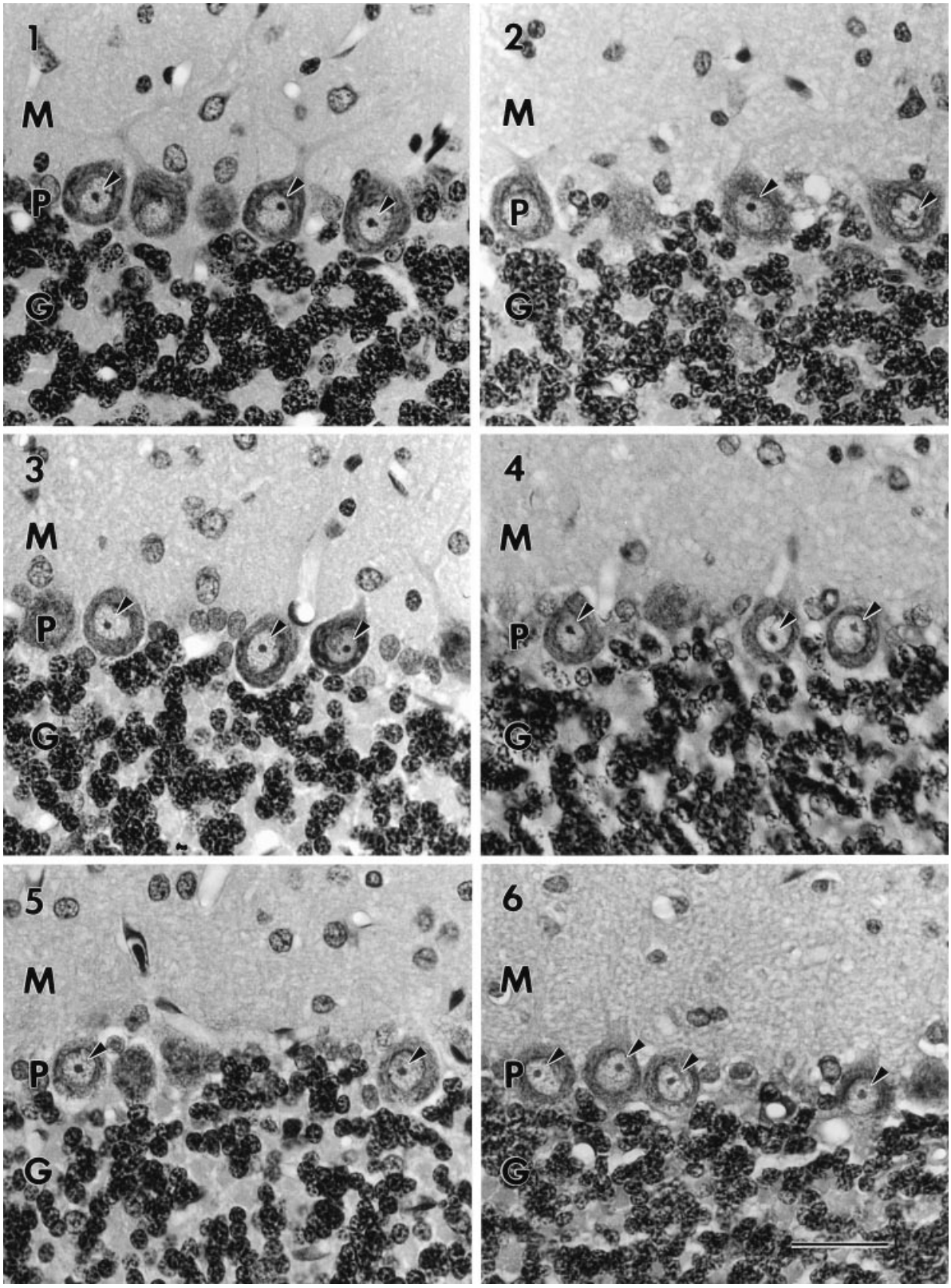
Table 3. *Mean ± S.E.M. forebrain† (g) and cerebellar weights (mg) of control and ethanol treated rats aged 30 d*

Group	n	Forebrain weight	Cerebellar weight
PND 4–9			
MRC	9	1.35 ± 0.01	260 ± 9
SC	9	1.41 ± 0.03	280 ± 8
ET	9	1.04 ± 0.03	140 ± 11
PND 10–15			
MRC	9	1.35 ± 0.03	230 ± 3
SC	9	1.29 ± 0.01	230 ± 2
ET	9	1.18 ± 0.02	210 ± 3
Two-way ANOVA of above data			
Forebrain			
Group		df 2, 48	$F = 79.0$***
Age at treatment		df 1, 48	$F = 0.07$
Groups × Age at treatment		df 2, 48	$F = 18.8$***
Cerebellum			
Group		df 2, 48	$F = 86.54$***
Age at treatment		df 1, 48	$F = 0.02$
Group × Age at treatment		df 2, 48	$F = 41.19$***

† Whole brain minus brainstem and cerebellum; n, number of animals examined; *** $P < 0.001$.
MRC, mother reared control; SC, separation control; ET, ethanol treated animal; PND, postnatal day; ANOVA, analysis of variance; df, degrees of freedom; *F*, *F* value.

Purkinje cells

Representative light micrographs of sections through the cerebella from the different groups of rats are shown in Figures 1–6. There were no obvious qualitative morphological differences in the appearance of the sections from animals in the different groups.



Figs 1–6. Representative light micrographs of 6 μm cresyl violet-stained wax sections from 30-d-old rat cerebella from each group. Figs 1 and 2 are from mother-reared rats; Figs 3 and 4 are from separation control rats and Figs 5 and 6 are from ethanol-treated animals. Figs 1, 3 and 5 were from animals treated between PND 4 and 9 and whilst Figs 2, 4 and 6 were from rats treated between 10 and 15 d of age. In all such sections Purkinje cells (P) profiles were arranged in a single row at the junction of molecular (M) and granule (G) cell layers. Prominent nucleoli (arrowheads) were visible in most (but not all) of the Purkinje cell nuclear profiles. Bar, 30 μm.

Table 4. *Mean ± S.E.M. Purkinje cell number in ethanol treated and control animals aged 30 d*

Group	n	Purkinje cell number ($\times 10^3$)	
PND 4–9			
MRC	9	236 ± 11	
SC	9	234 ± 9	
ET	9	128 ± 20	
PND 10–15			
MRC	9	258 ± 16	
SC	9	258 ± 13	
ET	9	254 ± 18	
Two-way ANOVA of above data			
Purkinje cell			
Group		df 2, 48	$F = 9.24$***
Age at treatment		df 1, 48	$F = 22.54$***
Control × Age at treatment		df 2, 48	$F = 7.77$**

n, number of animals examined; *** $P < 0.001$, ** $P < 0.01$. MRC, mother reared control; SC, separation control; ET, ethanol treated animal; PND, postnatal day; df, degrees of freedom; *F*, *F* value.

The mean ± S.E.M. total number of Purkinje cells of rats from PND 4–9 and PND 10–15 groups are presented in Table 4. In the PND 4–9 groups the MRC rats had about 235 000, the SC rats had about 234 000 and the ET rats had about 128 000 Purkinje cells. In contrast, in the PND 10–15 groups all 3 treatment groups had about between 254 000 and 258 000 Purkinje cells (Table 4). Two-way ANOVA of these data at 30 d revealed significant main effects of Group, Age at treatment and Group × Age at treatment (Table 4). Post hoc tests showed that in PND 4–9 groups the total number of cerebellar Purkinje cell in ET rats was significantly fewer than their MRC and SC controls. There was no significant differences amongst the 3 groups of rats in PND 10–15 groups (Table 4).

DISCUSSION

Previous studies (Bauer-Moffett & Altman, 1977; Barnes & Walker, 1981; West et al. 1986; Pierce et al. 1989; Bonthius & West, 1990) have demonstrated that neuronal cell loss as a result of ethanol exposure is dependent both on the timing of the exposure and the level of the blood alcohol concentrations (BAC) achieved. High BACs were more detrimental than the same amount of alcohol given over a lengthy period, resulting in relatively low BACs. In the present study both the PND 4–9 and PND 10–15 groups of rats had BACs at about 420–430 mg/dl immediately after the ethanol exposure. The rats remained intoxicated for a few hours following the exposure. Despite these relatively high BACs the PND 10–15 rats showed no significant loss of Purkinje cells although body, forebrain, and cerebellar weights were affected. On the other hand, the PND 4–9 group of rats showed marked loss of cerebellar Purkinje cells as well as deficits in body and brain weights.

These findings strongly suggest that Purkinje cells are particularly vulnerable during the period up to about 9 d of age. After this age they are somewhat protected against the effects of ethanol ingestion, at least in terms of their capability to survive following ethanol exposure (see West et al. 1984). In a previous study we (Pauli et al. 1995) reported that exposing rats to a high (7.5 g/kg body weight) dose of ethanol over an 8 h period on PND 5 was capable of affecting Purkinje cell numbers whilst similar exposure on PND 10 had no such effect. In this previous study we found that 5 and 10-d-old ethanol treated rats had about 138 000 and 217 000 Purkinje cells respectively. Our present results support and extend these previous findings. It is of particular significance that extending the period of ethanol exposure from 1 to 6 days still had no significant affect on Purkinje cell numbers as long as the commencement of the period of exposure did not occur until the rats were 10 postnatal days of age. This indicates that as the cerebellum matures the Purkinje cells become relatively resistant to at least some of the adverse effects of ethanol exposure. The exact changes which bring this about are unknown at present.

This finding does not imply that other features of the cerebellum including Purkinje cell morphology are not vulnerable in other ways not examined in this study. Indeed, the observation that cerebellar weights are markedly smaller in the ethanol treated animals compared with their age-matched controls argues in favour of there being potentially serious problems for optimal cerebellar development in rats exposed to ethanol during either period examined in this study. The possible functional implications of this deficit in cerebellar weight (and the implications that this may have for cerebellar morphology) are uncertain at present. However, it is interesting to note that Thomas et al. (1998) recently found that rats exposed to high doses of ethanol on PND 4 and 5 or 8 and 9 had a deficit in motor coordination as tested on a parallel bar apparatus. Furthermore, this deficit was found to correlate with the extent of the loss of cerebellar Purkinje cells. Nairn et al. (1989) and Mayhew (1991) have shown that there is a positive correlation between the numbers of Purkinje cells as estimated by modern stereological techniques and cerebellar weights in man (Nairn et al. 1989) as well as in a range of other mammals (Mayhew, 1991).

Loss of cerebellar Purkinje cells due to exposure to alcohol has been demonstrated in previous studies (Bauer-Moffett & Altman, 1977; Borges & Lewis, 1982; Phillips & Cragg, 1982; Bonthius et al. 1988; Pierce et al. 1989; Bonthius & West, 1990, 1991; Goodlett et al. 1990; Hamre & West, 1993; Marcussen et al. 1994). The recent study by Hamre & West (1993) in which neonatal rats were subjected to 2 d periods of ethanol exposure at the rate of 6.6 g/kg/d between PND 4 and 13 is of particular interest. The ethanol was given to the rats by means of an intragastric cannula. The rats were killed at 21 d of age and the number of Purkinje cell nuclear profiles in 3 'midline vermal sections' counted. Hamre & West (1993) found that the greatest effect on numbers of Purkinje cell profiles occurred in the rats given alcohol on PND 4–5 whereas no such effects were observed in rats given alcohol after 7–8 d of age. Whilst overall this result is consistent with our present findings it is unfortunate that the quantitative procedures used by Hamre & West (1993) were somewhat limited in their scope and liable to yield biased results (Gundersen, 1986; Mayhew, 1991, 1992). Modern stereological techniques are capable of not only producing relatively unbiased estimates of numerical densities but also allow the derivation of the total numbers of cells (e.g. Purkinje cells) in an organ. Such values are easier to conceptualise and compare than arbitrary counts such as the number of profiles in 3 (relatively undefined) 'midvermal sections' as described by Hamre & West.

In another study by West's group (Marcussen et al. 1994) the number of Purkinje cell profiles in a single midsagittal section was counted from rats which had been exposed to ethanol either during gestation (embryonic d 13–18) or between PND 4–9. The former period was selected to coincide with Purkinje cell genesis whilst the latter period is at a time of early Purkinje cell differentiation. Marcussen et al. (1994) found that the density of Purkinje cell profiles was significantly smaller in the postnatally treated rats whilst those exposed before birth were not significantly different from controls. This may indicate that Purkinje cells are more vulnerable during this phase of differentiation than they are just after being generated. If true, this is an extremely interesting finding with many implications about the possible mechanisms involved in the teratogenic effects of ethanol. Unfortunately, cell density measurements in the brain are notoriously difficult to interpret (Bedi, 1984; Braendgaard & Gundersen, 1986) in terms of total number due to the changing relationships between its components during development. The findings of Marcussen et al. (1994) are therefore in need of further confirmation using modern stereological methods to estimate total cell numbers rather than placing reliance on cell density estimates.

This fact has been now recognised by West's group as in their most recent publication (Thomas et al. 1998) they have used the relatively recently developed and largely unbiased optical fractionator method to estimate Purkinje cell numbers rather than just relying on estimates of numerical density. In this more advanced study they have examined artificially-reared rats which had been given high doses of ethanol either on (postnatal) d 4 and 5 or 8 and 9 or on d 4, 5, 8 and 9. They found that there was some Purkinje cell loss in all 3 ethanol-treated groups compared with controls. The extent of the loss was greater in animals treated on d 4 and 5 compared with those just treated on d 8 and 9. This finding has led them to modify their previous view (Hamre & West, 1993) that ethanol exposure after 7 d of age has no significant effect on Purkinje cells.

The mechanisms of alcohol's teratogenic actions on the brain are not understood at present. It may be that the ethanol, or one of its metabolites, is acting directly on the Purkinje cells in some way to cause their cell death. Alternatively, the loss of Purkinje cells may only be a secondary effect brought about as a result of the effect of alcohol on some other developmental feature. For example, it may be due to a disruption in the generation and migration of granule cells, or the process of Purkinje cell dendritic growth or synaptogenesis within the cerebellar cortex. A recent study by West et al. (1990) using a monoclonal antibody to microtubule associated protein 2 (anti-MAP2) showed that cerebellar regions undergoing rapid dendritic development may be more vulnerable to the effects of ethanol exposure than other regions. However, alcohol affects a number of different target sites in the CNS and may therefore influence several systems in parallel. Furthermore, the mechanisms of its actions may differ during the various stages of CNS development (West, 1994). For example, ethanol may act by affecting the endocrine system (Weinberg et al. 1986), CNS neurotransmitters (Druse, 1986, 1992) and brain microvasculature (Kelly et al. 1990) at different stages of development. It may be that there is no single mechanism that can account completely for the impairment of CNS neurons following ethanol exposure.

Giving alcohol to neonatal rats in a vapour chamber necessarily involves removing them from their mother for a period of time. This and any period that they spend intoxicated exposes them to a degree of

malnutrition or undernutrition. These conditions are known to influence various morphological features in the brain including granule-to-Purkinje cell ratios and synapse-to-neuron ratios in the cerebellum (Bedi, 1987; Bedi et al. 1980*a*, *b*; Bedi & Warren, 1988; Warren & Bedi, 1988, 1990). However, we (Bedi et al. 1992) have shown that even relatively lengthy periods of undernutrition during early postnatal life do not affect the total number of cerebellar Purkinje cells. We therefore believe that it is unlikely that the deficit in Purkinje cell numbers observed in the present study can be attributed to any secondary nutritional effects brought about as a consequence of the exposure to alcohol. After all, this exposure to alcohol occurred for only a few hours a day for a period of just 6 d.

Finally, our estimates for the total number of cerebellar Purkinje cells in 30-d-old control rats ranged between about 235000 and 258000. These values are in close agreement with most other studies (Braendgaard & Gundersen, 1986; Gundersen, 1986; Warren et al. 1988; Mayhew, 1991; Bedi et al. 1992; Pauli et al. 1995) on adult rats where modern stereological procedures have been used. An exception is the recent study by Thomas et al. (1998) mentioned above, which found there to be $\sim$ 450000 Purkinje cells in both gastrostomy and suckle control rats at 55 d of age. The exact reason for the discrepancy between Thomas et al. (1998) and the other researchers cited above is uncertain.

In conclusion, we have demonstrated that the long-term viability of rat cerebellar Purkinje cells is markedly affected by ethanol exposure between PND 4 and 9 but not in the subsequent period of PND 10 and 15. This period before 10 d of age therefore seems to represent a critical period in Purkinje cell development. The exact changes which must occur either in the cerebellum and/or in the Purkinje cells themselves to alter this state of vulnerability after about 10 postnatal days of age are unknown. The vulnerability within this critical period leads to significant deficits in the total number of Purkinje cells in rats exposed to relatively high doses of ethanol. The mechanisms involved in the loss of these Purkinje cells due to ethanol exposure are presently the subject of further research.

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