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Morphometric analysis of the postnatal mouse optic nerve following prenatal exposure to alcohol

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ABSTRACT

Pregnant female mice were divided on day 12 post coitum into a control and an experimental group. The experimental group was given a single intraperitoneal dose of 0.015 ml/g body weight of 25% solution of alcohol in distilled water while the control group was exposed to a similar weight related dose of normal saline. The optic nerves were isolated from the offspring of both control and experimental groups at wk 2, 3 and 5 (i.e. during the juvenile period of postnatal development) and analysed by light and electron microscopy. Although in both groups the optic nerve grew in size rapidly during the period studied, the rate of growth in the experimental groups lagged behind that of the controls. The difference was initially significant but tailed off, so that by wk 5 it was no longer significant. The time of initial onset and progression of myelinogenesis in the optic nerve of alcohol exposed mice also lagged behind that of controls. In both groups the size distribution of the myelinated nerve fibres in the optic nerve was unimodal with a positive skewing for all ages. The spectrum of size distribution of the nerve fibres was, however, broader in controls than in the corresponding experimental groups. With increasing age the proportion of small and medium size fibres was greater in the experimental group than in the controls, while for the large diameter fibres the reverse was observed. It is suggested that this study may shed light on the teratogenic effect of 'binge' drinking during pregnancy and that it is the critical period when exposure occurs that is more important than the duration of administration.

Key words: Morphometry; optic neuropathy; myelinogenesis; fetal alcohol syndrome.

INTRODUCTION

Since the clinical entity, the fetal alcohol sydrome, was first described in man (Jones & Smith, 1973; Jones et al. 1973, 1974), a number of clinical and experimental studies have been carried out to investigate its pathogenesis. This syndrome appears to be both species and strain dependent (Schwetz et al. 1978; Schenker et al. 1990; Zajac & Abel, 1992), with rodents being used in the majority of these studies (Abel et al. 1981; Garro et al. 1991; Middaugh & Boggan, 1991; Kotch et al. 1992; Pinazo-Duran et al. 1993). Considerable strain differences in sensitivity to alcohol have been reported in the mouse (Chernoff, 1977, 1980; Cook et al. 1987; Gilliam et al. 1990). For example, using 3 different strains of mice Chernoff (1980) observed that the CBA embryo was the most sensitive to prenatal alcohol exposure followed respectively, by the C3 and the C57BL strains of mice, while the C57BL/6J strain was more sensitive than the *long-sleep* or the *short-sleep* mouse (Gilliam et al. 1990).

The degree of sensitivity of individual organs to the teratogenic effects of alcohol varies considerably, the critical factors being the time of exposure during the prenatal period and the dose and frequency of exposure (Abel et al. 1981; Webster et al. 1983; Cook et al. 1987; Adickes, 1990; Michaelis, 1990; Kotch et al. 1992; Zajac & Abel, 1992; Ashwell & Zhang, 1994). The central nervous system (CNS) is particularly sensitive and the optic nerve in particular has been extensively studied to determine the effects of alcohol on this system (Cohen, 1967; Ehyai & Freemon, 1983; Pinazo-Duran et al. 1993). Although some morphometric studies have been carried out on the long-term effect of acute prenatal exposure to

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alcohol on the optic nerve in the mouse (Ashwell & Zhang, 1994; Parson et al. 1995), no detailed analysis of the findings during the early postnatal period is presently available. The aim of the present study was to investigate the effect of exposure to a single prenatal dose of alcohol on the early postnatal development of the mouse optic nerve.

MATERIALS AND METHODS

Brother × sister matings of superovulated (C57BL × CBA) F_1 hybrid mice were carried out. The presence of a vaginal plug observed the next morning was considered as evidence of mating and this was designated as day (d) 0.5 post coitum (pc). Pregnant mice were maintained under a 14 h–10 h light–dark cycle with food and water available ad libitum. On d 12 pc the pregnant females were divided into an experimental and a control group. Each mouse in the experimental group was given a single intraperitoneal (i.p.) dose of 0.015 ml/g (volume:weight) 25% solution of alcohol in distilled water. This dose has been shown to produce a peak blood alcohol level of 500–600 mg/100 ml 4–4.5 h after injection (Webster et al. 1983; Ashwell & Zhang, 1994). The control group was treated with 0.9% saline in a similar way. Offspring of both experimental and control groups were used in this study. Those that were to be used after the age of 3 wk were weaned at this age and maintained on a diet similar to that of the parents.

Five mice from each age group of both control and experimental animals were studied at wk 2, 3 and 5 postnatally. The 5th postnatal week was selected because we have already demonstrated that this is the time when the phase of rapid postnatal development of the optic nerve in the mouse is completed, with the period from birth to the end of the 5th postnatal week corresponding to the juvenile period of life in this species (Hogan et al. 1994; Dangata et al. 1996). Each animal was deeply anaesthetised following an i.p. injection of 0.02 ml/g body weight of a 1.2% solution of tribromoethanol (Avertin) in 0.9% saline. The heart was then exposed and, using a 23G needle, intracardiac perfusion of fixative was carried out by giving 2.0 ml/g body weight of a 2.5% solution of glutaraldehyde in 0.1 M phosphate buffer through the left ventricle while the heart was still beating.

The optic nerve was immediately, but carefully, dissected out avoiding traction on the nerve. Both nerves from each animal were put in the same prelabelled bottle containing 2.5% glutaraldehyde and 1 M paraformaldehyde fixative in 0.1 M phosphate buffer and left for a total of 12 h. The nerves were then washed in 0.1 M phosphate buffer and transferred into a secondary fixative consisting of 1% osmium tetroxide in 0.1 M phosphate buffer for a further 2 h. After this they were dehydrated in a graded alcohol series and then embedded in Araldite.

From each nerve, semithin sections ($\sim$ 1 μm) were cut perpendicular to the long axis of the nerve using a Reichert-Jung Ultracut E microtome. Sections were stained with 1% toluidine blue in 1 % borax for light microscopy. Ultrathin sections ($\sim$ 80 nm) thickness were then cut for electron microscopy. These were picked up on copper grids (200) and subsequently stained with 0.2 % lead citrate solution and a saturated solution of uranyl acetate (Reynolds, 1963).

From the ultrathin sections, a selection of photomicrographs was taken at an initial magnification of × 750 from centre to periphery of each nerve using a Philips EM301 transmission electron microscope. The objective here was to include as many representative profiles of each nerve as possible for the sampling procedure without having an overlap of the sampled fields. The micrographs were developed and printed to a final magnification of × 3000 in order to estimate the total number of myelinated nerve fibres present in each nerve. Additional micrographs were taken at a magnification of × 20000 as it was possible to clearly visualise distinct myelin lamellae present associated with each nerve fibre within the individual nerves.

Using the semithin sections, the cross-sectional area (csa) of each nerve, excluding its meningeal coverings (Williams et al. 1989) was measured in a Magiscan image analysis system (Applied Imaging). Other detailed morphometric measurements of the nerves were also carried out on the photomicrographs of the ultrathin sections using the image analyser. A systematic random sampling method (Mayhew, 1990) was used to determine the number and diameter spectrum of the myelinated nerve fibres present in each nerve. This was done by locating the centre of the nerve from which point sectors of 10° were drawn over different areas of the nerve. A grid of approximately 1 cm × 1 cm squares (equivalent to 7.8 μm² of nerve csa) was placed over each sector. Using the sampling method indicated above, and starting from the centre of each nerve, every 4th square falling completely within each sector in each direction (i.e. 1 in 16) was sampled for the estimation of nerve fibre counts and fibre size distribution. Only myelinated nerve fibres whose centres fell within a sample square were included in the analysis. Mayhew (1988, 1990) and Mayhew & Sharma (1984*a*, *b*) have shown that it is only necessary to count in the region of 150–200 nerve fibres to obtain an estimate of within 95% of

the actual number of nerve fibres present in the whole nerve (also see Dangata et al. 1994, 1995, 1996; Dangata & Kaufman, 1997; Parson et al. 1995). The Magiscan allowed a histogram of the diameter profile of the sampled nerve fibres of each nerve studied to be plotted. From this a summary histogram of the nerve fibre diameter profile for each age group of mice was also plotted. The number of myelinated nerve fibres present in each whole nerve was calculated using the ratio technique (Matheson, 1970; Mayhew, 1988, 1990). From the figures obtained the mean myelinated nerve fibre count of the optic nerve for each group of mice was then calculated.

A Student's 2-tailed t test was performed on the results of the morphometric parameters measured in the corresponding age groups of experimental and control mice to establish whether there was a significant difference in these parameters between the groups. Level of significance was taken as $P \leqslant 0.05$ (also see Dangata et al. 1994, 1995; Parson et al. 1995).

Our preliminary findings showed that the optic nerves of the experimental groups were generally greater in diameter than those of the corresponding age groups of the controls, although the difference in csa was significant between only the corresponding 2 and 3 wk old groups of both control and experimental mice. This finding, however, was in contrast to an earlier report by Ashwell & Zhang (1994) who noted a decrease in the csa of the optic nerve of 15 d old C57BL/6 mice following acute prenatal exposure of pregnant mothers to alcohol on d 8 pc. They used 2 similar (consecutive) doses of alcohol instead of the single dose used in the present study. There was an interval of 4 h between the 2 doses. This difference in the csa finding between that observed in the present study and that reported by Ashwell & Zhang made us curious to investigate whether our finding was a true deviation from that previously observed by them (see Discussion). In order to investigate this in detail the optic nerves from each group of 5 mice isolated at 2 and 3 wk of age from an additional series of alcohol-treated pregnant F_1 mothers were analysed as indicated above.

Although it has been reported (Dangata et al. 1996) that myelinogenesis in the mouse begins during the 5th postnatal day (pnd), the present study was undertaken to establish whether prenatal exposure to alcohol affects the time of onset of this process. In order to do this, 2 additional mice each at 8, 7, 6 and 5 pnd were isolated from both experimental and control groups of mice and their optic nerves processed for electron microscopy as indicated above. Photomicrographs of the optic nerve in these latter groups were taken at a magnification of $\times 20000$ and printed to a final magnification of $\times 40000$. This was undertaken principally in order to ensure visualisation of any myelin sheaths at this critical period of postnatal development that would not otherwise have been seen at the lower magnification.

RESULTS

Cross-sectional area (μm^2)

In both alcohol and saline-treated mice the optic nerve could, with little difficulty, be dissected from eyeball to optic chiasma. The meningeal coverings around the nerve were well defined in both groups of mice. The csa of the optic nerve grew rapidly during the early part of the juvenile period but began to decline towards the end of this period. The maximum rate of growth was observed between the 2nd and 3rd weeks of postnatal life, though this was invariably greater in the control than in the experimental group. Thus in the controls the increase in csa between the 3rd and the 5th wk was approximately 53% of the increase between the 2nd and the 3rd wk. This was in comparison with 14% for the experimental group over the same period of time. The optic nerves of the experimental groups were, however, generally greater in diameter than those of the corresponding age groups of the controls. The difference in csa between the corresponding age groups of 2 and 3 wk old control and experimental groups of mice was significant, but by the 5th wk it was no longer significant (see Table). The csa findings of the optic nerves from the additional series of 2 and 3 wk old alcohol-treated mice were comparable with those previously analysed. Therefore the 2 groups of 2 wk alcohol-treated mice were pooled together; similarly, both groups of 3 wk alcohol-treated mice were pooled.

Myelinated nerve fibre counts

In the control group, the first evidence of myelinogenesis was observed in $\times 40000$ micrographs of optic nerves analysed on the 5th pnd due to the presence of promyelin around the largest diameter fibres. Myelinogenesis was more obvious by the 6th pnd as it was possible at this age to distinguish up to 2 distinct myelin lamellae loosely wrapped around some of the largest axons; by the 8th pnd up to 3–4 myelin lamellae could be counted around some of the largest diameter axons. In the experimental group, only the presence of promyelin could be observed around some

Table. *Morphometric parameters analysed in optic nerve of alcohol and saline-treated mice during the juvenile period of postnatal development*

Age (wk)	Parameter analysed (±S.E.M.)								
	Mean cross-sectional area (μm²)			Mean myelinated nerve fibre count			Mean myelinated nerve fibre density (fibres per 1000 μm²)		
	Alcohol*	Saline*	*t* test	Alcohol*	Saline*	Student's *t* test	Alcohol*	Saline*	Student's *t* test
2	41070±1813	35607±2516	$0.01 \leqslant P \leqslant 0.05$	18375±1599	20114±1579	n.s.	444±29	563±19	$0.01 \leqslant P \leqslant 0.05$
3	46921±2553	41569±1201	$P \leqslant 0.01$	35795±1892	40616±3338	$P \leqslant 0.01$	770±22	977±74	$0.01 \leqslant P \leqslant 0.05$
5	48527±1706	47860±2474	n.s.	55606±2364	62987±5019	$P \leqslant 0.01$	1150±39	1304±57	$P \leqslant 0.01$

* Number of optic nerves analysed: alcohol-treated: 2 wk = 16, 3 wk = 18, 5 wk = 10; saline-treated: 2 wk = 9, 3 wk = 7, 5 wk = 8; n..s., no significant difference.

of the largest diameter fibres by the 6th pnd and by the 8th pnd not more than 1–2 myelin lamellae could be seen per axon. The myelin lamellae in both groups of mice at this stage were loosely wrapped around the axons. Similarly, in both groups, myelinogenesis progressed rapidly following the onset of this process, with the maximum rate of increase seen between the 2nd and the 3rd wk; by the 5th wk myelinated nerve fibres dominated the optic nerve in both groups. The myelin lamellae became more densely packed around their axons in both groups of mice with time, although, after the 2nd wk there was no significant change in the packing density of the myelin lamellae around the axons (see Fig.). As for the csa (see above), myelinogenesis progressed faster in controls than in the experimental group. Initially there was no significant difference between control and experimental groups, but from the 3rd wk onwards the difference was significant (see Table).

Numerical density of myelinated nerve fibres (fibres per 1000 μm²)

In both groups of mice there was a rapid increase in the numerical density of the myelinated nerve fibres with age. The mean values for the myelinated nerve fibre density for controls were always higher than those of the alcohol-treated mice. The difference in density between the corresponding age groups of control and experimental animals was significant for all the age groups analysed (see Table).

Myelinated nerve fibre diameter spectrum

The smallest myelinated nerve fibres in any of the series measured 0.16 μm in diameter. The largest fibres measured were 1.60 μm and 1.36 μm in diameter, respectively, for the control and experimental groups, and these values were noted in the 5 wk old mice. The distribution of the myelinated nerve fibre diameters in the optic nerve was unimodal with a positive skewing for all ages in both groups. By the 5th wk a shift in the spectrum of distribution of the myelinated nerve fibres to the right, i.e. in favour of the large diameter fibres could be observed. This shift was more obvious in the control series than the corresponding age groups of the experimental series. At each of the ages studied, the medium diameter fibres dominated the myelinated nerve fibre spectrum of the optic nerve in both control and experimental groups. With increasing age the proportions of both small and medium size fibres increased in the experimental group compared with the controls. The reverse phenomenon was observed in the case of the large diameter fibres. These differences were not, however, statistically significant.

DISCUSSION

The optic nerves of the offspring of F_1 hybrid mice given a single intraperitoneal injection of 25% (v:w) solution of 0.015 ml/g body weight of alcohol in distilled water on d 12 of gestation and appropriate saline-injected controls were analysed using light and electron microscopy. A significant effect on various morphometric parameters of the optic nerve was observed when these were assessed at 2, 3 and 5 wk after birth. It was observed that in this experimental system, exposure to a single dose of alcohol taken at a critical period of gestation had a significant teratogenic effect. Children with the characteristic features of the fetal alcohol syndrome (FAS) are known to have been born to mothers who had taken only a single dose of alcohol during pregnancy

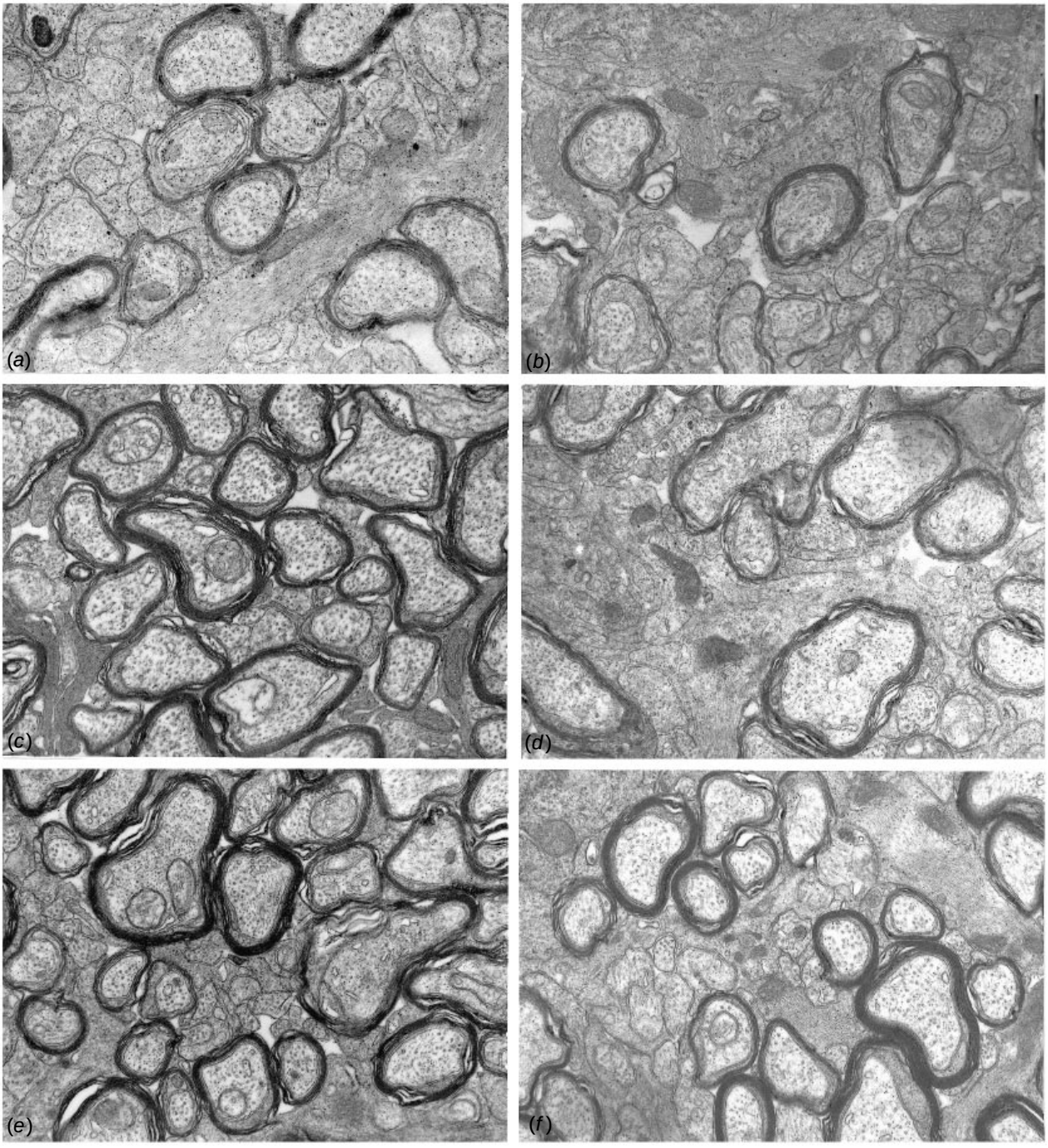

Fig. (*a–f*) Electron micrographs of transverse sections of the optic nerve from both alcohol-treated and control mice to show the progression of myelinogenesis during the juvenile period of postnatal development (*a*, *b*: 2 wk old control and alcohol-treated mice, respectively; *c*, *d*: 3 wk old control and alcohol-treated mice, respectively; *e*, *f*: 5 wk old control and alcohol-treated mice, respectively). A progressive increase in the number of myelinated nerve fibres was observed in the optic nerve with age. This was associated with a corresponding decline in the population of unmyelinated nerve fibres, so that by the 5th wk myelinated nerve fibres dominated the optic nerve in both controls and alcohol-treated mice. At each of the age groups studied, more myelinated nerve fibres were present in the optic nerve of controls than in the corresponding age groups of the alcohol-treated series. The myelin lamellae were distinct in all age groups of both control and experimental groups of mice. In each series of mice the number of myelin lamellae per axon increased and became more compact with age. Although the myelin lamellae became more compact around the axon with age, there was no significant difference in their degree of compaction with age. There was a linear relationship between axon size and the number of myelin lamellae in both control and experimental groups, i.e. the number of myelin lamellae around an axon is proportional to axon diameter. (All micrographs $\times 20000$.)

(Kronick, 1976; Herrmann et al. 1980; Clarren, 1981; Padmanabhan et al. 1984).

In both controls and experimental groups of mice in this study the optic nerve grew rapidly in calibre during the juvenile period of development. The maximum rate of growth was seen between the 2nd

and the 3rd wk of postnatal life. At all of the age groups studied, the rate of growth in the calibre of the optic nerve was greater in controls than in the experimental groups, although at decreasing level of significance with age; up to the 3rd wk the calibre of the optic nerve of the offspring of alcohol-treated mice was significantly larger than that of controls, but by the end of the 5th postnatal week, the difference had ceased to be significant. Ng & Stone (1982) noted that the csa of the optic nerve reflected a number of factors, such as the total number of axons in the nerve, axon diameter, the extent of myelinogenesis of each axon and the volume of the glial component present in the nerve. The present observations suggest that factors other than myelinogenesis alone, which is more intense in the controls than in the alcohol-treated series (see later), had a greater effect on the overall csa of the optic nerve during the juvenile period of postnatal development.

It is not clear why the csa of the optic nerve in the experimental group was initially significantly larger than that of the matched control group, although this may reflect a degree of tissue oedema. This finding contrasts with the small calibre optic nerve observed in the rat at this time by Pinazo-Duran et al. (1993). In their study, alcohol was administered in the diet to female rats 6 wk before pregnancy and throughout the gestation period, rather than as a single acute (or 'binge') exposure as in the present study. Similar findings to those reported by Pinazo-Duran et al. with regard to the size of the optic nerve were also reported by Ashwell & Zhang (1994) following acute exposure of C57BL/6 pregnant mice to alcohol on d 8 pc. This finding strongly suggests that in the study of alcohol teratogenicity the exact timing of exposure during pregnancy is probably more important than the duration of administration. For example, in these 2 previous studies alcohol was administered *before* rather than *after* the formation of the optic stalk (as in the present study); the optic stalk is the precursor of the optic nerve (Kaufman, 1979, 1992; Rugh, 1990; Dangata et al. 1994) and may play a role in determining its eventual size.

In both experimental and control groups, myelinogenesis progressed rapidly during the early part of the juvenile period, although both its onset and the rate of its progression in the experimental group significantly lagged behind that of the controls. This was clearly shown by the presence of a significant difference between the 2 groups of mice in their mean myelinated nerve fibre count after the 2nd wk as well as a significant difference in their mean nerve fibre density throughout the juvenile period. Although it has previously been reported that myelinogenesis in the optic nerve of the mouse is entirely a postnatal event (Gyllensten & Malmfors, 1963; Gyllensten et al. 1966; Dangata et al. 1996), the present findings suggest that prenatal factors (such as exposure to alcohol) may have a significant influence on its time of onset and progression. Oligodendrocytes play a critical role in the formation of the myelin sheath in the central nervous system (CNS) (Matthews & Duncan, 1971; Tennekoon et al. 1977; Dentinger et al. 1985; Asou et al. 1994, 1995; Umemori et al. 1994). These cells develop from glial cell precursors following their cytodifferentiation. The mature forms, i.e. the type-3 oligodendrocytes, are characterised by the presence of numerous processes and are first seen to be present shortly before the onset of myelinogenesis (Asou et al. 1994, 1995). These cells also synthesise myelin basic protein (see also Tennekoon et al. 1977) which constitutes approximately 30% of the protein in the CNS and is important for CNS function (Warrington et al. 1993).

In the present study the delay in the onset and the rate of progression of myelinogenesis, together with the reduction in mean myelinated nerve fibre count and density suggests that alcohol probably exerts a significant teratogenic effect either on the glial precursors of oligodendrocytes or on their cyto-differentiation, and that this could result in both a reduction in their number and/or their competence to synthesise myelin. It is worthy of note that Phillips and his coworkers (Phillips, 1989; Phillips et al. 1991; Phillips & Krueger, 1992) observed a delay in oligodendroglial cell maturation as well as a reduction in the number of oligodendrocytes produced in the optic nerve of the rat following exposure to alcohol. This resulted in a delay in the onset of myelinogenesis. They also reported a decrease in the myelinated nerve fibre count as well as the overall myelin thickness per axon. The impairment in the development of myelin and consequently the formation of the myelin sheath around the axons in the optic nerve could explain some of the neurological signs and severe visual dysfunction commonly associated with prenatal alcohol exposure in infants diagnosed as having the FAS (Phillips et al. 1991; Phillips & Krueger, 1992).

Experimental studies involving the histological analysis of other regions of the CNS have given similar findings to those here, and in comparable studies on the rat optic nerve cited above with regard to the teratogenic effect of alcohol on myelinogenesis. Following chronic prenatal exposure of pregnant rats to dietary alcohol, Miller & al-Rabiai (1994) observed a decrease in the space occupied by axons in the

pyramidal tract in the alcohol-treated compared with that in control rats. The individual diameters of the myelinated nerve fibres were smaller and each had a proportionately thinner myelin sheath. These observations confirmed an earlier finding on alcohol-exposure in relation to the histological features of the cerebral cortex (Miller, 1993). In 2 earlier reports, Lancaster et al. (1982, 1984) observed a decrease in brain myelin and a delay in brain myelin synthesis following exposure of rats to acute doses of alcohol. These findings further confirm that, given at an appropriate critical period, an acute dose of alcohol can significantly affect CNS development.

Although a relatively higher proportion of large diameter fibres in both controls and experimental groups was seen early in life, in the 2 wk old mice, the medium diameter fibres dominated the optic nerve fibre spectrum at any age. As myelinogenesis progressed, those fibres in which it had already started continued to acquire additional myelin lamellae until they had attained the maximum number proportionate to their mature size. The result of this phenomenon was the progressive shift of myelinated nerve fibres from one part of the myelinated nerve fibre spectrum to another and this may account for the shift in the spectrum to the right. The present findings clearly indicate that, in alcohol teratogenicity, there is a delayed onset of myelinogenesis. It should be noted that, as in the rat, myelinogenesis has yet to be completed in the optic nerve of the mouse by the end of the juvenile period (Black et al. 1982; Lancaster et al. 1984; Dentinger et al. 1985; Dangata et al. 1996; Dangata & Kaufman, 1997).

The findings from this study indicate that prenatal exposure of the conceptus to alcohol has a significant influence on the postnatal development of the optic nerve. We have no direct evidence from the present study that there is an effect on the prenatal development of the optic nerve. Indirect evidence, however, suggests that myelinogenesis is probably affected due to interference with oligodendroglial cytodifferentiation, the net result of which is likely to be a delay in the onset of myelinogenesis and its subsequent progression.

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