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Morphometric study of the optic nerve of adult normal mice and mice heterozygous for the *Small eye* mutation (*Sey*/+)

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ABSTRACT

The Small eye (*Sey*) gene, which has been mapped to chromosome 2 in the mouse, is known to cause variable malformations of the eye and nose. The effect of the gene in the heterozygous state is mainly on the eye. A combined electron microscopy and morphometric analysis of the optic nerve in adult littermates with a normal (+/+) and heterozygous mutant (*Sey*/+) genotype was carried out. The optic nerve could be dissected from the posterior pole of the eyeball to the optic chiasma in all the mice examined. The results of morphometric analyses carried out in this study show that the *Sey* gene indirectly affects the normal morphogenesis of the optic nerve in the heterozygous mutant *Sey* male mouse to a significant degree compared with its male normal littermate. The heterozygous mutant *Sey* female mouse is also affected, but not significantly so when compared with its normal female littermate. The mean nerve cross-sectional area and mean nerve fibre counts for the *Sey* strain are lower than those observed in other strains of mice that have been studied. The nerve fibre densities and the spectrum of nerve fibre sizes encountered are, however, similar to those seen in other strains of mice. We believe that the findings indicate that the smaller mean nerve fibre counts observed in the heterozygous mutant (*Sey*/+) mice compared to their normal (+/+) siblings is unlikely to have resulted from primary retinal dysgenesis, but is a consequence of the reduced size of their neural retina, and total retinal ganglion cell population.

Key words: *Sey* gene.

INTRODUCTION

The gene mutation in the mouse causing Small eye (*Sey*) arose spontaneously in Edinburgh in 1967 (Roberts, 1967). Since then the gene, which is allelic to the aniridia gene in the human, has been mapped to chromosome 2 (Hogan et al. 1986, 1988; Jong et al. 1990; Hill et al. 1991; Ton et al. 1992). Other genes known to cause developmental abnormalities of the eye also located on the same chromosome include *Ocular retardation* (or^J), *Dickie's small eye* (*Dey*) and *coloboma* (*Cm*) (Theiler et al. 1976, 1978, 1980; Theiler & Varnum, 1981). These are semidominant genes except the *Ocular retardation* gene which is recessive. Homozygous *Small eye* (*Sey*/*Sey*) embryos develop to term but do not develop either eyes or nose, and die soon after birth because of the breathing problems associated with the absence of the nose (Hogan et al. 1986). The effect of the *Sey* mutation is believed to be limited to the growth and differentiation of the lens placode and nasal placode, and it is the failure of the former to develop normally that has secondary consequences on the rest of the ocular apparatus in both the homozygous in particular and, but to a considerably lesser degree, the heterozygous state.

It is the eyes that are mainly affected in the heterozygous mutant *Sey* mice (Hogan et al. 1986). Abnormalities of the eyeball associated with the presence of the mutant gene, when in the heterozygous state, include microphthalmos often associated with cataracts, partial or complete absence of the iris,

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absence of the lens and retinal abnormalities (Jong et al. 1990). No evidence is yet available to indicate whether the gene exercises its ocular effect on the eyeball and its contents alone, or influences, either directly or indirectly, the development of the optic nerve and other parts of the visual system; and, if the optic nerve is affected, to what extent. This study has set out to establish whether the presence of the *Sey* gene influences the development of the optic nerve, and attempts to quantify the extent of the influence in relation to the parameters of the optic nerve measured.

MATERIALS AND METHODS

A breeding colony of *Small eye* mice was obtained from Dr Ruth Clayton, formerly of the Department of Genetics, University of Edinburgh, and maintained by brother × sister matings of heterozygous male and female mice. The latter were easily distinguished from their normal siblings due to the fact that their eyes were significantly smaller than those of their genetically normal (i.e. +/+) littermates.

Eleven week old normal (i.e. +/+) and heterozygous mutant *Sey* mice (i.e. *Sey*/+) of both sexes were studied. A total of 5 mice of each sex of normal, and 8 females and 5 males of heterozygous mutant *Sey* mice were used. Each animal was deeply anaesthetised by an intraperitoneal injection of 0.02 ml/g body weight of a 1.2% solution of tribromoethanol (Avertin) in 0.9% saline. The heart was exposed, and using a 21G needle, intracardiac perfusion 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 whole length of the optic nerve was immediately, but carefully, dissected out avoiding traction on the nerve. Each nerve was put into a prelabelled bottle containing 2.5% glutaraldehyde 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 (~ 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 (~ 80 nm) thickness were then cut for electron microscopy. These were picked up on copper grids and subsequently stained with 0.2% lead citrate solution and a saturated solution of uranyl acetate. A selection of photomicrographs was taken from the centre to the periphery of each nerve using a Philips EM301 transmission electron microscope. The micrographs were developed and printed to a final magnification of × 3000.

Using the semithin sections, the cross-sectional area (*csa*) of each nerve excluding its epineurium was measured in a Magiscan image analysis system (Applied Imaging). From the measurements of the *csa* of the individual nerves, the mean *csa* of the optic nerves from each group of mice was calculated. 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 μm^2 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. Also, only myelinated nerve fibres whose centres fell within a sampled square were included in the analysis. Mayhew & Sharma (1984*a*, *b*) have shown that it is only necessary to count in the region of 200 nerve fibres to obtain an estimate of within 95% of the actual number of nerve fibres present in the whole nerve. The Magiscan allowed a histogram of the diameter profile of the sampled nerve fibres of each nerve studied to be plotted. A summary histogram of the nerve fibre diameter profile for each group of mice was then plotted by hand.

The number of myelinated nerve fibres present in the whole nerve was calculated using the ratio technique (Matheson 1970; Mayhew, 1988, 1990), and from the figures obtained the numerical density of fibres per 1000 μm^2 was calculated for each nerve. The mean nerve fibre count and fibre density of the optic nerve for each group of mice was also calculated.

A Student's 2-tailed t test was performed on all results to establish whether there was a significant difference in the parameters measured between the left and the right optic nerve of both males and females, and between males and females in each of the two groups of mice studied. This test was carried out to compare corresponding sexes of the two groups of

Table 1*a*. *Optic nerve cross sectional area for normal (+/+) and heterozygous mutant (Sey/+) adult mice*

Group/sex of mouse		Mean cross-sectional area (μm^2)		
		L (Left) or R (Right)		Pooled (L+R)
Normal (+/+)	Male (n = 9)	L	49464±5335	49397±4259
		R	49343±6932	
	Female (n = 10)	L	42154±4867	43996±2571
		R	45838±257	
Heterozygous mutants (Sey/+)	Male (n = 9)	L	34411±4968	33179±2778
		R	31636±1897	
	Female (n = 16)	L	41985±2420	40535±1685
		R	39085±2387	

Table 1*b*. *Level of significance between optic nerve cross sectional areas of normal (+/+) and heterozygous mutant (Sey/+) adult mice*

Normal (+/+) male (n = 9)			
n.s.	Normal (+/+) female (n = 10)		
(0.01 < *P* < 0.05)	xxxxxx	Mutant (Sey/+) male (n = 9)	
xxxxxx	n.s.	(0.01 < *P* < 0.05)	Mutant (Sey/+) female (n = 16)

n.s., not significant.

Table 2*a*. *Mean myelinated nerve fibre counts of normal (+/+) and heterozygous mutant (Sey/+) adult mice*

Group/sex of mouse		Mean myelinated nerve fibre count		
		L (Left) or R (Right)		Pooled (L+R)
Normal (+/+)	Male (n = 9)	L	71392±3394	70021±4886
		R	68924±8836	
	Female (n = 10)	L	61241±8472	62589±4305
		R	63937±3210	
Heterozygous mutants (Sey/+)	Male (n = 9)	L	47584±7204	48605±4062
		R	49881±3352	
	Female (n = 16)	L	54943±4190	54558±2856
		R	54173±4161	

Table 2*b*. *Level of significance between mean nerve fibre count of normal (+/+) and heterozygous mutant (Sey/+) adult mice*

Normal (+/+) male (n = 9)			
n.s.	Normal (+/+) female (n = 10)		
(0.01 < *P* < 0.05)	xxxxxx	Mutant (Sey/+) male (n = 9)	
xxxxxx	n.s.	n.s.	Mutant (Sey/+) female (n = 16)

n.s., not significant.

Table 3*a*. *Mean myelinated nerve fibre densities in the optic nerve of normal (+/+) and heterozygous mutant (Sey/+) adult mice*

Group/sex of mouse		Mean myelinated nerve fibre density (per 1000 μm²)		
		L (Left) or R (Right)		Pooled (L+R)
Normal (+/+)	Male (n = 9)	L	1470±88	1441±75
		R	1417±123	
	Female (n = 10)	L	1437±78	1416±41
		R	1395±34	
Heterozygous mutants (Sey/+)	Male (n = 9)	L	1373±114	1469±83
		R	1590±109	
	Female (n = 16)	L	1309±69	1349±50
		R	1389±74	

Table 3*b*. *Level of significance between mean nerve fibre densities of normal (+/+) and heterozygous mutant (Sey/+) adult mice*

Normal (+/+) male (n = 9)			
n.s.	Normal (+/+) female (n = 10)		
n.s.	xxxxxxx	Mutant (Sey/+) male (n = 9)	
xxxxxx	n.s.	n.s.	Mutant (Sey/+) female (n = 16)

n.s., not significant.

mice to determine whether a significant difference existed between them. Level of significance was taken as $P < 0.05$.

RESULTS

Cross-sectional areas (μm²)

The optic nerve, which had a well defined dural sheath in both the normal and the heterozygous mutant *Sey* mice, could be dissected from the eyeball to the optic chiasma. One nerve from the male series of each group of mice was lost during the course of dissection. No significant difference was observed in *csa* between the left and the right optic nerves of either sexes of each of the two groups of mice. For this reason the sample size for each sex in each of the two groups of mice was pooled for the purpose of the statistical analysis of the mean *csa* for each sex. The female heterozygous mutant *Sey* significantly differed from their male littermates with respect to their *csa* ($P < 0.05$), but in the normal series no significant difference was observed between the two sexes. The inter-group comparison of the *csa* between corresponding series revealed a significant difference between heterozygous mutant *Sey* males and normal *Sey* males ($0.01 < P < 0.05$). No such difference was seen between females of the two groups of mice (see Tables 1*a*, *b*).

Total myelinated nerve fibre counts and nerve fibre density per 1000 μm²

The statistical analysis of the mean myelinated nerve fibre counts showed no significant difference between the left and the right optic nerve of either males or females of the same group of mice. Because of this, all samples for each sex in each of the *Sey* groups studied were pooled together for the purpose of the statistical analysis of the mean myelinated nerve fibre count. There was also no significant difference between males and females in each group of mice with respect to the mean myelinated nerve fibre count. The heterozygous mutant *Sey* series consistently had lower values for the mean myelinated nerve fibre count compared to their normal littermates. The heterozygous mutant

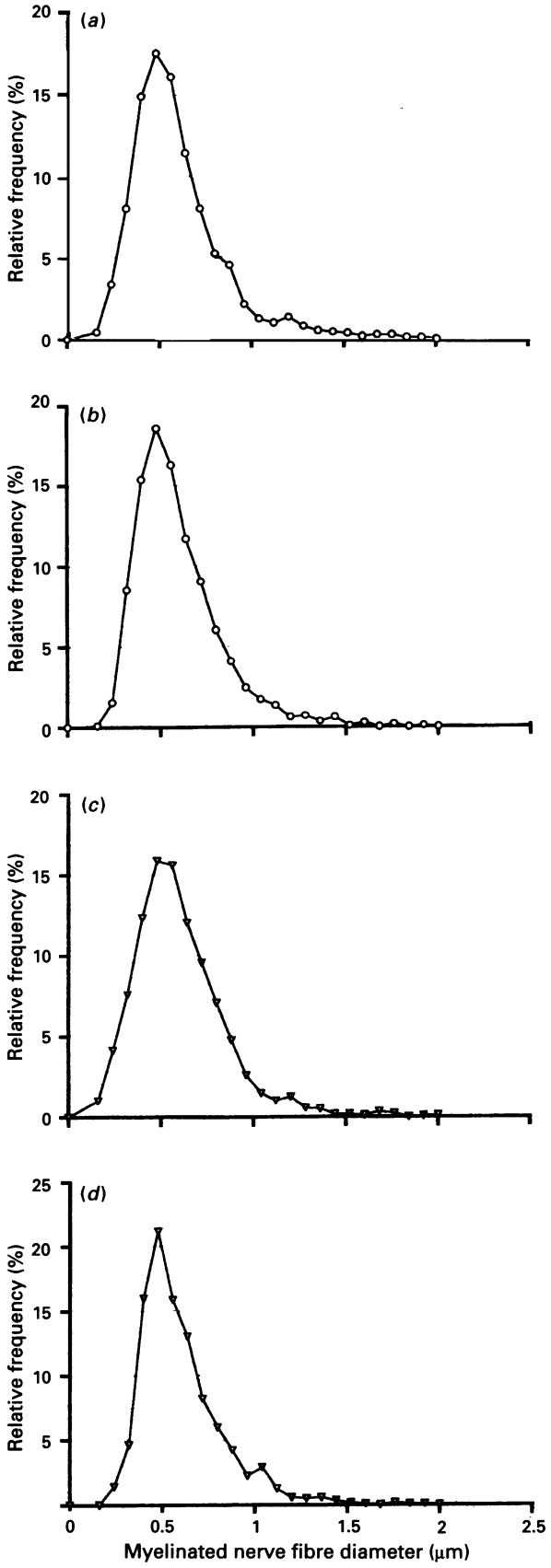

Fig. 1. Graphs showing distribution of myelinated nerves of different fibre diameters within the optic nerve of normal *Sey* (+/+) female (*a*) and male (*b*) mice, and heterozygous mutant *Sey* (*Sey*/+) female (*c*) and male (*d*) mice.

Sey males significantly differed from their normal male littermates ($0.01 < P < 0.05$) though in the case of +/+ and *Sey*/+ females, the difference observed was not significant (see Table 2*a*, *b*). There was no significant difference in the mean myelinated nerve fibre density either within the groups or between them (see Table 3).

Myelinated nerve fibre diameter spectrum

More than 90% of the myelinated nerve fibres analysed in each group of mice were less than 1.0 μm in diameter, with the largest nerve fibres measured in each group being slightly less than 2.0 μm in diameter. The distribution of the fibres in both groups was unimodal, with a modal diameter of 0.48 μm. In each case, there was a positive skewing of the nerve fibres in favour of the small diameter fibres (see Fig. 1*a–d*).

Representative electron micrographs which display the appearance of transverse sections through nerve fibres within the optic nerve of normal *Sey* (+/+) and heterozygous mutant *Sey* (*Sey*/+) mice, are presented in Figure 2*a* and *b*, respectively.

DISCUSSION

The findings from this study suggest that the influence of the *Sey* gene, in the heterozygous mutant mouse, is primarily on the development of the eyeball, but also extends secondarily to the optic nerve. From earlier studies, other mutant genes have been shown to have a primary effect on the development of the optic nerve in the mouse (Theiler et al. 1976; Robb et al. 1978; Franz & Besecke, 1991). The small eyeballs generally observed in the heterozygous mutant *Sey* were always associated with corresponding small-calibre optic nerves. This is highlighted by the significant difference that was observed in the mean *csa* and the mean myelinated nerve fibre counts between the male series of both *Sey* groups of mice involved in the study. Although the difference in these two parameters between the female series of both groups of mice was not significant, values of the parameters for the heterozygous mutant *Sey* females were always considerably lower than those of their normal female littermates. The reason for the greater effect of the gene on the heterozygous mutant *Sey* male mouse compared to their female siblings is, however, not clear.

Earlier reports on the effect of the *Sey* gene on the development of the eye have been mainly concerned with the effect of the gene on the lens and contents of the orbit in the homozygous mutant during the early

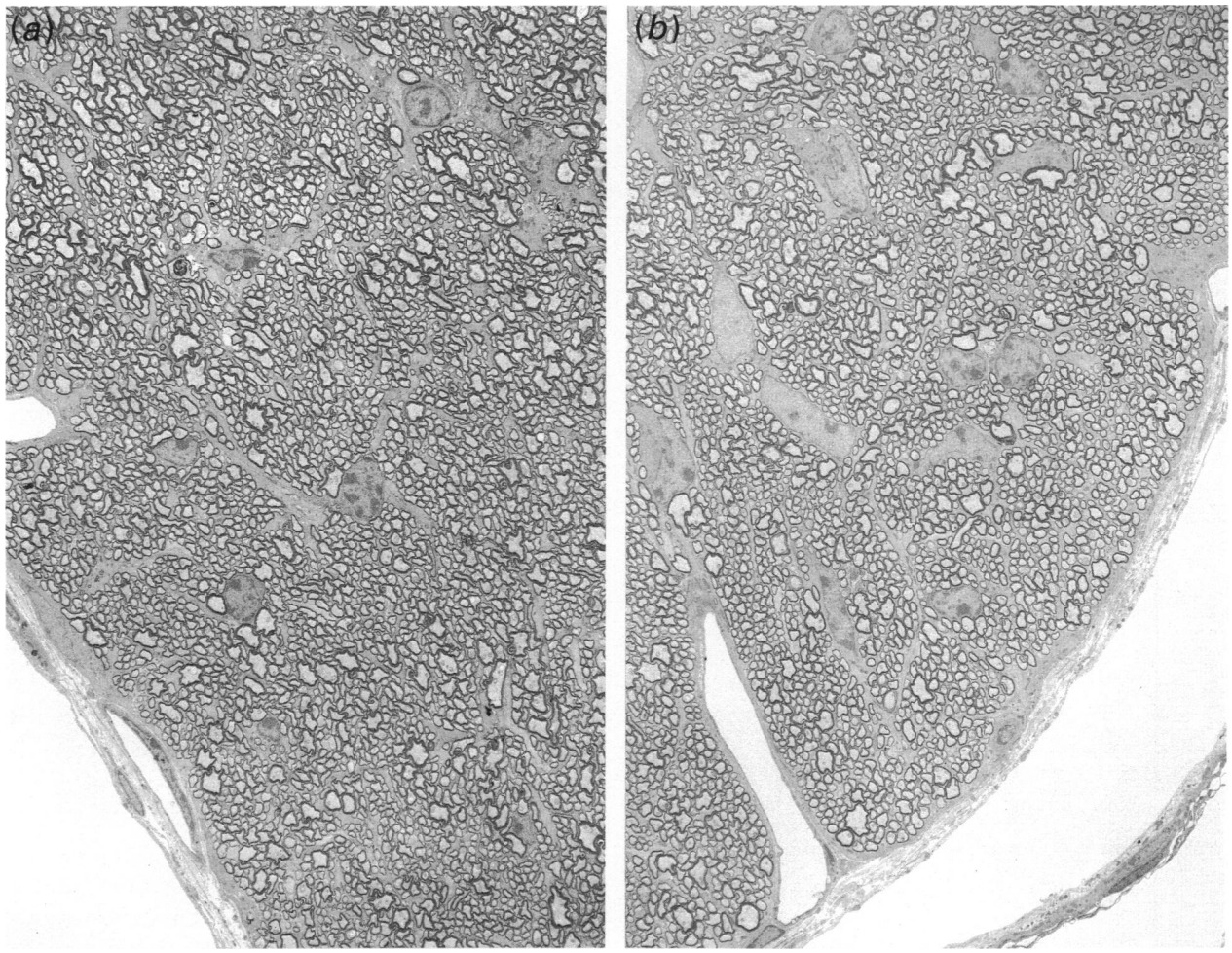

Fig. 2. Representative low-magnification transmission electronmicrographs of transverse sections through the optic nerve of a normal *Sey* (+/+) mouse (*a*), and a heterozygous mutant *Sey* (*Sey*/+) mouse (*b*). ×750.

embryonic period. Little information is available on its effect on the development and contents of the optic nerve in the adult heterozygous mutant mouse. In the homozygous mutant, while an irregularly shaped optic vesicle initially forms, in the absence of an inductive influence from the lens vesicle, it soon degenerates, and no remnant of the optic apparatus beyond the optic chiasma is generally seen (Hogan et al. 1986, 1988). A similar situation is seen in another mutant involving the eye, namely the *Ocular retardation* gene (or^J) when present in the homozygous state (see Truslove, 1962; Robb et al. 1978). Theiler et al. (1976) observed that in the adult (*or*/*or*) mutant, where the *or* gene was believed to be allelic but not identical to the or^J gene, small eyes are present with closed lids. This was associated with abnormalities of the retinal layers and absence of the optic nerve, the latter being replaced by a thin layer of connective tissue.

Robb et al. (1978) observed that in normal (+/+) *Ocular retardation* mice, by d 14.5 postcoitus (p.c.) the eyeball increases in volume and the optic nerve increases in diameter as a result of the differentiation of large numbers of ganglion cells within the neural retina which send axons to the brain. This is in contrast to the situation in homozygous or^J mutant mice of about the same age, where they observed that despite the presence of a substantial retinal neuroblastic layer in which it was difficult to distinguish ganglion cells and nerve fibres, no axons exited from the eye. Furthermore, they suggested that another factor that might have induced regression of the optic stalk was the absence of vascularisation of the optic nerve (Robb et al. 1978).

In the homozygous mouse mutant *extra-toe* (X^t/X^t), Franz & Besecke (1991) observed that the optic nerve was either completely absent or at most only partly developed. No explanation is yet available, however, for the variable phenotype observed in the (X^t/X^t) mice, nor has the developmental mechanism that leads to the ocular pathology seen in these mice yet been established. Theiler et al. (1978, 1980), in their

work on *Dickie's small eye* (*Dey*) found that the morphogenesis of the eye in the heterozygous mutant animals was delayed compared to that observed in controls on d 12 p.c. This apparent lagging in development generally starts between d 10 and 11 p.c. though, in some instances, it was observed as early as d 7 p.c. and was invariably noted by d 12 p.c. The ocular abnormalities noted in adult (*Dey*/+) heterozygotes were small eyes with coloboma, small or absent lens, with cataract if present, abnormal folding of the retina and reduction in the pigment layers, with often absence of the anterior chamber. Homozygotes invariably die during the early post-implantation period, often at the egg cylinder stage.

The normal development of the eye in the mouse begins with the invagination of the central region of the optic placode to form the optic pit and its progressive differentiation, in association with cephalic neurulation, to form the optic vesicle. This forms from the neuroepithelium in the diencephalic region of the forebrain during d 8–9 p.c. By about d 9.5 p.c., an optic stalk is present and the optic cup has begun to indent, while between about 9.5–11 d p.c., the lens placode indents to form the lens pit and subsequently differentiates to form the lens vesicle. This then separates from the surface and progressively indents the optic cup so that it is now seen to have an inner and an outer layer which are destined, respectively, to form the neural and pigment layers of the retina (Kaufman, 1979, 1992; Rugh, 1990).

The normal process of ocular development is believed to be influenced by the process of embryonic induction by which regions of the anterior head ectoderm become progressively specified as the presumptive optic apparatus. Similar mechanisms are believed to be involved with regard to the differentiation of the nasal placode. The mechanism of embryonic induction may itself be affected by gene mutation (Jong et al. 1990). Direct contact between the neuroepithelium of the optic vesicle and the overlying surface ectoderm is believed to be a necessary prerequisite for the induction of the lens placode (Bard, 1990). Other factors that influence normal morphogenesis include programmed cell death (apoptosis) and cell resorption, the appropriate location of component tissues at the time of cell–cell interaction, and the availability of an adequate blood supply (Truslove, 1962; Silver & Hughes, 1974; Silver & Robb, 1979; Perry et al. 1983). In the optic stalk, cell death and cell resorption allow access for the ingrowing nerve fibres that exit from the neural retina with the eventual formation of the optic nerve, while the blood supply that facilitates the nutrition of this process mainly comes from the hyaloid vessels. Truslove (1962) observed that in both normal and homozygous mutant (or^J/or^J) mice the blood supply was adequate up to d 11 p.c., but from d 12 p.c. the choroid fissure in the mutant mice gradually became obliterated. A progressive reduction in the size of the eyeball was then apparent, and this was eventually followed by the resorption of the optic stalk. When an inadequate blood supply was observed in this mutant strain of mice, this was generally associated with the failure of the optic vesicle to form a proper cup. The relationship between these two events, however, is not entirely clear.

The presence of the *Sey* gene in the homozygous state is believed to interfere with the normal mechanism of embryonic induction necessary for the development of the lens vesicle (and nasal apparatus), and results in the formation of a smaller lens vesicle than normal in the heterozygous mice bearing this mutation. Although it is the lens that is primarily affected, there appears to be a consequential effect on other components of the eyeball, as well as secondary effects on the optic nerve. Had the effect of the *Sey* gene (in the heterozygous mutants) been primarily on the development of the optic nerve, through a failure of optic vesicle and stalk formation, then it is likely that a similar effect to that seen in the or^J mutant would have been observed (Truslove, 1962).

It is logical that, since nerve fibres from the neural retina are directed into the optic stalk, the calibre of the optic nerve will necessarily reflect the number of nerve fibres which it contains (Ogden & Miller, 1966; Potts et al. 1972*a*, *b*; Hughes, 1977). Such a direct relationship between the calibre of the nerve and its nerve fibres has been demonstrated in this study by the *csa* and the mean nerve fibre count. Despite the presence of a significant difference in both mean *csa* and mean nerve fibre counts between the normal *Sey* male series and their heterozygous mutant *Sey* littermates, their mean nerve fibre densities, which reflect the actual relationship between calibre and the number of nerve fibres of a nerve, were not significantly different. The distribution of nerve fibres within the nerves was similar in both normal and heterozygous mutant *Sey* series. This was demonstrated by the similarity of the nerve fibre diameter spectrum in both the sexes of both groups of mice, with each having a modal diameter of 0.48 μm. Not surprisingly, if the overall size of the neural retina is reduced, as it is in the heterozygous mutant *Sey* mice, then the diameter of the optic nerve is also likely to be smaller than that of its normal littermates.

The similarity in myelinated nerve fibre diameter

spectrum as well as the mean myelinated nerve fibre densities between the normal and the heterozygous mutant *Sey* mice is an indication of the fact that myelinogenesis is not affected by the *Sey* gene. Myelination itself does not occur in the optic nerve of the mouse before the 5th day of postnatal life (Gyellensten & Malmfors, 1963; Gyellensten et al. 1966), and this is well after the process of embryonic induction. It would seem the primary inhibitory effect of the gene is on the induction of the lens vesicle, and this indirectly affects the size of the eyeball and the calibre of the optic nerve, but does not appear to influence to any degree retinal differentiation or myelination of the nerve fibres within the optic nerve. Mylination in the *Sey* mice does not appear to lag behind that observed in normal strains of mice. This is evident in the density of myelinated nerve fibres in the normal and *Sey*/+ groups of mice which are comparable with that observed in F1, C57BL and CBA strains of mice of the same age (Dangata et al. 1994).

The *Sey* gene is believed to be analogous to the human aniridia gene (Glaser et al. 1990). In the mouse, the effect of the mutant gene in the homozygous state primarily causes inhibition of the inductive mechanism necessary for the formation of the lens vesicle (Harch et al. 1978). In the heterozygous state, it influences the overall size of the eyeball and its component structures such as the iris and retina, while any effect on the optic nerve is likely to be secondary to that on the eye. Optic nerve hypoplasia which is observed in about 75% of aniridic patients is believed to be a consequence of poor macular and/or retinal development. These effects of the gene on the development of the eye in the human manifest themselves in a variety of ways. A reduction in the size of the iris leads to narrow angle glaucoma. The reduced visual acuity and nystagmus observed have been attributed to macular hypoplasia rather than being due to abnormalities of the iris (Hittner, 1989). Histological findings in the human include hypoplasia of the iris, absence of trabecular meshwork and Schlemm's canal (Hittner, 1989). From the results obtained for the myelinated nerve fibre spectrum and density for the *Sey* mouse in this study, it appears that the hypoplasia of the optic nerve observed in heterozygous mutant *Sey* mice is unlikely to have resulted from primary retina dysgenesis. It is likely to be secondary to a smaller total number of ganglion cells present in the neural retinal resulting from an overall reduction in the size of the eyeball, which itself is a consequence of the inhibitory effect of the *Sey* gene on embryonic induction.

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