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The primary ultrastructural defect caused by anophthalmic white (*Wh*) in the Syrian hamster

(dominant spotting mutation/pleiotropic effects/retention of cilia)

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ABSTRACT **Anophthalmic white (*Wh*) of the Syrian hamster is a highly pleiotropic dominant spotting color mutation causing homozygotes to be deaf, blind, and white. An ultrastructural analysis of embryonic tissue reveals that *Wh* causes the retention of cilia by cells of opposing layers of the embryonic retina and by the lens fiber cells. Previous ultrastructural analyses indicate that *Wh* also causes the retention of cilia by secretory cells of the anterior lobe of adult pituitaries. We propose that the primary ultrastructural defect caused by *Wh* is the retention of cilia by embryonic cells. These retained cilia are hypothesized to interfere with normal cell–cell interactions and subsequent cell differentiation.**

Within many of the mammalian genera, there exists a class of dominant spotting mutations with a common triad of pleiotropic effects. The less severely acting mutations cause the animals to be deaf, blind, and white (1–3). More severely acting mutations cause abnormalities of the brain and spinal cords (3, 4). These mutations, which appear to cause homologous abnormalities, have been observed in mice, hamsters, mink, man, cats, dogs, horses, and cattle (2, 5–7). One generally accepted working hypothesis used to explain the effects of these highly pleiotropic mutations is that each mutation primarily alters the differentiation of neural crest cells. Because of the major contribution that neural crest cells make to very many different developing organ systems (8), this hypothesis has, in the past, seemed the simplest explanation.

Anophthalmic white (*Wh*) of the Syrian hamster is a dominant spotting mutation. Homozygotes are deaf, blind, and white (6, 9–13). A series of recently published observations has led us to formulate a new hypothesis with regard to this mutation and others like it. First, anophthalmic white hamsters are sterile. Ultrastructural analyses of the adult testis indicate that *Wh* halts sperm development at the early spermatid stage of spermiogenesis—the luteinizing hormone–follicle stimulating hormone-dependent stage (14). Second, nearly all of the physiological functions regulated by the pituitary are significantly altered by *Wh* (6). Third, ultrastructural analyses of adult pituitaries from normal, heterozygous, anophthalmic white and normal, experimentally blinded hamsters show that *Wh* causes cytological changes of the anterior lobe of the pituitary. These changes include a 33% reduction in the number of cells in the anterior lobe of the pituitary and, more important, the presence of numerous ciliated secretory cells deep within the tissue (15).

With these observations in mind, a single unifying hypothesis was presented by Asher (6) suggesting that the primary action of *Wh* is to alter the development of all structures derived from the embryonic diencephalon. We began a study of the development of the structures derived from the diencephalon, which include the posterior lobe of the pituitary, the eyes, and the pineal body. We present here a portion of the results of a light and electron microscopic analysis of *Wh/Wh* embryos. The data indicate that the likely primary ultrastructural defect caused by *Wh* is the retention of cilia by embryonic cells. We propose that ultrastructural analyses of dominant spotting mutations such as microphthalmic white (Mi^{wh}) and splotch (*Sp*) in the house mouse and the Waardenberg syndrome of man will demonstrate that these mutations generally cause the retention of cilia by embryonic cells.

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MATERIALS AND METHODS

Adults. The hamsters used in this study were derived from the strain AN/As-*Wh* now at 16 generations of full sibling mating in which at least one parent at each generation was *Wh/wh*. The strain is also homozygous for *e* (cream). Because of the strong epistatic interaction between *Wh* and *e*, all three genotypes are distinguished easily: (*i*) *wh/wh, e/e*—cream; (*ii*) *Wh/wh, e/e*—black-eyed white; and (*iii*) *Wh/Wh, e/e*—anophthalmic white. Color illustrations of these hamsters were published by James *et al.* (14).

The animal room was kept under a regime of 13 hr of light and 11 hr of dark and held at 70°C with a relative humidity of ≈50%. Hamsters were fed Wayne Breeder Chow and distilled water ad lib.

Embryos. Because of the extensive developmental analysis of the eyes of *Wh/Wh* embryos, as published by Jackson (16), we present here a light and transmission electron microscopic (TEM) analysis of embryos from 11½ days of gestation. Our choice of this age was determined by the fact that the gene appears to inhibit the closure of the choroid (optic) fissure which normally occurs at ≈11½ days. A scanning electron microscopic (SEM) analysis of normal (*wh/wh*) embryos at 7½, 8, and 8½ days was also performed. Because of the infertility of *Wh/Wh* hamsters (14) and the inability to determine genotypes prior to 10 days of gestation, known *Wh/Wh* embryos at 7½, 8, and 8½ days were not available for SEM analysis.

Female hamsters were placed nightly with males at ≈3 hr after the onset of darkness. Mating was allowed to continue for 30 min. Matings were made *inter se* by using normal or black-eyed white hamsters. Gestational age was computed from the time of copulation. At 7½, 8, 8½, and 11½ days of gestation, females were killed by cervical dislocation. Embryos were dissected free of the uterus in physiological saline (pH 7.3–7.4) and fixed by three different procedures depending upon their ultimate use. Whole embryos or embryonic tissues were removed and fixed in Bouin's fluid (17), phosphate-buffered Karnovsky's fixative (18), or 4% gluteraldehyde in 0.1 M phosphate at pH 7.3. Homozygous (*Wh/Wh*) embryos (from crosses between heterozygotes) were easily recognized at 11½ days by the com-

Abbreviations: TEM, transmission electron microscopic; SEM, scanning electron microscopic.

plete absence of the pigment ring of the eye. Prior to 10 days of gestation, the genotypes of embryos from this cross, at present, cannot be determined.

After fixation overnight in Bouin's fluid, heads were dehydrated, embedded in Paraplast, serially sectioned at 10 μm, and stained with Harris' hematoxylin and eosin. Complete serial cross and sagittal sections were obtained from eight *wh/wh* and seven *Wh/Wh* embryos.

After a light microscopic analysis of these embryos, four additional 11½-day embryos of each genotype were prepared for TEM analysis. After ≈1 hr of fixation in Karnovsky's fixative, the embryonic eyes were dissected from the heads and fixed for an additional 2–3 hr. Eyes were postfixed in 1% osmium tetroxide in phosphate buffer and embedded in Epon/Araldite. Semithin cross and sagittal sections (1 μm) were cut and stained with a 1.0% solution of toluidine blue 0 and examined with a Zeiss Universal microscope. After verifying that the tissues were correctly oriented, thin sections were cut, stained with uranyl acetate and lead citrate, and examined in a Philips 300 electron microscope at 80 kV.

Six whole *wh/wh* embryos at 7½, 8, and 8½ days of gestation were fixed in phosphate-buffered 4% gluteraldehyde for a total of 4 hr. The embryos were washed in phosphate buffer and dehydrated in a graded series of ethanol to 100% ethanol. The dehydrated embryos were then dried in a Balzers critical point dryer, coated with gold, and examined in a JEOL JSM-35C scanning electron microscope at 15 kV. Tracheae from normal (*wh/wh*) adult hamsters were similarly fixed and prepared for SEM analysis. The ciliated tracheal epithelium was used as a standard of comparison when viewing ciliated embryonic cell surfaces.

RESULTS

Light microscopic analyses of serial cross and sagittal sections of eight *wh/wh* and seven *Wh/Wh* 11½-day embryos indicated the following differences. (*i*) All normal (*wh/wh*) eyes contained a sensory and pigmented layer of the retina (Fig. 1*A*). (*ii*) The choroid (optic) fissure of most *wh/wh* eyes was closed to the level of the optic cup. (*iii*) The central cavity of the optic stalk from the level of the eye to its connection with the diencephalon was completely closed in all normal eyes examined. (*iv*) All *wh/wh* lenses, with one exception, were completely filled with lens fiber cells (Fig. 1*A*). By contrast, all anophthalmic white (*Wh/Wh*) eyes lacked a pigmented layer of the retina. In its place was a very thickened cell layer resembling a second, nonpigmented neural layer (Fig. 1*B*). In addition to this obvious defect, the *Wh/Wh* eyes were always smaller in diameter, contained lenses that were invariably open vesicles, and always possessed open choroid (optic) fissures. These observations are in agreement with the results of Jackson (16). The eyes of *Wh/Wh* embryos exhibited two additional defects not mentioned by Jackson (16). (*i*) The central cavity of the optic stalk was invariably open from the posterior part of the eye to its connection with the diencephalon—in marked contrast with the normal cavity which is completely sealed off. (*ii*) We observed that the layers of the mutant retina, toward the rear of the eye, were often widely separated and that the opening between the two layers appeared to be filled with cilia-like material (Fig. 1*C*).

We next began a TEM analysis to determine if the cilia-like material seen in Fig. 1*C* contained the ultrastructural elements characteristic of cilia and whether cilia were found on all surfaces separating the two layers of the retina. A TEM analysis showed several points of interest. First, the sensory (neural) and pigmented layers of normal (*wh/wh*) retinas were separated by microvillar projections (Fig. 1*D*). Extensive examination of the interface between these two layers revealed very few ciliated cells. When present, cilia were only found on cells of the neural layer of the retina. By contrast, almost every cell of the outer and inner (neural) layers of *Wh/Wh* retinas contained at least one cilium (Fig. 1*E*). On the other hand, cells of the choroid (optic) fissure were without cilia and appeared to have completely normal ultrastructure.

Because of the presence of cilia between the layers of the retina, we predicted that the inner surface of the lens should be similarly ciliated. TEM analysis of normal (*wh/wh*) lenses revealed sporadic evidence (see arrows) for cilia retained by the lens epithelial and lens fiber cells (Fig. 2*A*—from the area in

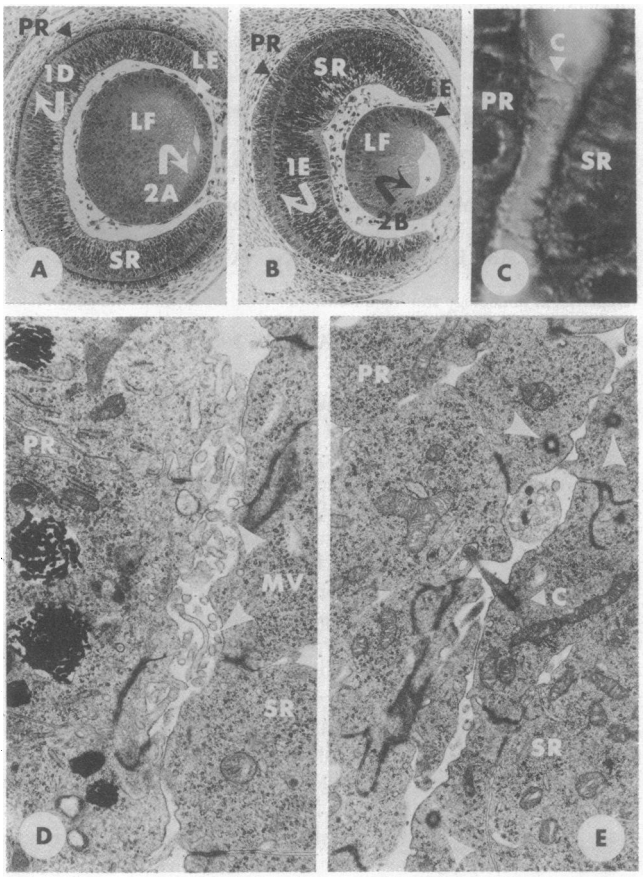

FIG. 1. Micrographs representing sections of normal (*A* and *D*) and mutant (*B*, *C*, and *E*) embryonic eyes at 11½ days of gestation. (*A*) Light micrographs of a semithin section through an Epon-embedded normal (*wh/wh*) eye showing the position of the pigmented retina (PR), sensory retina (SR), lens fiber cells (LF), and lens epithelial cells (LE). (×65.) (*B*) Light micrograph of a semithin section through an Epon-embedded mutant (*Wh/Wh*) eye showing improper differentiation of the pigmented (PR) retinal layer. (×65.) (*C*) Nomarski photomicrograph of a 10-μm hematoxylin and eosin-stained section through a paraffin-embedded mutant (*Wh/Wh*) eye. The micrograph of an area similar to that indicated by arrow 1E in *B* shows a very large gap between the sensory (SR) and pigmented (PR) layers of the retina which appears to be filled with cilia (*C*) projecting from both layers (photographed by Steven R. Heidemann). (×950.) (*D*) Electron micrograph from the area represented by arrow 1*D* in *A* showing the junction between the pigmented (PR) and the sensory (SR) layers of the retina that contain numerous microvillous projections (MV) which interdigitate the two layers and presumably have allowed the normal induction and differentiation of the pigmented retina (PR). (×5,600.) (*E*) Electron micrograph from the area represented by arrow 1E in *B* showing the junction between the two abnormal retinal layers. Inhibition of the proper differentiation of a normal pigmented retina (PR) was presumably due to the presence of cilia (*C*). (×5,600.) Arrowheads indicate the presence of numerous cilia.

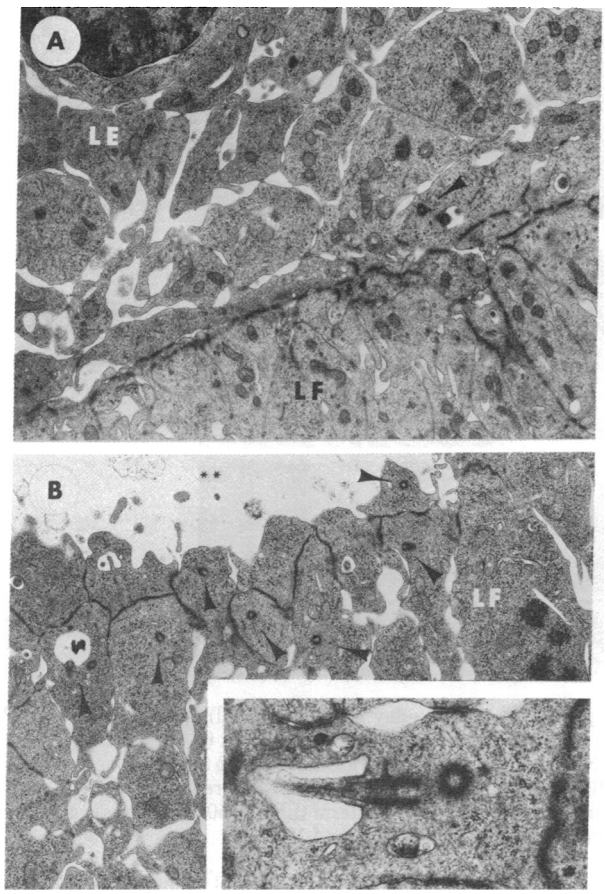

FIG. 2. Micrographs of the lens from normal (*A*) and mutant (*B*) embryos at 11½ days of gestation. (*A*) Electron micrograph from an area represented by the arrow labeled 2A in Fig. 1*A* showing a normal (*wh/wh*) lens with lens epithelium (LE) and lens fiber (LF) cells. (×5,100.) (*B*) Electron micrograph from the area represented by arrow 2B of Fig. 1*B* showing lens fiber (LF) cells of a mutant (*Wh/Wh*) which appear to have retained their cilia. (×5,900.) Asterisks indicate the opening of the lens vesicle. (*Inset*) One cilium and centriole in a lens fiber cell next to the area shown in *B*. (×16,800.) Arrowheads indicate the presence of cilia.

Fig. 1*A* indicated by arrow 2A). By contrast, nearly every lens fiber cell of *Wh/Wh* hamsters was found to be ciliated (Fig. 2*B*—from the area in Fig. 1*B* indicated by arrow 2B).

The TEM analyses of 11½-day embryos demonstrated that *Wh* caused the presence of cilia on opposing cells of the two layers of the retina and on cells of the inner surface of the lens fibers. It is possible that these cilia were produced as a consequence of abnormal differentiation or were retained by cells that were normally ciliated prior to the formation of the optic cup and lens vesicle. To distinguish between these two possibilities, we performed a SEM analysis of six 7½-, 8-, and 8½-day normal (*wh/wh*) embryos and normal adult tracheal tissue. The analysis (Fig. 3) demonstrated three facts: (*i*) the cells of the anlage of the optic cup, otic vesicle, and lens (not shown) appear to be covered with cilia-like projections prior to 11½ days of gestation; (*ii*) the density of these projections normally decreases between 7½ and 8½ days; and (*iii*) in the areas where tissue fusion occurs (at the crest of the neural folds and edges of the otic pit) cells appear to have resorbed these cilia-like projections and have instead a few ruffled membraneous processes (not shown). This latter observation is in agreement with Watermann's observations (19). Note also the bulbous nature of the tips of the projections in the SEM micrographs of Fig. 3 *C*, *G*, and *J* and compare this with the sectioned cilium in the TEM micrograph in Fig. 1*E*. A more complete SEM and TEM analysis of these projections will be published at a later time.

DISCUSSION

The results reported here document two newly recognized effects of the gene *Wh*. The gene causes (*i*) the presence of numerous cilia between the neural and pigmented layers of the retina and (*ii*) the presence of cilia on the inner surface of the lens fiber cells. We have previously reported that *Wh* causes the presence of cilia on secretory cells of the adult anterior lobe of the pituitary (15).

At least two possible interpretations can be made as to the primary action of *Wh*: (*i*) the gene causes the abnormal production of cilia or (*ii*) the gene inhibits resorption of preexisting cilia. To determine which of these alternative explanations is correct, we performed SEM analyses of normal embryonic tissue. These analyses demonstrate that the primordia of normal (*wh/wh*) optic vesicles appear to be covered with cilia at 7½ days and that cilia density within the optic vesicle appears to decrease by 8½ days of gestation. The presumptive lens ectoderm overlying *wh/wh* optic vesicles at 8½ days is similarly highly ciliated. TEM analysis of 11½-day *wh/wh* embryos indicates that cilia are nearly lost from the lens and retinal layers. Thus, the development of the eye begins with ciliated ectodermal cells. By 11½ days, the cilia are lost. Because the outer segments of rods and cones are modified cilia (20, 21), cilia must reappear as the neural layer of the retina differentiates. Because fertile *Wh/Wh* hamsters are very rare and we cannot recognize *Wh/Wh* embryos prior to 10 days of gestation, we have not produced *Wh/Wh* embryos for SEM analysis. However, TEM analyses at 11½ days of gestation indicate that *Wh/Wh* cells from the lens and retina are ciliated. Because the normal sequence of events is to lose these cilia, we conclude that *Wh* blocks this resorptive process.

The function of ciliated ectodermal cells is suggested by an experiment performed by Nace. Using embryos of *Rana pipiens*, Nace (personal communication) attempted to incorporate fluorescent gamma globulin into the neurocoel of neuralating embryos. This reagent appeared to be swept out of the neural tube as the neural folds fused. Thus, by immersion, it was not possible to incorporate these molecules into the developing neural tube. We suggest that cilia of the neural plate, optic vesicle, otic pit, and lens pit serve the same housekeeping function.

To explain all of the pleiotropic effects of the gene *Wh* (6), we propose the following sequence of events. As the neural tube is formed, a portion of the cilia at the edges of the neural ectoderm must be resorbed to ensure proper fusion of the neural folds (19). We postulate that the gene *Wh* alters this resorptive process in such a way that fusion of the neural folds is still permitted; however, one product of fusion—the neural crest—is in some way defective. As a possibility, the neural crest cells may retain their cilia and thus are unable to migrate to their proper final location within the epidermis. Thus, hamsters would lack melanocyte-derived pigmentation. The retention of cilia—by a few chick embryo neural crest cells—has been reported recently (22).

The neural tube thus formed would be lined with ciliated ependymal cells. In the anterior portion of the tube (the diencephalon), four evaginations appear and produce the optic cups, pineal, and posterior lobe of the pituitary. In each case, cilia would sweep these evaginations free of debris. Certain cells would then lose their cilia and allow specific cell layers to fuse. If *Wh* prevents resorption of cilia at this time, the cilia would interfere with intimate cell contact, thus inhibiting the closure of the passage way between the eye and the diencephalon which

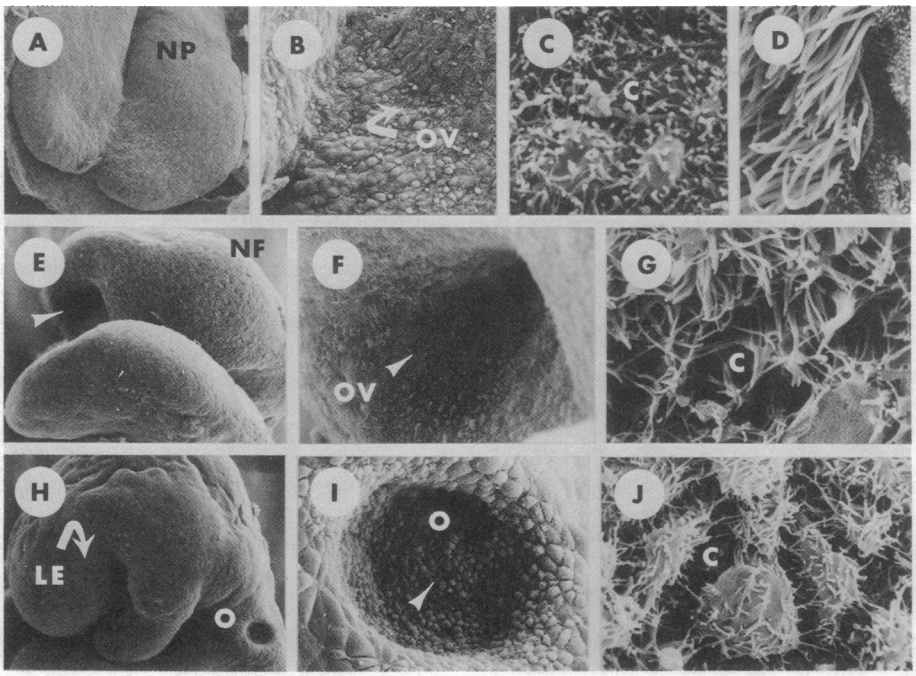

FIG. 3. Scanning electron micrographs of hamster tissue. (*A*) The neural plate (NP) of a 7½-day *wh/wh* (normal) embryo. (×95.) (*B*) Presumptive neuroectoderm of the optic vesicle (OV) from *A*. (×280.) (*C*) Cilia (C) of cells from the area represented in *B*. (×4,100.) (*D*) Ciliated (C) epithelial cells of (*wh/wh*) adult trachea. (×4,100.) (*E*) The open neural folds (NF) of an 8-day *wh/wh* (normal) embryo showing the optic vesicle (arrow). (×80.) (*F*) The optic vesicle represented in *E*. (×370.) (*G*) Ciliated (C) cells of the inner surface of the optic vesicle represented in *F*. (×4,100.) (*H*) The head of an 8½-day *wh/wh* (normal) embryo showing the otic pit (O) and presumptive lens ectoderm (LE). (×50.) (*I*) The otic pit (O) represented in *H*. (×270.) (*J*) Ciliated (C) cells of the surface of the otic pit indicated by the arrowhead in *I*. (×4,100.)

runs through the center of the optic stalk. We postulate that because this canal is completely open, pressure between the layers of the optic cup builds up and thus the choroid fissure cannot close. The two layers of the optic cup cannot interact properly because of the presence of cilia and elevated internal pressure which would tend to separate the layers. Thus the pigmented layer of the retina fails to differentiate (21). These retained cilia would also interfere with the proper development of the posterior lobe of the pituitary and pineal.

Finally, the inner ear, lens, and anterior lobe of the pituitary are derived from ciliated epidermal cells. These structures begin with invaginations to produce the otic pit, lens pit, and Rathke's pocket, respectively. These ectodermal invaginations are first swept clean of debris by active cilia movement. Next, cells lining the outer edges of these invaginations must resorb their cilia to ensure proper cell contact which leads to the formation of the closed vesicles. It is postulated that the gene alters all of these resorptive processes. The organs so differentiated would contain large numbers of abnormally ciliated cells.

The hamsters thus developed would be deaf, blind, and white, show some abnormal pituitary functions, and be fertile or sterile, depending upon the severity of the effects of the gene upon pineal development and function. Because of the negative influence of the pineal upon reproduction, hamsters with defective pineals should be fertile, whereas those with normal pineals should be sterile (23).

The above hypothesis has several ramifications. *Wh* is a dominant spotting mutation and shares many of its characteristics with numerous other mutations in other mammalian species (1). We propose that many of these other mutations alter cilia resorption and thus cause their pleiotropic effects by inhibiting or altering proper cell–cell contact, cell interaction, and subsequent cell differentiation. In this regard, several mouse mutations appear to be homologous with *Wh* and include Mi^{wh}, *Sp*, Dreher (*dr*), and Kreisler (*kr*). These mutations appear to cause the entire range of phenotypes expected from mutant genes that may alter cilia resorption and include—as an obvious prediction—mutations which inhibit proper neural tube closure. We suggest that many of these mutants function to alter resorption of cilia in specific areas and at specific times. The consequence of the action of a mutation of this kind is the production of a phenotype having many coordinate pleiotropic effects that are temporarily and spatially disconnected during development.

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