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Brain Development in Hydrocephalic-Polydactyl, a Recessive Pleiotropic Mutant in the Mouse*

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Summary. Animals homozygous for the recessive, pleiotropic, mutation *hpy* (hydrocephalic-polydactyl) progressively lag behind their wild-type littermates in increase in body weight and brain dry weight over the period from 1–40 days post-partum; many homozygotes die within the first 14 days after birth. Light microscope observations of serial sections of brains revealed a mild to severe dilation of the entire ventricular system and damaged ependyma. Ciliated ependymal cells appeared reduced in number and destruction of ependymal cells over wide areas of the ventricular surfaces was observed. Preliminary scanning electron microscope studies confirmed the light microscope observations and revealed large numbers of erythrocytes and phagocytes associated with the ependymal surface. Neither the histological studies nor experiments involving intracerebral injections of tracer dyes demonstrated obstruction or stenosis of the aqueduct of Sylvius. Individual neurons appeared to be present in normal numbers and to be developing normally and at the same rate as in wild-type animals.

Key words: Brain development – Hereditary hydrocephalus – Non-obstructive hydrocephalus – Recessive pleiotropic mutant – Mouse.

Introduction

A number of mutations in the mouse giving rise to various types of development-related defects, so-called genetic diseases, serve as important models in attempts to elucidate similar pathological conditions and syndromes in man. In addition

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they provide insight into the nature of normal processes. Several mutations which give rise to hydrocephalus have been reported in the past but, unfortunately, most of them appear to have been lost (c.f., Green, 1966; Borit and Sidman, 1972). Brain development in two different, more recently discovered mutants, has been described by Green (1970) for the mutation *ch,* and by Borit and Sidman (1972) for the mutation *oh.* In *ch/ch* animals Green reported that hydrocephalus first became evident at 11 days post-partum apparently as a result of the retarded development of the subarachnoid space; obstruction or stenosis of the aqueduct of Sylvius did not appear to be involved. However, Borit and Sidman reported that hydrocephalus in *oh/oh* animals involved stenosis of the aqueduct – a fairly common finding in human cases (Bickers and Adams, 1949; Alvord, 1961). The present paper reports our findings for another mutation producing post-partum hydrocephalus, originally discovered by Hollander in 1964 in a stock derived from x-irradiated mice (Hollander, 1966). It is a recessive, pleiotropic mutation and the homozygous condition is easily detected at birth on account of the associated pre-axial polydactyly. Many of the mutants, presumably those with the most extreme manifestations of the syndrome, die during the first two weeks after birth although among the survivors animals with a severe doming of the cranium (severe hydrocephalus) are not infrequent. This mutation, symbolized *hpy* (hydrocephalic-polydactyl) is located in linkage group XI (on chromosome six) and has been described in detail by Hollander (1976). Here we report our observations on the growth and development of the brain and other neural manifestations of the mutation. As will be seen, the findings are of interest in regard to studies of the hydrocephalic condition in general, especially since despite the overall similarity in the end result the development of hydrocephalus induced by this mutation differs significantly from the cases described by others.

Materials and Methods

Animals. The breeding stock was derived from Bryan's strain E (a multiple mutant line) and was composed of 12 heterozygous males (male homozygotes are sterile) and 26 females, some of which were homozygotes. For these studies 220 litters were raised constituting a total of 1906 offspring of which 388 (20.4%) were homozygous *hpy.* Surviving mutants together with appropriate numbers of wild-type littermates (a total of 263 mice) were used in the various experiments described below as follows: 164 for the brain dry weight determinations, 39 for the tracer dye studies, and 60 for histological preparations. Animals were sacrificed at various intervals over the period from the day of birth to 40 days post-partum.

Brain Dry Weight. Changes in the brain dry weight with age served as an index of brain development. After determination of body weight animals were killed either by decapitation (neonates) or by cervical dislocation. The removed heads were skinned and in older animals the brain case carefully opened to facilitate penetration of fixative. Fixation was necessary to harden the brains, especially of neonates, to allow their removal intact. After an overnight fixation in Zenker's fixative (Humason, 1972) heads were washed in tap water to remove excess dichromate and mercuric chloride. If necessary the tissue was further hardened by an overnight soaking in 70% ethanol. The hardened brains were carefully removed, rinsed in water and gently blotted dry and weighed. Isolated brains were then dried in an oven to constant weight (three days at 40–50°C), and returned to the

oven for an additional 24 h and reweighed. This was done because the dried brains were extremely hygroscopic; if the two final weights differed the mean was used in the computations.

Injection Studies. In order to determine if obstruction or stenosis of the aqueduct was involved, the circulation of cerebrospinal fluid was checked with the aid of tracer dye injections after the procedure of Borit and Sidman (1972). Older animals were anesthetized with sodium pentobarbital (i.p.) before injection while those less than about 10 days of age were injected without anesthesia. A lateral ventricle was tapped with a 30-gauge needle and the appropriate amount (0.01–0.25 ml) of a 1% dye solution in 0.9% saline was injected. Solutions of azosulfamide (Sigma) or bromophenol blue (Fisher) served as visual tracers, while uranine (sodium fluorescein, Fisher) was used in a fluorescent procedure. Except for a few of the older animals from which urine samples were collected after recovery from the anesthetic, the mice were decapitated between 10 and 60 s after injection. Blood smears were also made from the caudal vein of uranine-injected mice 60 s after injection. The smears and urine samples were examined for the presence of dye with a Zeiss fluorescence microscope. After decapitation the brains of azosulfamide- or uranine-injected animals were carefully removed and cut in various planes to determine the distribution of dye, while the brains of bromophenol blue-injected mice were exposed and fixed in neutral formalin for 24 h before further dissection.

Histological Studies. The precise routine depended on the nature of the material to be processed. Thus, brains of mice of less than about one week of age were not removed. Instead, intact heads were fixed and embedded. Brains of older animals were fixed and washed in situ and then removed for further processing. In general, mice were anesthetized with chloroform vapor and decapitated while submerged in 0.9% saline. Heads were rapidly freed from skin and placed in vials of degassed fixative (Zenker stock solution A was placed under vacuum until all dissolved air had been removed, then the vacuum was released very slowly and the required volume of glacial acetic acid added to complete the fixative). During the first two hours of fixation the fixative was replaced with fresh, degassed, solution every 30 min. After 12 h the fixative was replaced once more. For those specimens where some mineralization of the calvaria was anticipated, decalcification was accomplished by the addition to the fixative of concentrated formic acid to a final concentration of 10% (v/v). Specimens were fixed for a total time of 24 h at room temperature after which they were enclosed in small cheesecloth bags and washed in running tap water for 24 h. All further processing through embedding in wax was carried out under vacuum. Dehydration was performed with a graded ethanol series (70%, 80%, 95%, 100%) with four one-hour changes of each alcohol. Brains of older animals fixed in situ were dissected out in 70% alcohol and then processed as just described. After dehydration all specimens were cleared in: three graded mixtures (1:2, 1:1, 2:1) of 100% ethanol-tertiary butyl alcohol, pure tertiary butyl alcohol, three graded mixtures (ratios as above) of tertiary butyl alcohol-benzene and, finally, in pure benzene. Each reagent was used for 4 h at room temperature. Following this, specimens were infiltrated with paraffin wax ("Paraplast" or "Paraplast Plus")-benzene mixtures (ratios and times as above) and then in pure paraffin wax. After 12 h in pure wax, the specimens were cast in blocks and sectioned at 7 μm. Serial sections were stained with a safranin-fast green mixture as described by Bryan (1955). Selected sections were photographed on Kodak Panatomic-X roll film in a Graflex Rapid-Vance 120 film holder with a negative size of 2.25×2.25 inches at a primary magnification of $6.5\times$.

Scanning Electron Microscopy. These preliminary studies were undertaken in an attempt to corroborate and extend the light microscope studies. Two different preparative routines were investigated: (1) Initially, wax-embedded brains (or intact heads) of normal and mutant littermates were sectioned until regions of interest were reached. Then a 1 mm thick piece of the block was removed and dewaxed in several changes of warm xylene. After dewaxing, samples were washed in several changes of absolute alcohol, critical point dried and mounted on SEM stubs with silver cement. They were then coated with gold or gold-palladium, (2) the ventricles of anesthetized animals were irrigated with saline followed by 2.5% phosphate-buffered glutaraldehyde (pH 7.2). After fixation, regions of interest were removed, washed in buffer, dehydrated, critical point dried and coated with metal as before. The coated specimens were examined in a Kent-Cambridge Stereoscan microscope at 10 KV.

Results

At birth many *hpy*-homozygotes were noticeably smaller and less vigorous than their non-*hpy* littermates. In general, at about six days after birth, the cranium developed a distinct domed appearance indicative of hydrocephaly. Animals exhibiting the most extreme manifestations usually died within the first two weeks after birth. Homozygotes which survived to weaning also possessed an abnormal gait suggestive of disturbed motor functions. In these animals hind limbs worked in concert giving the mouse a hopping or rabbit-like gait. As can be seen from Figure 1A, mutants progressively lagged behind their wild-type littermates in increase in body weight with age. Differences in brain weights show a similar time-course but are less pronounced (Fig. 1B). Because of this, the relationship of the curves is reversed when brain weight is computed in terms of per cent of body weight (Fig. 1C). Prior to the observable onset of the condition, histological sections of the brain did not reveal any marked irregularities. With the dissecting microscope a slight enlargement of the ventricular system (c.f., Fig. 2) was detected in some animals three days after birth, and by six days a domed appearance of the cranium was clearly evident. Thereafter, there was a progressive dilation of the ventricles of the cerebral hemispheres. Coupled with this was increased development of the associated vasculature. In about 30% of the cases this was manifested as an extensive outgrowth of vascular tissue which filled the cerebral fissures and covered the calliculi. There were also numerous adhesions between the meninges and the surface of the brain.

The displacement and ensuing abnormal development of brain tissue in the cortex resulted in the hemispheres becoming extremely thin-walled sacs. In addition to displacement of tissue in the posterior region, a forward displacement was also observed. The olfactory lobes appeared truncated or foreshortened; this deformation appeared to result from the anterior region of the brain being forced against the anterior wall of the cranium. The diencephalon appeared to be elongated in the vertical plane rather than in the horizontal plane as in wild-type mice. In terms of overall shape, the brain of the immature mutant mouse more closely resembles that of the normal adult than the brain of a same age wild-type animal.

The circulation rate and distribution of tracer dyes in the nervous system of mutants of less than 8–10 days of age was identical to that of their wild-type littermates. Dye appeared to be confined to the ventricular system and no discoloration of underlying tissue was observed. In older mutants dye appeared to be distributed as rapidly as in wild-type animals but, especially in the more mature mutants, staining of the periventricular tissue was also detected.

Examination of histological sections revealed what appeared to be defects in the lining of the ventricles. The ependymal cells appeared flattened in the mutant brain compared to those of the wild-type, and there also appeared to be edema of the white matter of the hemispheres (Figs. 3–6). In some instances, the ependymal layer appeared intact but its surface coat seemed to be denuded over large regions. In others, the ependymal layer appeared to have lost its association with the underlying tissue and hung free in the ventricular space.

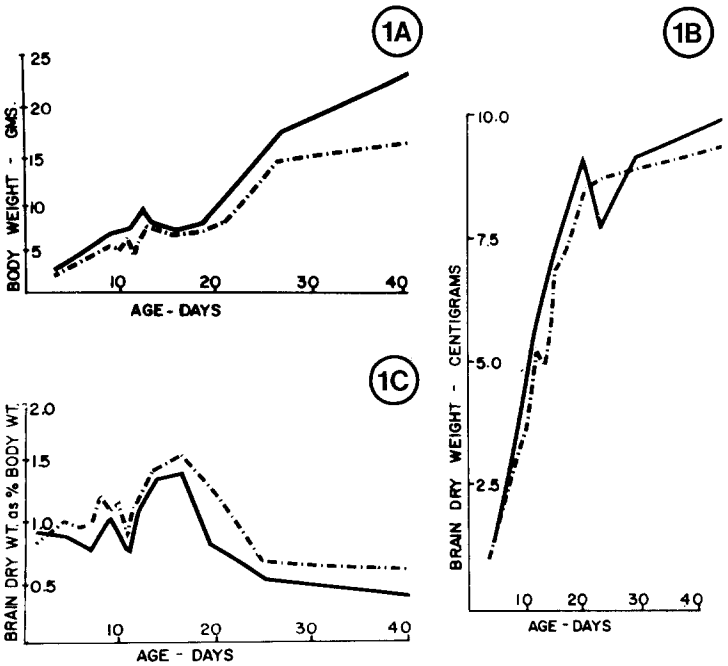


Fig. 1A–C. Post-natal development of mutant and wild-type littermates. Mutant animals designated by the dotted line. Each point is an average of at least four animals. Figure 1A charts the changes in body weights with increasing age, Figure 1B the change in brain dry weights, and Figure 1C shows brain dry weight as a per cent of body weight

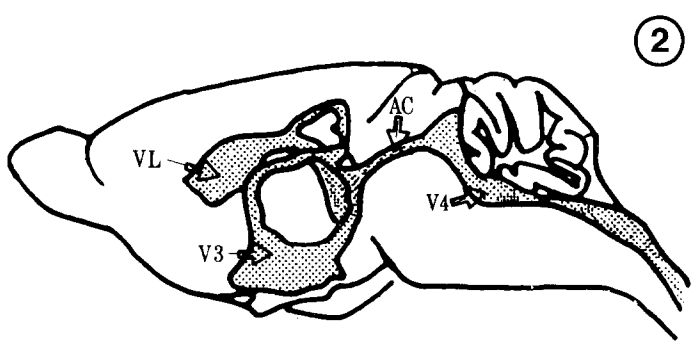
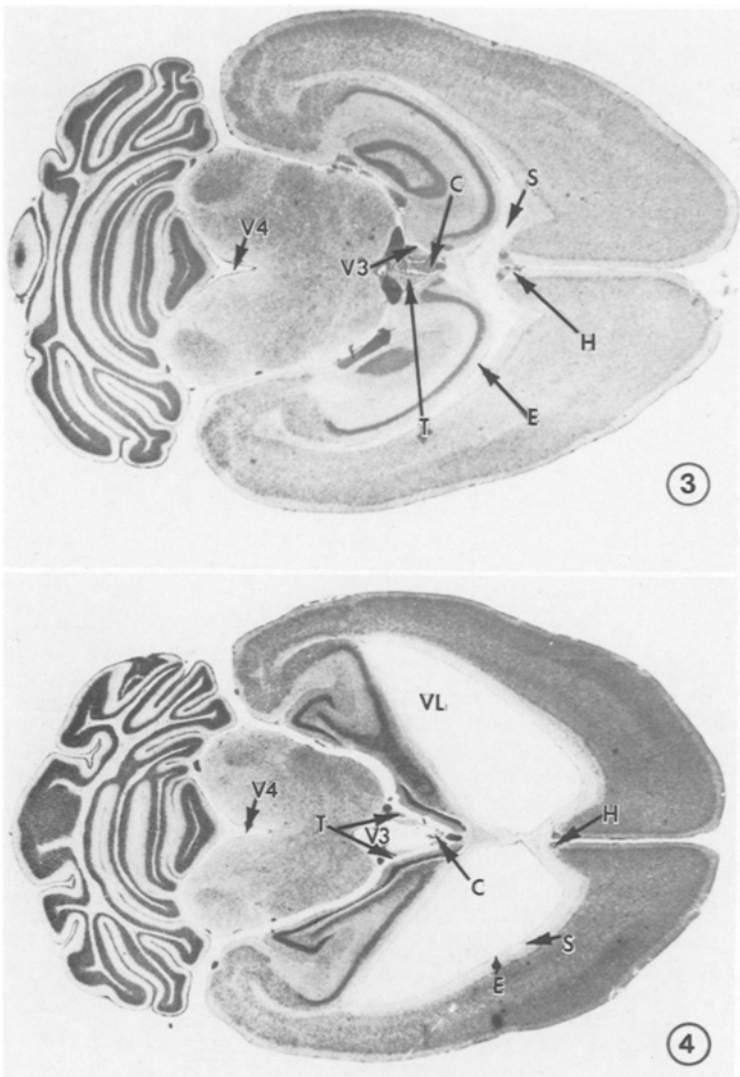


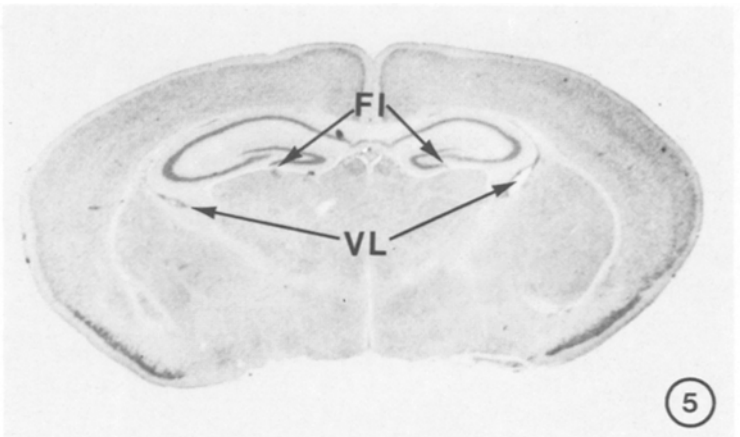
Fig. 2. A diagrammatic representation of the ventricular system of the mouse brain: *VL* lateral ventricle; *V3, V4* third and fourth ventricles; *AC* aqueduct of Sylvius. (Redrawn from: Sidman, et al., 1971, and Rapräger and Röder, 1975)

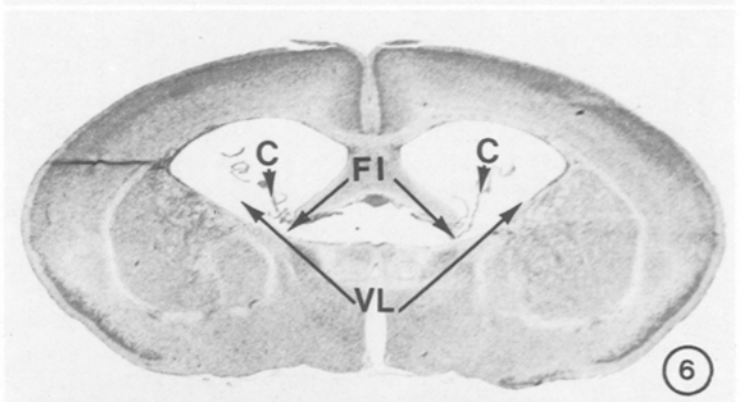
This latter finding did not appear to be a consequence of poor fixation since this defect was not observed in non-*hpy* specimens processed together with mutant material. Breaks were also seen in the subependymal layer and sometimes they extended into the adjacent white matter. In some mutants the degradative

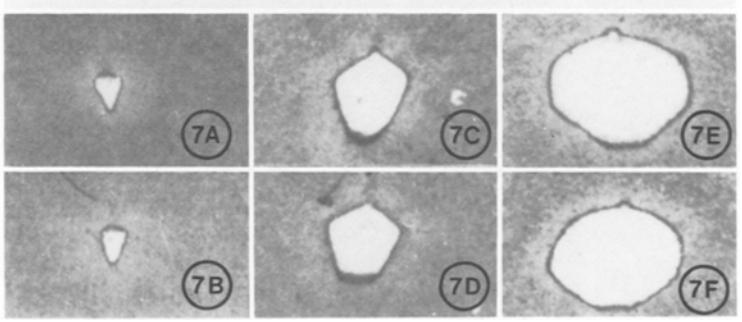


Figs. 3 and 4. Horizontal sections of 12-day old mouse brains. Figure 3 shows the typical appearance of the brain of wild-type animals while Figure 4 illustrates the typical appearance of mutant brains. Ventricles labeled as in Figure 2; *C* choroid plexus, *E* ependymal lining, *H* hippocampi, *S* subependymal layer, *T* the thalamus. Both figures × 20

process led to the formation of large ventricular diverticula which were not surfaced with ependymal cells. The corpus callosum was reduced to a thin band of tissue. The fornices and adjacent regions of the septal area were also markedly reduced in thickness or, in some instances, absent. The total amount of hemispheric white matter was severely reduced particularly in the posterior aspects.







Figs. 5 and 6. Cross sections of brains of non-mutant (Fig. 5) and mutant (Fig. 6) animals. *FI* intraventricular foramina. Both figures × 20

Fig. 7A–F. Cross sections through the aqueduct of Sylvius at 6 days (**A** and **B**), 8 days (**C** and **D**), and 12 days (**E** and **F**) post-partum. A, C, E = wild-type; B, D, F = mutant. All figures × 60

Appropriate sections of brains were examined to determine the status of the aqueduct in animals of different ages. No obvious differences between mutants and their wild-type littermates were detected (e.g., Fig. 7). In other words, no histological evidence favoring occlusion or stenosis of the aqueduct in young or more-mature *hpy*-homozygotes was obtained. Individual neurons appeared

to be developing in a normal manner and at the same time as in wild-type animals. Although quantitative measurements were not made, there did not appear to be any significant reduction in the number of differentiating neurons during, at least, the early phases of such activity.

In general, the SEM studies served to confirm the light microscope findings reported above. In addition, it appeared that both the frequency of ciliated cells and the number of cilia per cell in ependyma of mutants appeared reduced in comparison with wild-type tissue. Also, large numbers of erythrocytes and of presumed phagocytes were observed on the ependymal surfaces of both Zenker-fixed and glutaraldehyde-fixed mutant brains.

Discussion

For convenience of description, at least, the development of the brain can be considered in a number of phases based on the status of cellular differentiation and/or levels of functional activity such as reflex responses. Fox (1965) has defined five such periods of neurologic development in the mouse: perinatal (birth to three days), neonatal (three to nine days), postnatal transition (9–15 days), pre-juvenile (15–26 days), and juvenile (26 days to sexual maturity). By 15 days brain-to-body ratios are well established, as are EEG activity and seizure patterns, and total brain growth and axo-dendritic development are within adult levels of activity. Other workers (cited by Fox, 1965) have reported that cell density, retinal activity, and cholinesterase content of the CNS have reached mature levels by the end of the second week of life. These various findings point to the post-natal transition period as being a most critical phase in the developmental timetable. It is during this period that the manifestation of hydrocephaly becomes markedly evident in *hpy*-homozygotes and, moreover, it is during this same period that many of the affected individuals die.

The overall pattern of brain damage resembles that reported for *oh* by Borit and Sidman (1972). Thus, white matter appeared to be more affected than gray matter. There also appeared to be a graded or progressive reduction in the degree of damage to the periventricular tissue along the anterior-posterior axis of the brain. Most severe changes were noted in the tissue surrounding the lateral ventricles with the least damage being observed in the more caudal regions of the brain. As pointed out by Borit and Sidman, this graduated distribution of damage could be brought about by pulsatile forces emanating from the rhythmic pumping action of the heart and transmitted to the CSF by the choroid plexuses. It would then follow that regions closely apposed to the most extensive choroid plexus (i.e., regions surrounding the lateral ventricles) should be the most severely affected. Furthermore, as the lateral ventricles become further distended their increasing surface area should potentiate the pulsatile effects. At the same time, any stenosis of the aqueduct should serve to further exacerbate the effects of the hemodynamic forces. However, despite the extensive dilation of the cerebral hemispheres noted in the *hpy*-induced condition, neither the tracer dye studies nor the histological investigations furnished any evidence favoring obstruction or stenosis of the aqueduct. In

this respect the hydrocephalus induced by the *hpy* mutation differs significantly from that induced by *oh* (Borit and Sidman) which involves a secondary stenosis of the aqueduct of Sylvius. By the same token, *hpy*-induced hydrocephalus differs from the commonly encountered condition in man and from that induced in post-natal-transitional animals by intracerebral injection of viruses (for references see Chew-Lim and Webb, 1976). Instead, the condition is more akin to that produced in rats by injection of anti-rat-kidney serum (ARKS) into pregnant females on the 9th day of gestation in which the hydrocephaly is not associated with obstruction or stenosis of the aqueduct (Duckett, et al., 1974). It is of interest that the hydrocephalic effect of ARKS peaks on days 7–9 of fetal life which, in the rat, corresponds to a time shortly before the so-called "hydrocephalic" or vesicular stage of brain development (Brent, 1970). Thus, whatever the nature of the immunologically-induced damage may be, it appears that it prevents the normal distention of the ventricles from receding according to schedule so that their dilated condition is evident at birth. Although the hydrocephalus induced by *hpy* is not congenital, it is not inconceivable that the etiology may be very similar. Such a conclusion receives support from the findings that ciliary degeneration (and other indications of damaged ependymal cells) and sub-ependymal damage are commonly encountered pathological changes in hydrocephalic brains (Duckett, 1972; Chew-Lim and Webb, 1976; and others). Furthermore, according to Duckett (1972), ependymal damage can result in a reduction or blockage of the transfer of CSF across the CSF-ependymal interface. As reported in the results section, clear evidence of ependymal damage was obtained from the various histological studies. However, at this time, it is not clear which pathological changes might be causal in nature and which the results of the condition. Inhibition of transfer of CSF together with the presence of normal (or hypertrophied) choroid plexus tissue could result in an excessive accumulation of CSF in the ventricular system.

Although quantitative determinations were not attempted, the amount of choroid plexus tissue in *hpy* brains was judged to be not less than that characteristic of comparable wild-type animals. Thus, it would appear logical to infer that in hpy-homozygotes the hydrocephalus results from a net increase in the amount of CSF. This could be accounted for most simply by postulating an overproduction of CSF by the choroid plexuses. Alternatively, the level of production of CSF could remain within the range for non-hydrocephalic animals while trans-ependymal flow of CSF could be severely impaired as a result of the damage to the ependymal cells. However, we noted in numerous specimens that the ependymal lining had become divorced from its typical association with the sub-ependymal tissue. Therefore, and unless trans-ependymal flow of CSF depends almost exclusively upon intact ependymal cells (active transport of CSF), the latter postulate would appear to be somewhat less than satisfactory. To examine this question further, detailed information concerning the ultrastructure of the cell types constituting the ependyma, and choroid plexuses of mutant and wild-type brains will be required. Unfortunately, our preliminary studies with the SEM were not completely satisfactory in meeting these requirements. Thus, although excellent cytological preservation at the light microscope level was obtained with Zenker's fixative, indications of fixation-induced damage

to cell membranes were detected with the SEM. On the other hand, while cell preservation was adequate following fixation with gluteraldehyde, appreciable amounts of coagulated material (apparently derived from CSF) adhering to the ventricular lining tended to obscure ciliary and cell surface detail. Appropriate ultrastructural studies with the SEM and TEM are now in progress and the findings will be reported elsowhere. In the meantime, the precise nature of the factor(s) responsible for the development of this gene-mediated example of non-obstructive hydrocephalus must remain an open question. In any event, it is becoming clear that despite their different etiologies, the various types of hydrocephalus (hereditary; viral-induced; obstructive or non-obstructive) manifest as a common feature the progressive destruction of the ependyma. It would therefore appear that such overt pathological changes may be largely the consequences of an initial impairment of defect in ependymal function which in turn leads to the generation of a self-reinforcing condition condusive to further degradation of this important cell layer.

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