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Sporadic Obstructive Hydrocephalus in *Aqp4* Null Mice

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Abstract

Aquaporin-4 (Aqp4) is a water transport protein expressed in glia and ependymocytes in brain. We report here the unexpected occurrence of severe obstructive hydrocephalus in a random subset of Aqp4 knockout mice. Of 612 Aqp4 knockout mice produced by heterozygote–heterozygote or knockout–knockout breedings, 9.6% of offspring manifested progressive encephalomegaly. Encephalomegaly was never seen in wild-type or Aqp4 heterozygous mice. Examination of the subset encephalomegalic mice revealed marked triventricular hydrocephalus (lateral ventricle size ~500 mm³), elevated intracranial pressure (19 ± 3 vs. 6.1 ± 0.6 mm Hg), and death by age 6 weeks, with a median survival of 28 days. Intraventricular dye injection studies revealed total obstruction of the cerebral aqueduct. Evans blue extravasation studies indicated an intact blood–brain barrier in the hydrocephalic mice. Brain histology revealed reduced ventricular size and ependymocyte disorganization in some nonhydrocephalic Aqp4 null mice. Our studies establish Aqp4 deletion as a predisposing factor for the development of congenital obstructive hydrocephalus in mice. We suggest that AQP4 polymorphisms might also contribute to the development of aqueduct stenosis in humans.

Keywords

aquaporin; aqueduct stenosis; *Aqp4* hydrocephalus; ependyma

Aquaporin-4 is a water-selective transport protein expressed strongly in astrocytes throughout the central nervous system, and in ependymal cells lining brain ventricles in contact with cerebrospinal fluid (CSF) (Nielsen et al., 1997; Rash et al., 1998). Phenotype analysis of *Aqp4* null (*Aqp4*^{−/−}) mice has revealed the involvement of *Aqp4* in brain water balance, glial cell migration, and neural excitation phenomena (reviewed in Verkman et al., 2006; Tait et al., 2008). With regard to brain water balance, *Aqp4* deletion in mice reduces brain water accumulation in cytotoxic (cell swelling) brain edema, including water intoxication, ischemic stroke, and bacterial meningitis (Manley et al., 2000; Papadopoulos and Verkman, 2005). *Aqp4* also facilitates movement of excess water out of the brain, with greater brain water accumulation seen in *Aqp4*^{−/−} mice in models of vasogenic (leaky vessel) brain edema, including intraparenchymal fluid infusion, cortical freeze injury, brain tumor, and brain abscess (Papadopoulos et al., 2004; Bloch et al., 2005).

Various causes of hydrocephalus in rodents, including congenital hydrocephalus in the H-Tx rat (Shen et al., 2006) and hydrocephalus produced by kaolin injection into rat cisterna magna (Mao et al., 2006), are associated with increased *Aqp4* expression. We suggest that the increased *Aqp4* expression is a compensatory phenomenon that facilitates brain water clearance in hydrocephalus. This idea is supported by the finding of accelerated ventricular enlargement and elevation in intracranial pressure (ICP) in *Aqp4*^{-/-} mice after intracisternal kaolin injection, which produces severe obstructive hydrocephalus in mice (Bloch et al., 2006).

We report here an unexpected observation of sporadic occurrence of obstructive hydrocephalus in a small subset of *Aqp4*^{-/-} mice. The spontaneously hydrocephalic mice were characterized in terms of their genetic pattern, brain structure, ICP, and blood-brain barrier integrity. Our data establish *Aqp4* deletion as a novel model of progressive obstructive hydrocephalus and *Aqp4* as a new determinant in the pathogenesis of hydrocephalus.

MATERIALS AND METHODS

Mice

Aqp4^{-/-} mice used in this study were generated by Fan et al. (2005). The mice were maintained in a specific-pathogen-free-grade animal facility on a 12-hr light/dark cycle. All experiments were performed on age-matched wild-type and *Aqp4*^{-/-} mice in a CD1 genetic background. The following mouse breedings were set to quantify the incidence of hydrocephalus phenotype: 1) *Aqp4*^{-/-} × *qp4*^{-/-}; 2) *Aqp4*^{+/-} × *Aqp4*^{+/-}; and 3) *Aqp4*^{+/-} × *Aqp4*^{-/-}. Offspring was genotyped on postnatal day 10 by polymerase chain reaction that used the following primers: 5'-ACCATAAACTGGGTGGCTCAG-3'; 5'TAGAGGATGCCGGCTCCAATGA-3'; and 5'CACCGCTGAATATGCATAAGGCA-3'. The following polymerase chain reaction conditions were used: 94°C for 5 min, then 30 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 45 sec. Hydrocephalic mice were identified by their enlarged head, which was confirmed in all cases by brain sectioning. Protocols for mouse experiments were approved by the Committee on Animal Research of Northeast Normal University.

ICP Measurement

Mice were anesthetized with intraperitoneal pentobarbital sodium (60 mg/kg, Sigma-Aldrich, St. Louis, MO) and immobilized in a stereotactic frame (Benchmark, Neurolab, St. Louis, MO). A 0.5-cm midline incision was made over the vertex of the skull. A 1-mm burr hole was drilled 3 mm posterior and 3 mm lateral to the bregma with a micromotor drill (Foredom, Bethel, CT). A polyethylene catheter (PE-10) with a 0.6-mm outside diameter connected to a pressure sensor was inserted to a depth of 2 mm. ICP was recorded with a BL-420S physiological recording system (Chengdu Technology Market, Chengdu, Sichuan, China). During ICP measurements, rectal temperature was maintained at 37–38°C with a heating pad. ICP values were averaged over 5 min.

CSF Circulation

A 0.5-mm burr hole was drilled 3 mm posterior and 3 mm lateral to the bregma on the head of anesthetized hydrocephalic and control *Aqp4*^{-/-} mice, as described above. Evans blue dye solution (5 µl, 4% in phosphate-buffered saline, Sigma-Aldrich) was injected into the right lateral ventricle through the hole by a 30-gauge needle attached to a 10-µL glass syringe. After 1 hr, mice were humanely killed with an overdose of pentobarbital sodium, and whole brains with a portion of the spinal cord were surgically removed and fixed in 4% formalin solution for 48 hr. The fixed brains were coronally sliced at 0.5-mm intervals from the

anterior horn of the lateral ventricles to the medulla oblongata with a brain metal matrix. The blockage site of CSF flow pathway in hydrocephalic mice was determined from the Evans blue dye distribution.

Histology

Age-matched *Aqp4*^{-/-} (with and without hydrocephalus) and wild-type mice were anesthetized and perfused with 20 ml of 4% formalin solution by puncture of the left cardiac ventricle. The brains were removed and immersed in the formalin solution for 48 hr and then embedded in paraffin blocks. For examination of ependymal cells, serial coronal sections (4–5 μ m) were prepared from the paraffin blocks with a microtome (SM2000R, Lecai Instruments). For assessment of ventricular size, serial 5- μ m sagittal brain sections were prepared. The sections were stained with hematoxylin-eosin and imaged under a light microscope (IX41, Olympus) equipped with a CCD camera.

Blood–brain Barrier Integrity

Evans blue dye (4% in saline, 160 mg/kg) was injected through a jugular venous catheter into anesthetized hydrocephalic and age-matched control mice. Ninety minutes later, the left cardiac ventricle was perfused with 20 ml phosphate-buffered saline. Brains were then removed and divided into two groups. One group was fixed in formalin solution for 48 hr. Serial coronal slices were made with a brain matrix to visualize the distribution of Evans blue dye in brain tissue. Another group was immersed in 1 ml formamide solution at 55°C overnight to extract the Evans blue dye. The extracted dye was quantified by optical absorbance at 610 nm against Evans blue/formamide standards.

Statistical Analysis

Data are presented as mean $\pm$ standard deviation (SD). Significance between experimental groups was determined by a two-tailed Student's *t*-test assuming a 95% confidence interval. All analyses were performed by Microsoft Excel software.

RESULTS

This study was based on anecdotal observations that a small (<2%) fraction of *Aqp4*^{-/-} mice generated by Ma et al. (1997) were hydrocephalic (data not shown). This observation was also made in *Aqp4*^{-/-} mice generated by Fan et al. (2005). A fraction of *Aqp4*^{-/-} offspring developed encephalomegaly that appeared spontaneously and randomly in *Aqp4*^{-/-} $\times$ *Aqp4*^{-/-} or *Aqp4*^{+/-} $\times$ *Aqp4*^{+/-} breedings (Fig. 1A). The brains were remarkably larger in these mice (Fig. 1B, top), with serial 1-mm coronal sections revealing marked triventricular enlargement (Fig. 1B, bottom).

Table I summarizes the incidence of the hydrocephalus phenotype. Of 612 *Aqp4*^{-/-} mice produced by heterozygote–heterozygote or knockout–knockout breedings, 9.6% of offspring manifested progressive encephalomegaly. Encephalomegaly was never seen in *Aqp4*^{+/+} or *Aqp4*^{+/-} mice. In most cases, the hydrocephalus phenotype became evident at age 2–3 weeks and progressed rapidly, resulting in death by postnatal week 6, with a median survival of 28 days (Fig. 2). Nearly all mice that developed hydrocephalus did so very early in life, with only one instance of an *Aqp4*^{-/-} mice developing hydrocephalus at age 12 weeks. ICP was elevated by >3-fold in the hydrocephalic mice vs. nonhydrocephalic *Aqp4*^{-/-} or wild-type mice (Fig. 3), indicating a high-pressure type hydrocephalus.

Intraventricular dye injection was performed to determine whether the hydrocephalus was obstructive or communicating. Figure 4A shows absence of Evans dye accumulation distal to the cerebral aqueduct in the brainstem. Histological analysis showed stenosis of the

cerebral aqueduct with marked ependymal cell disorganization at this site (Fig. 4B). These findings suggest the possibility that a primary ependymal cell abnormality in *Aqp4*^{-/-} mice might produce aqueduct stenosis and consequent obstructive hydrocephalus.

It has recently been reported that the blood–brain barrier is disrupted in *Aqp4* deficiency (Zhou et al., 2008). To determine whether blood–brain barrier integrity is disrupted in the hydrocephalic *Aqp4*^{-/-} mice, an Evans blue extravasation assay was done in which brains were analyzed at 90 min after intravenous Evans blue dye injection. In contrast to the prior report that used mice from the same source, we found no differences in dye staining of brain parenchyma in hydrocephalic vs. wild-type mice (Fig. 5A). Further, the amount of dye extracted from hydrocephalic brains did not differ significantly from that in brains of wild-type mice (Fig. 5B), indicating an intact blood–brain barrier in the hydrocephalic mice.

Interestingly, brain histology in a few nonhydrocephalic *Aqp4*^{-/-} mice revealed reduced ventricular size and occasionally seen regions of ependymal cell disorganization (Fig. 6). We have analyzed 10 nonhydrocephalic *Aqp4*^{-/-} mice by continuous coronal sectioning of the whole brain and identified regional ependymal disorganization in three mice. No such ependymal alterations were seen in 10 age-matched wild-type mice. The incidence of ependymal cell alterations was not determined in mice older than 6 weeks because hydrocephalus was rarely seen in these mice (only one instance of adult hydrocephalus was identified, as mentioned above). As discussed below, this sporadic abnormality may be involved in the pathogenesis of the hydrocephalus seen in some *Aqp4*^{-/-} mice.

DISCUSSION

The central observation of this study was the sporadic occurrence of spontaneous hydrocephalus in a small, apparently random subpopulation of *Aqp4* null mice, resulting in triventricular enlargement, progressive encephalomegaly, and mortality. The hydrocephalus was obstructive in nature, with elevated ICP and absence of dye communication between the third and fourth ventricular compartments, indicating aqueduct stenosis, which was confirmed by histological examination. Our findings, therefore, suggest *Aqp4* as a new determinant of aqueduct stenosis producing hydrocephalus. Prior indirect evidence has also implicated *Aqp4* in the progression, but not pathogenesis, of hydrocephalus (Bloch et al., 2006; Mao et al., 2006; Shen et al., 2006).

The etiology of aqueduct stenosis in some *Aqp4*^{-/-} mice that causes hydrocephalus is not clear because many of the pathological changes seen in affected mice are likely to be the result of the disease process. The apparent ependymal disorganization seen in some unaffected *Aqp4*^{-/-} mice suggests a primary ependymal abnormality sporadically causing aqueduct adhesions as one possible etiology of the obstructive hydrocephalus. However, the precise link between *Aqp4* deficiency and ependymal cell abnormalities is unclear. Another possibility is the existence of a modifier gene that primes the brain for development of hydrocephalus in *Aqp4* deficiency. Identification of such a putative hydrocephalus modifier gene presents a significant challenge.

Ependymal cells normally form a single-layered cuboidal/columnar, mostly ciliated epithelium separating the CSF-filled spaces of the central nervous system from its parenchyma (Bruni, 1998; Mathew, 2008). Ependymal cells do not constitute a homogeneous cell population but fall into different subtypes on the basis of differences in morphology (Bruni, 1998). The cuboidal cilia-bearing ventricular epithelial cells make up the largest subpopulation of ependymal cells. The most prominent feature of the ventricular epithelial cells is their bundles of cilia, which are thought to cause a streaming of the CSF and a dispersion of materials in this fluid, as well as maintaining the structural integrity of

the ventricles. The ependyma express *Aqp4* in orthogonal arrays in their basolateral membranes (Nielsen et al., 1997; Rash et al., 1998). Although the function of ependymal *Aqp4* is not understood, current thinking is that *Aqp4* may facilitate water movement between ventricular CSF and brain parenchyma (Papadopoulos et al., 2004; Verkman et al., 2006; Tait et al., 2008). Our study suggests that *Aqp4* may also be involved in other aspects of ventricular cell biology, such as maintenance of its structural integrity. We have observed discontinuities in the ependymal epithelium in *Aqp4* deficiency although technical artifacts can not be completely ruled out in the present study. It has been suggested that ependymal denudation is a key event that may cause aqueduct obliteration and subsequent obstructive hydrocephalus to develop (Wagner et al., 2003). Comparisons of *Aqp4*-deficient vs. *Aqp4*-expressing ependymal cell cultures may further clarify the role of *Aqp4* in the structural integrity of the ependyma.

It was recently reported that the blood–brain barrier is anatomically and functionally disrupted in *Aqp4* deficiency (Zhou et al., 2008). In contrast with that report, our experiments here did not find evidence for blood–brain barrier disruption in *Aqp4*^{−/−} mice by the Evans blue extravasation assay. We previously quantified ultrastructural features of cerebral capillary endothelia and pericapillary astrocyte foot processes (Manley et al., 2000; Papadopoulos and Verkman, 2005) and did not find evidence of blood–brain barrier disruption in *Aqp4* deficiency. In those studies no interendothelial tight junction opening or expansion of astrocyte foot processes was seen in the *Aqp4*^{−/−} mice.

Aqueduct stenosis, as found here in hydrocephalic *Aqp4* null mice, is a common cause for hydrocephalus in children and is sometimes seen in adults (Oi et al., 1996). The etiology of aqueduct stenosis is not known (Crews et al., 2004), but there are often adhesions within the aqueduct of Sylvius. Multiple conditions have been suggested to cause aqueduct stenosis, including infections, nutritional deficiencies, and genetic disorders (Wagner et al., 2003). The possibility that *AQP4* deficiency or functionally significant *AQP4* polymorphisms contribute to the pathogenesis of aqueduct stenosis in humans warrants further investigation.

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Fig 1.

Spontaneous hydrocephalus in *Aqp4* null mice. **A:** External appearance of an *Aqp4*^{+/+}, a nonhydrocephalic *Aqp4*^{-/-}, and a hydrocephalic *Aqp4*^{-/-} (H) mouse. **B:** Whole brain (top) and coronal sections (bottom) of brains from a hydrocephalic *Aqp4*^{-/-} (H), a nonhydrocephalic *Aqp4*^{-/-}, and an *Aqp4*^{+/+} mouse. Scale in centimeters. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Fig 2. Survival of *Aqp4* null mice. Curve labeled *Aqp4*^{-/-} represents nonhydrocephalic *Aqp4*^{-/-} mice, and curve labeled *Aqp4*^{-/(H)} represents mice with visible encephalomegaly by age 3 weeks.

Fig 3.

ICP measurements. ICP of hydrocephalic *Aqp4*^{-/-} (H) mice was significantly higher than *Aqp4*^{+/+} or nonhydrocephalic *Aqp4*^{-/-} mice. Mean $\pm$ SD, $n = 8$ per group. * $P < 0.001$.

Fig 4.

Aqueduct stenosis in *Aqp4* null hydrocephalic mice. **A:** Serial coronal sections of the brains from a hydrocephalic *Aqp4*^{-/-} (H) and an *Aqp4*^{+/+} mouse after injection of Evans blue dye into a lateral ventricle. Note absence of dye from the fourth ventricle of the *Aqp4*^{-/-} (H) mouse. L, lateral ventricle; 3rd, third ventricle; Aq, aqueduct; 4th, fourth ventricle. **B:** Hematoxylin–eosin–stained sections through the aqueduct in a hydrocephalic *Aqp4*^{-/-} (left) and an *Aqp4*^{+/+} (right) mouse. Green arrows show nuclei of ependymal cells arranged as a cuboidal/columnar epithelium. Red arrows indicate region with paucity of ependymal cells. Black arrow shows an abnormal-looking (pyknotic) ependymal cell nucleus forming a multilayered epithelium. **C:** Analysis of aqueduct shape (as major:minor axis ratio) and size (as area). Mean ± SD, *n* = 12 per group. **P* < 0.02. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Fig 5.

Blood–brain barrier integrity in *Aqp4* null hydrocephalic mice. **A:** Serial coronal brain sections from a hydrocephalic *Aqp4*^{-/-} (H) and an *Aqp4*^{+/+} mouse after intravenous Evans blue dye infusion and intravascular washout. **B:** Amount of extravasated Evans blue dye was not different in the three groups. Mean $\pm$ SD, $n = 8$ per group. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Fig 6. Ependymal abnormalities in selected *Aqp4* null mice. Magnified view of ependyma in *Aqp4*^{+/+} mice and selected nonhydrocephalic *Aqp4*^{-/-} mice where ependymal disorganization was seen. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE I

Incidence of Hydrocephalus in *Aqp4*-deficient Mice

Breeding	Number of offspring	<i>Aqp4</i> ^{-/-} offspring	Hydrocephalus			Incidence of hydrocephalus in <i>Aqp4</i> ^{-/-} mice (%)
			<i>Aqp4</i> ^{-/-}	<i>Aqp4</i> ^{+/-} or <i>Aqp4</i> ^{+/+}	Incidence (%)	
<i>Aqp4</i> ^{-/-} × <i>Aqp4</i> ^{-/-}	506	506	49	0	9.7	9.7
<i>Aqp4</i> ^{+/-} × <i>Aqp4</i> ^{+/-}	421	106	10	0	2.4	9.4
<i>Aqp4</i> ^{-/-} × <i>Aqp4</i> ^{+/-}	271	0	0	0	0	0