Mutation in Tg737 Gene Shortens Neuronal Cilia in Mice

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ABSTRACT

Cilia are organelles consisting of a microtubule array projecting from the basal body of the cell. They are divided into two groups: Motile cilia and non-motile cilia. The role of primary cilia, which are non-motile, in the mammalian nervous system is not yet well understood. These neuronal cilia are shortened or even absent in Tg737\textsuperscript{orpk} mice, which have an insertional mutation in the Tg737 gene. This mutation affects polaris, a product of the Tg737 gene. Current research indicates that polaris is involved in a microtubule transport system known as interflagellar transport (IFT). Prior experiments using the Tg737\textsuperscript{orpk} mice examined neuronal cilia in wildtype (wt) and mutant mice at postnatal days 14 and 31. Greater decreases in length of neuronal cilia in mutant postnatal day 31 mice than mutant postnatal day 14 mice indicate that IFT is necessary for the maintenance of primary neuronal cilia. To further explore regional variations in cilia loss, cilia length and density were qualitatively assessed in several brain regions at postnatal days 7 and 14 in wildtype and Tg737\textsuperscript{orpk} mice. Staining from the postnatal day 7 mutant mouse was inconclusive, but the postnatal day 14 mutant mouse exhibited shortened and absent cilia in the hippocampus and piriform cortex characteristic of the Tg737 mutation.
INTRODUCTION

Background information

Cilia have a wide variety of functions in cells. Motile cilia, which have a characteristic pattern of nine microtubule doublets surrounding a central pair, constantly beat back and forth. This simple movement has many important functions in the human body, from removing particles in the lungs and moving cerebrospinal fluid throughout the brain, to providing fluid flow crucial to organogenesis in the developing embryo (1).

Non-motile or primary cilia also likely have a variety of functions. They lack the central doublet of motile cilia, and thus lack the ability to beat. They are found throughout the body (2), including in the central nervous system, but they were often considered functionless or vestigial. Recent research, however, has uncovered that primary cilia may have many important chemosensory functions (3). The recent characterization of intraflagellar transport (IFT) shows that even non-motile cilia are constantly transporting particles called “rafts” along the microtubules to the end of the cilium and back (4). These rafts carry a variety of proteins and subparticles to and from the end of a cilium (5). Since cilia cannot synthesize proteins, they rely on IFT for formation and maintenance.

Mutations that affect IFT can lead to defects in the cilia and disrupt their function. This occurs in the transgenic Oak Ridge polycystic kidney (Tg737orpk) mouse. The product of the Tg737 gene is polaris, an IFT protein involved in retrograde transport (6). The mutation is achieved by inserting exogenous DNA into an intron (7), resulting in a mutation that reduces levels of but does not completely eliminate the polaris protein. Homozygous mutants exhibit shortened cilia in many places, including the kidney, retina, and embryonic node (6, 8). Because
of this, they exhibit many defects, including retinal degeneration, polycystic kidney disease (PKD), and polydactyly (8).

Recently, cilia have been linked to several diseases, such as Bardet-Biedl syndrome (9) and PKD (10). These links led to several studies concerning PKD using the Tg737<sup>orpk</sup> mouse. One finding is that the cilia in renal tubules act as flow meters (11), and that the disruption of IFT necessary for normal cilia leads to cell death and ultimately the large kidney cysts characteristic of the disease. However, it is likely that neuronal primary cilia have a different function than those of the kidney, given that the cilia of the kidney extend into the lumen of renal tubules and experience the passing flow of urine (12), while neuronal cilia only project into the surrounding extracellular space and rarely project into the cerebrospinal fluid. Additionally, though they are found throughout the central nervous system, neuronal cilia have different morphology and chemistry across different brain regions. This implies that they may have varied effects according to the region they inhabit. Measuring and mapping how neuronal cilia are distributed throughout the brain in normal and mutant mice may give clues to their ultimate function.

**Specific aims**

This project was designed to further explore the relationship between Tg737<sup>orpk</sup> wildtype and mutant mice using immunolocalization at two different ages (13). The specific aims of this project are:

1. Examine the immunoreactive cilia in various brain regions of Tg737orpk wildtype and Tg737<sup>orpk</sup> mutant mice.
2. Compare the developmental changes in immunoreactive cilia from normal and mutant postnatal day 7 to normal and mutant postnatal day 14.
3. Compare the results from goal #2 to previous experiments measuring cilia from postnatal day 14 to postnatal day 31.

MATERIALS AND METHODS

Subjects

Subjects were bred from a heterozygous Tg737orpk (+/-) breeding pair originally obtained from Dr. Greg Pazour of the University of Massachusetts Medical School.

Tissue Preparation

Mice were deeply anesthetized with 20% urethane solution and transcardially perfused with a 0.9% saline solution followed by a 4% paraformaldehyde solution in 0.1 M phosphate buffer at pH 7.4. Brains were removed and post-fixed in the same fixative solution for 2 hours at 4°C. Tissues were then cryoprotected in 30% sucrose for approximately 24 hours, and frozen in -80 °C isopentane. The brain tissues were then stored at -80 °C until sectioned. Frozen tissue was sectioned coronally at a thickness of 30 μm using a sliding microtome. The sections were collected in Tris-buffered saline (TBS), pH 7.6. Series of sections were taken through the entire brain and stored in cryoprotectant solution (30% glycerol and 30% ethylene glycol in 0.1 M phosphate buffer) at -20 °C. Various wells were then processed for immunohistochemistry.

Immunohistochemistry

Selected tissue sections were removed from cryoprotectant and rinsed in TBS for 15 minutes three times each. All rinses and incubations were performed under continuous, gentle agitation. Sections were then placed in a pre-incubation solution (0.5% normal goat serum, 0.1% Triton X-100 in TBS) for 30 minutes at room temperature. Tissue sections were then incubated with the primary antibody adenylate cyclase III (AC3) at a 1:2000 dilution in pre-incubation
solution overnight at 4°C. Next, tissues were washed in TBS for 15 minutes three times followed by a 15 minute wash in pre-incubation solution. Sections were then incubated with the secondary antibody, biotinylated goat anti-rabbit, at a 1:500 dilution in pre-incubation solution for 1 hour at room temperature. After incubation, sections were washed in TBS twice for 15 minutes then placed in ABC solution (50 µl of Vector Standard A and B solutions in 2.5 ml of TBS) for 30 minutes. Sections were washed twice in TBS for 15 minutes before being placed in DAB solution (2.5 mg 3,3’-diaminobenzidine and 2 µl of hydrogen peroxide in 5 ml TBS) for 2 minutes. Next, tissues were washed twice in TBS for 15 minutes and mounted onto subbed slides. The slides were vacuum dehydrated overnight. Slides were then prepared for counterstaining by placing them in 70% ethanol for 20 minutes followed by 50% ethanol for 5 minutes. After a brief dip in dH2O, the sections were counterstained with thionin (1.25 g thionin, 100 ml of 1 N acetic acid, 18 ml 1 N NaOH, 382 ml dH2O). The sections were then placed in a series of ethanol solutions (50%, 70%, 95%, 100%) until differentiated and cleared with three 10 minute washes in xylene. The slides were then immediately coverslipped with DPX mounting medium and dried overnight.

Data Collection

After immunohistochemical processing, slides were viewed using a light microscope. In both the wild type and mutant, the presence of cilia was qualitatively examined, and various sections were drawn for comparison with the aid of a camera lucida under oil immersion (1000X magnification). All cilia visible in one field were drawn. Photographs of slides were taken using a digital camera.
Figure 1. Neuronal cilia from the piriform cortex of postnatal day 14 mice. Neuronal cilia are immunostained using AC3 (brown), and the cells are counterstained with thionin (blue). Cilia are reduced in number and length from the wildtype (A,C,E) to the mutant (B,D,F).
Figure 2. Neuronal cilia from the CA3 region of the hippocampus in postnatal day 14 mice. Cilia are reduced in number and length from the wildtype (A,C) to the mutant (B,D).
Figure 3. Neuronal cilia from the granular layer of the hippocampus in postnatal day 14 mice. Cilia are reduced in number and length from the wildtype (A,B,D) to the mutant (C,E).
DISCUSSION

The results of the immunohistochemistry were in part inconclusive, due to several obstacles which arose throughout the course of the immunological assays. For example, all initial attempts to localize immunoreactive cilia on either the wildtype postnatal day 7 or mutant postnatal day 7 mouse failed. Somatostatin-3 (sst3), a primary antibody used in previous experiments was then used in place of the AC3 antibody (13). Sst3 failed to produce results as well, including in the postnatal day 14 mice. Eventually the AC3 antibody was used again to successfully stain the postnatal day 14 mice. It was then inferred that a problem caused the postnatal day 7 mice to resist immunological staining. The cause of the resistance is unknown, although a problem with the fixation of the brain tissue is likely. Since the cilia of the postnatal day 7 mice could not be localized, some experimental goals were unreachable. The hypothesis that older mice have shorter cilia because the mutation affects cilia maintenance requires two different age sets and thus was not further explored.

The results obtained from the postnatal day 14 mice did reinforce the standard Tg737 mutant model. As previous experiments in the kidney and brain demonstrate, cilia in the postnatal day 14 mutant were significantly shorter than cilia in the postnatal day 14 wildtype across all regions. Due to time constraints, quantitative data could not be obtained regarding the exact decreases in cilia length and number. Qualitative assessment of cilia from a few areas of the brain, such as the hippocampus and piriform cortex, does support the hypothesis put forth that the mutation affects cilia in different brain regions with differing levels of severity. This suggests that the different ages and functions of neurons may affect how they grow and maintain cilia. Alternatively, some neurons may splice the Tg737 gene differently (14), leading to a difference in how severely the mutation affects the gene product. Lastly, it should be noted that
the pattern of cilia in the postnatal day 14 mouse brain does not entirely coincide with the pattern obtained in previous experiments using sst3 (15). This supports the theory that cilia in different regions contain different chemicals according to the function of different areas in the brain.

The effects of the Tg737<sup>orp</sup> mutation can be easily viewed in many organs. For example, the cysts of PKD are quite large. In the brain, morphological changes occur. Hydrocephaly is common, and, in the postnatal day 14 mutant mouse used, there was a marked change in the shape of the hippocampus. Unfortunately, determining the reason behind these morphological changes is more difficult than simply observing them. The overall purpose of neuronal cilia still remains unclear, although localizing cilia and measuring their changes is a step in the right direction. Further study may well elucidate their true function.
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REFERENCES


