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The effect of ethanol on three types of ependymal cilia in the brain lateral ventricle

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The Effect of Ethanol on Three Types of Ependymal Cilia in The Brain Lateral Ventricle

by

Alzahra Al Omran

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Master of Science Degree in

Pharmaceutical Sciences

Major in Pharmacology and Toxicology

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An Abstract of

The Effect of Ethanol on Three Types of Ependymal Cilia in The Brain Lateral Ventricle

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Ependymal cells are multiciliated cells that line the central canal of the brain ventricles and the spinal cord, contributing to cerebral spinal fluid (CSF) circulation. Motile cilia dysfunction can result in hydrocephalous due to the hindrance of CSF movement. Though, the cause of hydrocephalus has been intensively studied, we are the first to report three distinct groups of cilia responsible for the flow of CSF through the lateral ventricle. We classified the cilia based upon the beating frequency and movement angle into three types that localize in certain areas within the lateral ventricle. Moreover, we were able to localize *hydin,* a unique gene that is expressed in the central pair microtubules of motile cilia. Mutations in *hydin* are known to cause hydrocephalous. We also provide visual proof, through immunofluorescence, of polycystin-2 existing in the ependymal cilia. Our studies further show that ethanol can reduce the beating frequency of all three types, in both *ex-vivo* and *in-vivo* ethanol treatments.

Acknowledgements

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Table of Contents

List of Tables

List of Figures

Chapter 1

1. Introduction

1.1 Cilia

Cilia are microscopic organelles projecting from the apical surface of eukaryotic cells (Tissir, Qu et al. 2010, Jimenez, Dominguez-Pinos et al. 2014). The main structural components of the cilium are the basal body, which anchors the cilia onto the surface of the cell and the axoneme, which projects from the basal body into the lumen. The ultrastructure of the cilium axoneme is composed of nine doublet microtubules arranged obliquely and surrounded by a plasma membrane. Motile cilia consist of a more complex cyto-architecture. In addition to the nine doublet microtubules, they contain a single central microtubule, inner and outer dynein arms, and radial spokes that connect the peripheral microtubules to the central pair (Brooks and Wallingford 2014).

There are three types of mammalian cilia differentiated with regards to their microstructure and movement. The primary monocilia is characterized by the presence of nine microtubules and the absence of the central pair of microtubules (9+0 configuration). It is considered both, a mechanosensory and a chemosensory organelle

that is associated with the cell cycle and cell signaling (Abou Alaiwi, Lo et al. 2009, Tissir, Qu et al. 2010). The nodal cilia are monocilia with a 9+0 configuration like the primary cilia, but in contrast, they are motile displaying a rotatory motion as a result of having dynein arms. This motion is necessary to move morphogenetic cues within the embryonic node (Tissir, Qu et al. 2010, Brooks and Wallingford 2014). The third type of cilia is the motile cilia that appear in multiciliated cells beating in a synchronized manner. This later type of cilia will be the focus of our studies (**Figure 1**).

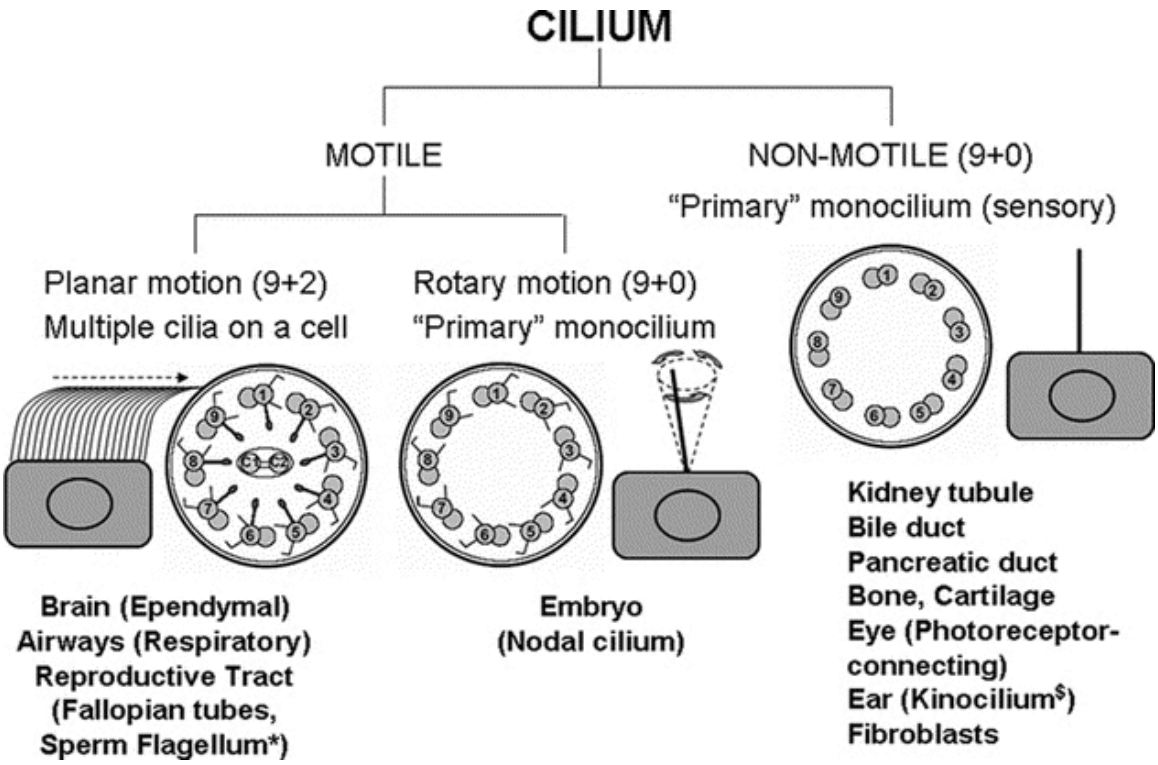

Figure 1. Different types of cilia

A cross-section of the ciliary axoneme ultrastructure divided into the two main types, non-motile sensory (primary cilia) 9+0 structure and motile consisting of planar motion 9+2 structure (motile cilia) and rotary motion 9+0 structure (nodal cilia). (Used

with permission from (Leigh, Pittman et al. 2009))

1.2. Motile cilia

The motility of cilia is essential for normal mammalian development and health. The motile ciliated cells are ubiquitous in the lung airways, the fallopian tubes in the uterus, the efferent ducts in the testes and sperm tail, as well as in the brain ventricles and spinal cord canal. A complex of large proteins forms the dynein domain, which is the core structure of the dynein arm and is responsible for motile cilia beating. The outer dynein arm is located closer to the boundary of the cilia while the inner dynein arm runs toward the central pair. By the sliding action of the outer and inner dynein arms, the doublets slide corresponding to each other and to the base of the axoneme, which cause the cilia to lean. The central pair, radial spokes, and dynein arms complex are fundamental components in cilia beating initiation and regulation. The motile cilia fluid dynamics have two stages, the power stroke where the cilia arc becoming horizontal to the cell surface, and the recovery stroke where the cilia reneges its initial upright position (Siyahhan, Knobloch et al. 2014).

There are several markers for the motile cilia each localized to specific areas. A mutation in one of these protein markers or altered localization is associated with motile cilia dysfunction.

Hydin is a hydrocephalous-inducing gene, that encodes for a central pair protein within the axoneme of motile cilia. A mutation in *hydin* leads to dislocation and eventual loss of the central pair, which disturbs the movement of cilia leading to lethal communication

hydrocephalous (Davy and Robinson 2003, Dawe, Shaw et al. 2007). *Hy3/hy3* mutant mice show early hydrocephalous onset and an enlargement of the lateral and third ventricles, as result of abnormal CSF flow caused by the impairment of cilia movement. The beating frequency of the ependymal cilia in the *hydin* mutant mice declines significantly due to changes in the location of the central pair microtubules leading to ineffective fluid movement. (Lechtreck, Delmotte et al. 2008).

Polycystin-2 (PKD2) is a membrane-associated protein known to be expressed in primary cilia that plays an important role in the mechanosensory and ciliogenesis functions of motile cilia (Jain, Pan et al. 2010). Polycystin-2 is also associated with sperm development and motility. *Pkd2* mutant Drosophila develops male sterility as a result of declining sperm movement (Kierszenbaum 2004). Polycystin-2 serves as a sensory protein that detects changes in fluid flow within the respiratory ciliated cells. Bronchiectasis and other respiratory diseases have been reported in polycystic kidney diseas e patient as a consequences of a mutation in PKD2 (Jain, Javidan-Nejad et al. 2012).

1.3. Cilia-related disease

Cilia are widespread in most mammalian cells, therefore any defect within the cilia structure or function could induce pathological phenomena. Mutations in any gene encoding for ciliary proteins may not only cause ciliogenic dysfunction and other cilia related manifestations, but it may also result in cellular pathophysiologies (Baker and Beales 2009). The ciliopathies in motile cilia differ from those resulting from primary

cilia structure perturbations. Anosmia, polycystic kidney disease, retinal degeneration, liver and pancreatic cyst formation are all phenotypes that result due to primary cilia dysfunction (Abou Alaiwi, Lo et al. 2009). On the other hand, primary ciliary dyskinesia (PCD), male infertility, and hydrocephalus are phenotypes that result from motile cilia dysfunction.

Primary ciliary dyskinesia is an autosomal-recessive respiratory disease resulting from mutations in the genes encoding for ciliary structural proteins. PCD is a heterogeneous disorder; there are upwards of 30 genes that may cause PCD according to the literature. These genes correlate with cilia microstructure such as central pair microtubules, radial spokes and inner or outer dynein arms. A mutation in one or more ultrastructural genes will generate defects in the ciliary formation, beating frequency, and pattern. As a result, there is an accumulation of mucus within the lung airway causing PCD patients to suffer from chronic and frequent respiratory infections (Lucas, Burgess et al. 2014).

Hydrocephalus is a combination of neurological and pathological manifestations characterized by an imbalance between the production and the absorption of cerebral spinal fluid (CSF), leading to an extreme accumulation of fluid in the brain ventricles. The brain ventricle cavities and subarachnoid spaces expanded in response to the increase in CSF (Banizs, Pike et al. 2005, Jimenez, Dominguez-Pinos et al. 2014). However hydrocephalus is classified into two types based upon the etiology, congenital and non-congenital. Congenital, also known as neonatal hydrocephalus, occurs due to genetic abnormalities in combination with other conditions such as cerebral hemorrhage,

radiation exposure during the fetal developmental phase, and pregnancy alcohol abuse (Jimenez, Dominguez-Pinos et al. 2014). Non-congenital hydrocephalus occurs after birth or at any point during adulthood as a result of an injury or infection of the brain ependyma. Moreover, hydrocephalus can also be divided into non-communicating or communicating among the CSF flow prospective. The non-communicating can be described as a blockage of the aqueduct that connects the ventricles preventing CSF circulation and causing ventriculomegaly. The communicating type, on the other hand, preserves the duct but has an increase in the production of CSF from the choroid plexus or a reduction in the reabsorption mechanism from the arachnoid spaces (Banizs, Pike et al. 2005).

The motile cilia dynamics deteriorate in hydrocephalic conditions for several reasons. First, any mutation within the essential proteins responsible for ciliogenesis or cilia ultrastructure may lead to a disruption in cilia motility. Study shows that the destruction of motile cilia microstructure by a mutation in *Mdnah5*, which encodes for the inner dynein arm, will impair cilia motility (Ibanez-Tallon, Pagenstecher et al. 2004). Furthermore, as a result of the increased cerebral pressure and dilatation of the ventricles, detachment of the ependymal cell layer may take place. Since ependymal cells do not proliferate at any developmental age, a gap could form in the ependymal layer as a consequence of ventricular expansion (Sarnat 1995).

1.4. Ependymal cells and cerebrospinal fluid

Ependyma is described as a solo-layer of multiciliated glial cells acquired from the

neuroepithelium, which coats the brain ventricles serving as a barrier between the brain parenchyma and brain ventricles. Each ependymal cell has around 16 motile cilia that are relatively 13 μm in length (Jimenez, Dominguez-Pinos et al. 2014, Siyahhan, Knobloch et al. 2014).

The ependymal cilia help distribute and circulate the CSF from its original source, the choroid plexus located in the lateral ventricle and the third ventricle. The CSF flows in a unilateral direction starting in the lateral ventricle, then moving through the third ventricle, then the fourth where it passes into the subarachnoid space covering the brain and spinal cord. The direction of the CSF flow depends upon the ependymal cell planar polarity orientation, which is dependent upon the primary cilia. The location of the basal body during ciliogenesis controls the orientation of the cilium microtubule of the motile cilia (Jimenez, Dominguez-Pinos et al. 2014).

CSF is a transparent fluid with water like viscosity and an electrolyte composition similar to that of blood plasma; however, there are excessively lower concentrations of glucose, protein, and other nutrients. The CSF plays an important role in the physical protection of the brain; it serves as cushioning that insulates the brain from the solid skull. It works as a detoxification system that clears and dilutes the waste and toxins from the brain. Also, during brain development, in the embryonic stage, the CSF transfers signals for neuronal cell proliferation and migration (Hladky and Barrand 2014). Recent studies show that CSF is a good tool for the early diagnosis of Alzheimer's disease. Protein metabolism levels vary among Alzheimer's patients; the CSF however can be tested for

the reduction of amyloid-β 42 levels about 10-15 years before the onset of dementia (Lehtinen, Bjornsson et al. 2013).

1.5. Pharmacological manipulations of motile cilia

There are numerous studies examining the effects of different pharmacological reagents on ependymal cilia movement, which could affect the role of cilia in brain viability.

Alcohol consumption has been reported to reduce respiratory performance due to its significant effect on mucociliary clearance. However, research shows that the beating frequency of cilia in the airway increases after exposure to alcohol as a result of an increase in the release of nitric oxide (Sisson 1995). The increase of the ciliary beating takes place when the dose is within the clinically approved limit; the beating decreases when the dose is above 1000 mM (Sisson 1995). On the other hand, a recent study conducted on *Chlamydomonas* cilia revealed that ethanol diminishes cilia motility and beating through a distinct pathway. It altered the phosphorylation of docking complex component (DCC1), an important protein in the outer dynein arm of the cilia (Yang, Pavlik et al. 2015). Previous studies from our lab had investigated the dynamics of ependymal cilia, in the third ventricle, and found a decrease in beating among the cilia of the third ventricle after ethanol incubation *ex vivo* (Liu, Jin et al. 2014).

Furthermore, various studies have investigated the role of acetylcholine on cilia motility at the cellular level and in the trachea. Acetylcholine augments the cilia movement by boosting the endogenous levels of cGMP and cAMP (Zagoory, Braiman et al. 2002).

Changes in ependymal cell structure occur after treatment with galactocerebrosides, the main glycolipid products of myelin, the biomarker in demyelination disease, indicating a disruption in the ependymal physiological function (Laabich, Graff et al. 1991).

Chapter 2

Materials and Methods

The Institutional Animal Care and Use Committee (IACUC) of The University of Toledo approved all of the procedures for animal use in accordance with the guidelines of the Institutional Animal Care and Use Committee at the National Institutes of Health and the Guide for the Care and Use of Laboratory Animals.

2.1. Live imaging

To conduct an ependymal cilia live imaging experiment, a wild type mouse strain C57BL/6 was euthanized by asphyxiation using a CO2 gas chamber for five minutes. Cervical dislocation was used as a second method of euthanasia to confirm death. A craniotomy was performed to collect the whole brain. A thin sagittal plane section was obtained and immediately placed in a glass-bottom petri dish, containing 37 ℃ Dulbecco's Modified Eagle Medium (DMEM)/High-Glucose (HyClone Inc.), 10% fetal bovine serum (FBS) (Fisher Scientific) (and 1% penicillin/streptomycin solution containing 10,000 units/mL of penicillin and 10,000 µg/mL of streptomycin. To provide the fresh live tissue section with the essential environment to survive, the microscope's enclosed chamber temperature had been adjusted to 37 °C with a gas mixture of 95%/5%

O2/CO2. Next, the cilia movement was recorded using a 60X objective oil immersion lens and a differential interference contrast (DIC) filter. Metamorph imaging software was used to adjust the camera to 1x1 binning with a 5-10 msec exposure time.

2.2. Beating frequency measurement

After recording the live cilia movement, each movie is analyzed by counting the number of cilia beating per one second; the power and recovery stroke is counted as one complete beat cycle. In order to accurately estimate the number of beats, the movie may be slowed to a manageable speed while using a cell counter to count the number of beatings. Each movie was recorded at 5-millisecond exposure time with at least 200 frames per second.

2.3. Determination of different types of cilia within the lateral ventricle

To determine the specific location of each type of cilia and their relation to each other, a video for each type was recorded, marking the region of each video within the ventricle. After recording and analyzing the videos, counting the number of beats per second and measuring the angle of movement determined the type of cilia. Each video is related to its area in the ventricle. Subsequently a map showing the distribution of the cilia types in the ventricle walls is drawn.

2.4. Immunofluorescence microscopy

The brain section was fixed with a phosphate buffered saline solution containing 4% paraformaldehyde (PFA) (Electron Microscopy Science Lab) and 2% sucrose (Sigma

Inc.) for 10 min. Alternatively, the whole brain can be fixed with 4% PFA and then sectioned into 60 μm sections using a cryostat. The brain slice was incubated with a solution of 0.1% Triton-X (Fisher Scientific) in 1X PBS for 5 minutes. Mouse primary antibody, anti-acetylated α-tubulin (Sigma, Inc.), anti-hydin (Novus Biologicals) or anti-polycystin 2 (Santa Cruz Biotechnology) was used at a dilution of 1:5,000, 1:30, and 1:250, respectively in a 10% FBS in 1X PBS solution for one hour at room temperature (RT) or overnight at 4 °C. The brain slice was then incubated in the secondary antibody, fluorescein anti-mouse IgG or texas-red anti-rabbit IgG (Vector Lab), at a dilution of 1:500 in a 10% FBS in 1X PBS solution for 1 hour at RT. Before observation under a fluorescent microscope, the section was counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Vector Lab) for 5 minutes to stain the nucleus/DNA. To minimize photobleaching, the sections were imaged immediately with the minimum exposure time possible.

2.5. *Ex-vivo* ethanol treatment

After dissection, a thin sagittal plane section of the mouse brain was obtained and incubated in 1 ml 37℃ DMEM/High-Glucose media. Then, the videos of the beating cilia were captured at an exposure rate of 5 milliseconds for at least 1 second (200 frames per second). To investigate the effect of ethanol in the ependymal cilia, the same section was incubated in 0.25% ethanol (190 proof) (Decon Lab) in 37℃ DMEM/High-Glucose media for 5 minutes. The cilia beating were recorded for the same areas that were recorded before ethanol addition. The movies were analyzed by counting the ciliary beating frequency for both the control and the ethanol-treated tissue.

2.6. *In-vivo* ethanol treatment

Two-month-old Wistar rats were received from Harlan Laboratories Company and housed in the Department of Laboratory Animal Resources at the University of Toledo. All animals were kept in standard plastic tube cage with free access to food and water. The animal's room temperature and relative humidity was maintained at 25℃ and 50%, respectively with 12-hour light/dark cycle. The body weight was monitored daily for the rats under treatment.

The animals were separated into two different groups. The control group, totaling six rats, was treated with water by oral gavage for seven days. The rats were deprived of food for two hours prior to gavage. The treatment group of six rats was given 95% ethanol, the equivalent of 190 proof, by oral gavage. The dose of ethanol is 6g per kg of body weight divided by the density of ethanol and diluted in DI water to the total volume of 3 ml.

2.7. Statistics

All the images and movies were analyzed using Metamorph software. All quantitative data were displayed as mean ± SEM. Statistical analysis using student t-test was performed to compare the effect of ethanol on the dynamics of ependymal cilia within the ventricles between ethanol-tretaed and control groups.

All statistical results were considered significant at a significance level of $p<0.05$.

Chapter 3

Results

3.1 Live imaging of ependymal cilia

We developed a novel technique to allow close observation of the movement of ependymal cilia within the brain ventricles. By obtaining a sagittal plane section from the mouse brain, we can expose the ependymal cilia with minimum reduction in vitality and image by using a high-resolution DIC microscopy system producing live images and movies **(Figure 2)**.

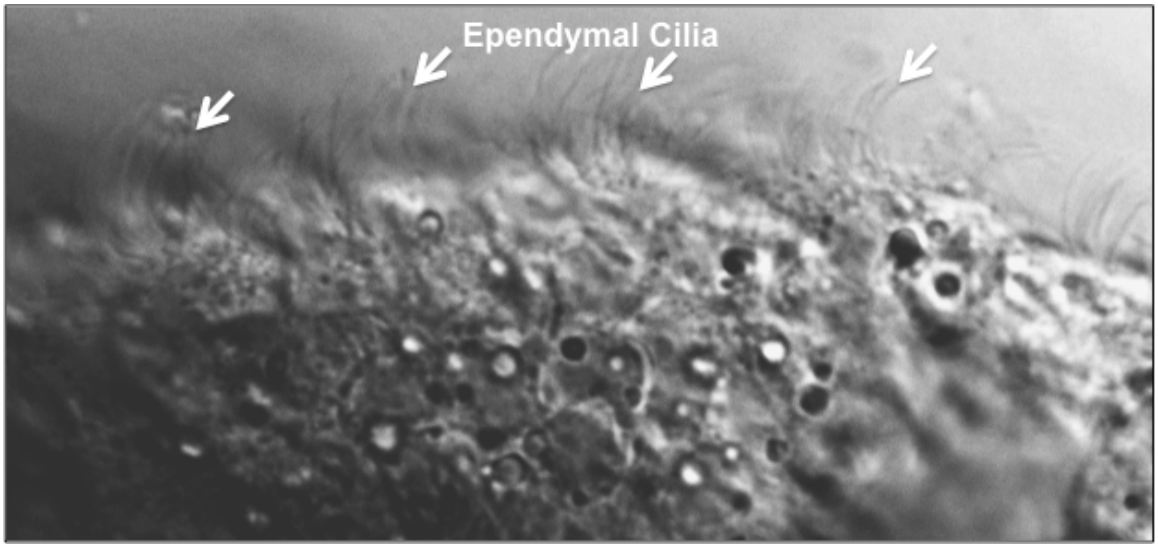

Figure 2. DIC image of the ependymal cells with the motile cilia.
Shown here are the ependymal cilia of the mouse lateral ventricle taken from a live imaging experiment.

3.2. Classification of the ependymal cilia

Through analysis of the live movies of the lateral ventricle cilia beating, we noticed remarkable variations in their beating frequencies. This guided us to classify the cilia into three distinct types with regards to their beating frequency, movement angle and pattern **(Figure 3)**. Type I cilia beat the fastest at >60 Hz and have the least volume replacement stroke with a beating angle of <90∘. On the other hand, the beating frequency of type II cilia was 30-60 Hz with a beating angle between 90∘ and 135∘, while type III cilia have the slowest beating frequency of <30 Hz but the largest volume replacement stroke with a beating angle of >135∘ **(Table 1) (Movies 1, 2 and 3).** The ciliary beating in the lateral ventricle mimics that of the cilia within the third ventricle, which has also been studied in previous work from our lab (Liu, Jin et al. 2014). Worth mentioning is that the three types of ependymal cilia are located in specific locations within the lateral ventricle **(Figure 4).**

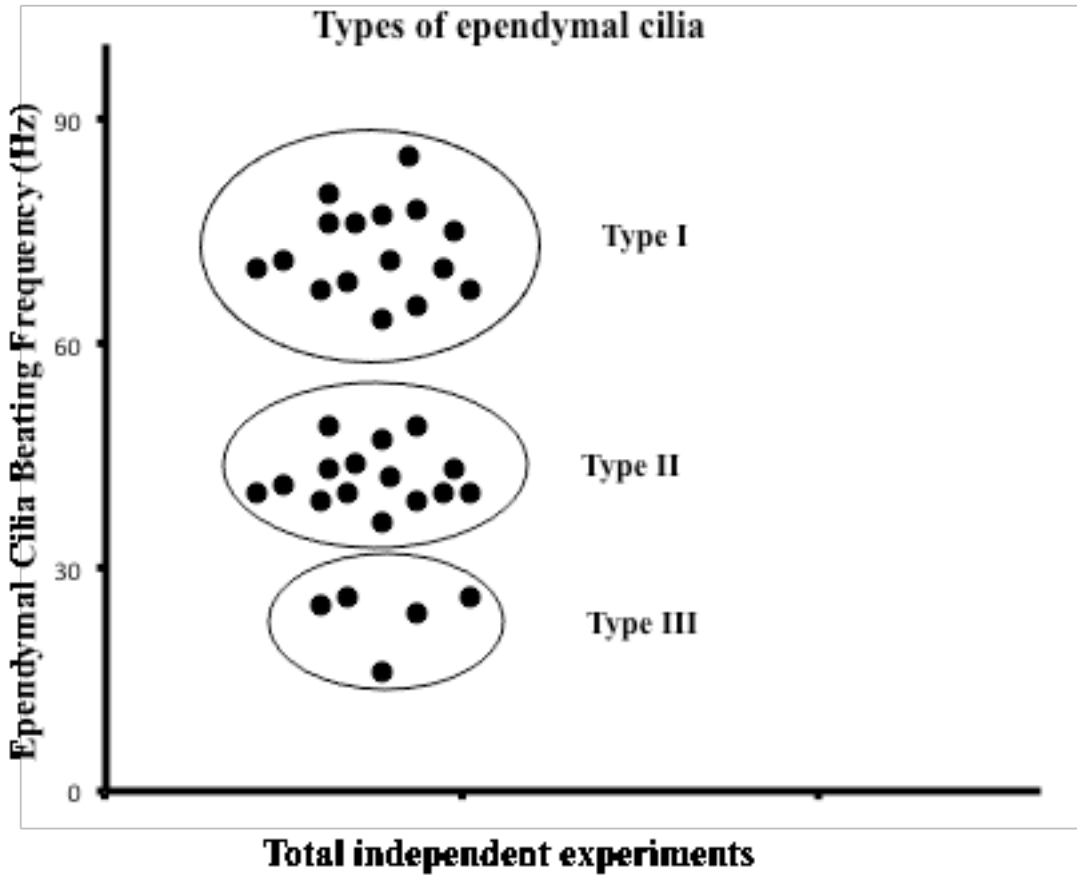

Figure 3. Types of ependymal cells in the lateral ventricle

Classification of ependymal cilia is based upon their beating frequency and angle. Each dot represents an independent experiment. Type I cilia are the fastest and has a beating frequency >60 Hz with a beating angle less than 90∘. Type II beating frequency is between 30-60 Hz with a beating angle between 90∘ and 135∘. Type III cilia have the slowest beating frequency <30 Hz and a beating angle > 135∘.

Ependymal Cells Types	Type I	Type II	Type III
Cilia beating frequency	> 60 Hz	30-60 Hz	< 30 Hz
Cilia beating angle	< 90°	90°-135°	> 135°

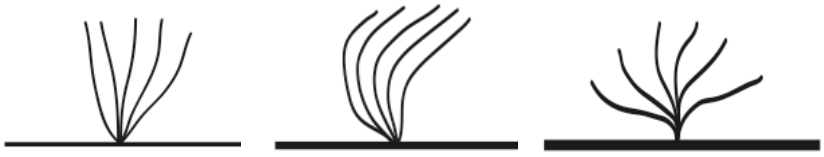

Table 1. Classification of ependymal cilia in the lateral ventricle based on their beating frequency and beating angle

This table displays the values we used for categorization of the ependymal cilia into three different types, and the respective figures below each cilia type that demonstrates the pattern of movement to each type.

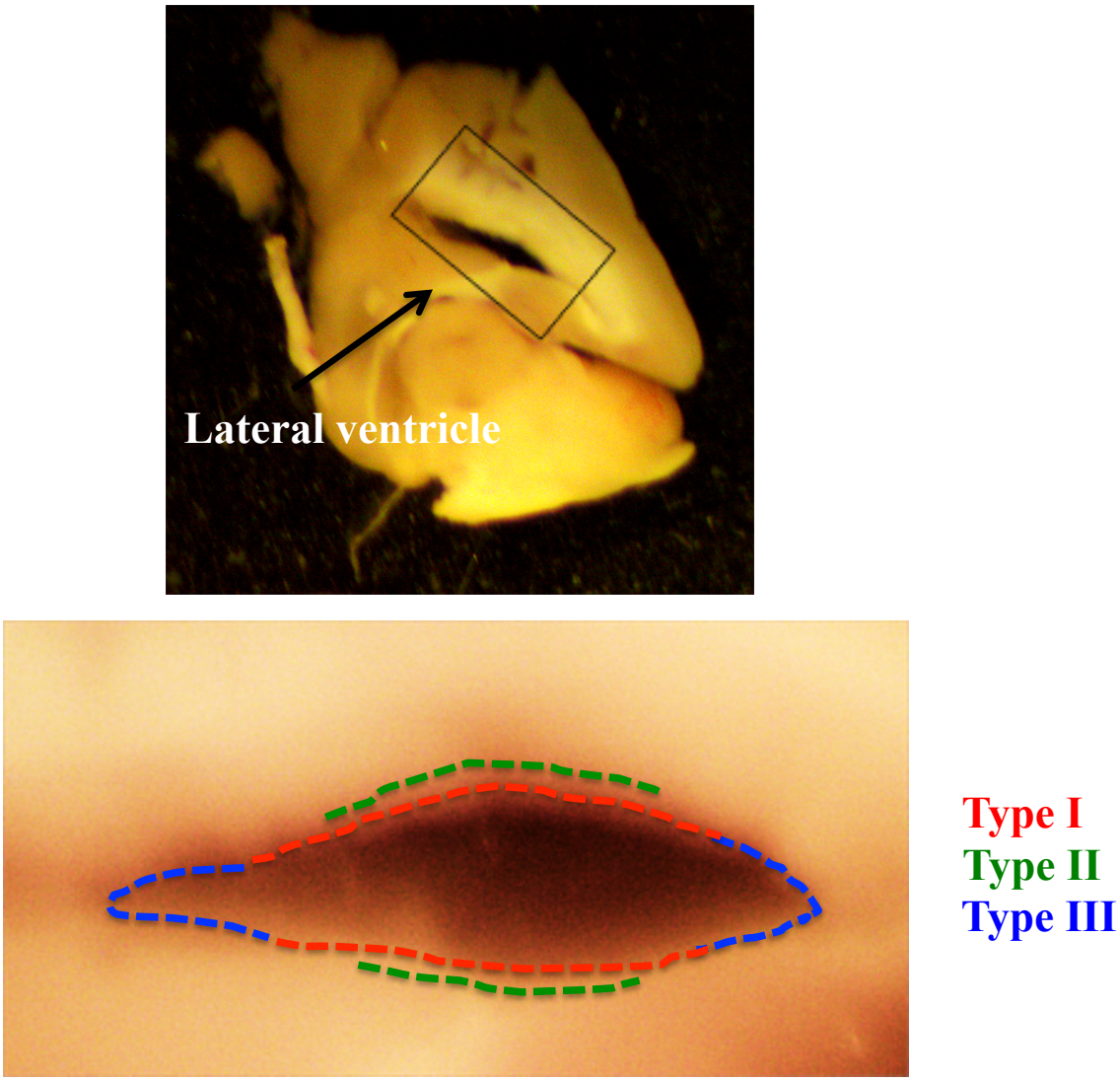

Figure 4. The differential localization of each type of ependymal cilia in the lateral ventricle

Each type of the ependymal cilia is localized in certain area in the lateral ventricle based on the beating frequencies and angle of movement. This figure shows a sagittal section view of the lateral ventricle.

3.3. Motile Cilia markers

In an effort to explore the mechanism or the structural differences that could explain the variation in the movement and beating pattern among the three types of the ependymal cilia, the ciliary localization of several key structural proteins that might account for the differences between the types of cilia was investigated for the first time. We were able to demonstrate the ciliary localization of the motile cilia protein, *Hydin* in both the lateral and the third ventricles. *Hydin* ciliary localization was also confirmed by co-staining with the ciliary marker acetylated-α-tubulin **(Figure 5a, 5b and 6).**

Hydin presented as a good candidate for a cilia marker to distinguish between the three types of cilia since it is located in the central pair of the motile cilia. Eventually, we were able to localize *hydin* in the ependymal cilia for the first time by immunofluorescence microscopy in the lateral ventricle **(Figure 7).**

Furthermore, since Polycystin-2 is expressed in the motile cilia of the airways, this suggested to us that it may also be expressed in the motile cilia of the ependymal cells (Jain, Javidan-Nejad et al. 2012). We were able to localize Polycystin-2 in the lateral ventricle by immunofluorescence **(Figure 8).**

Hydin and Polycystin-2 were localized in all of the three types of the ependymal cilia. Future studies will be conducted to further examine other motile cilia proteins that might be specific to each type of cilia. For example, CCDC115, CCDC114, and ARMC4, which are encode for outer dynein arm protein (Hjeij, Onoufriadis et al. 2014).

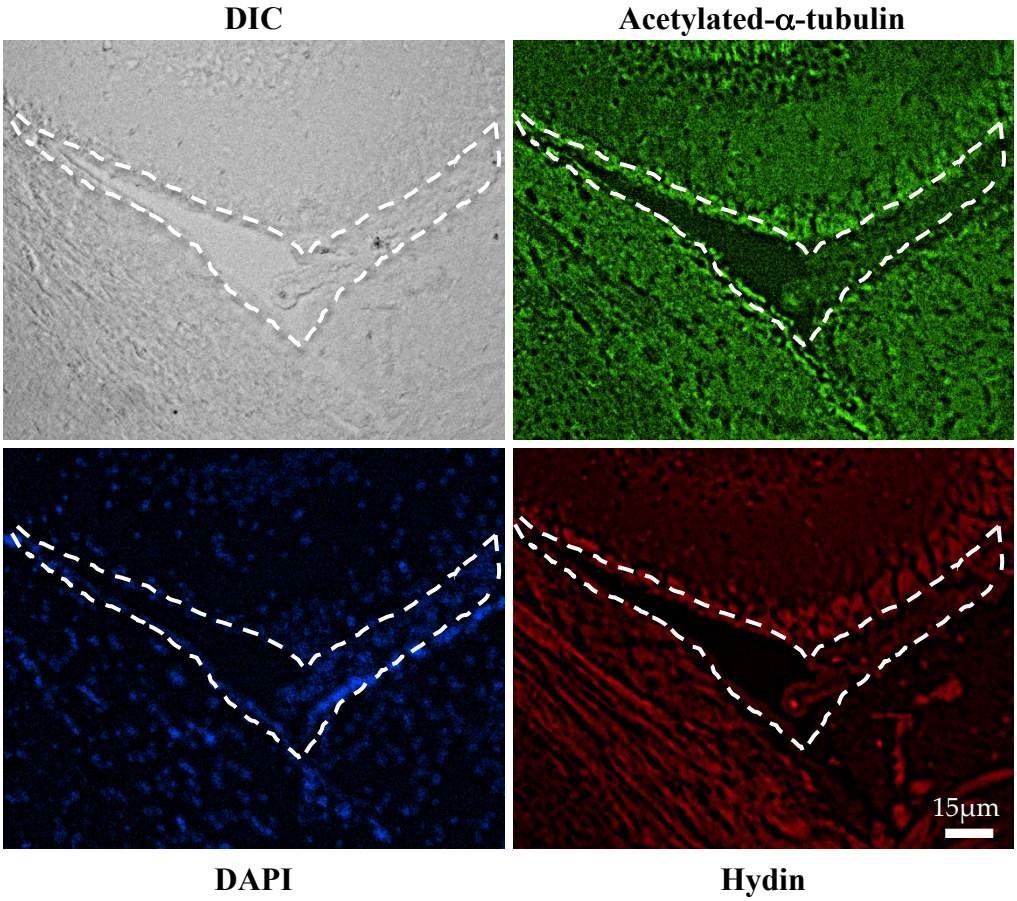

Figure 5a. Stained lateral ventricle section of the mouse brain

A section of the lateral ventricle of mouse brain marked with white dashed line. The section is presented in DIC and stained with anti-acetylated-α-tubulin (green), anti-*hydin* (red), and counter-stained with DAPI, the nuclear marker (blue) at low magnification (10x).

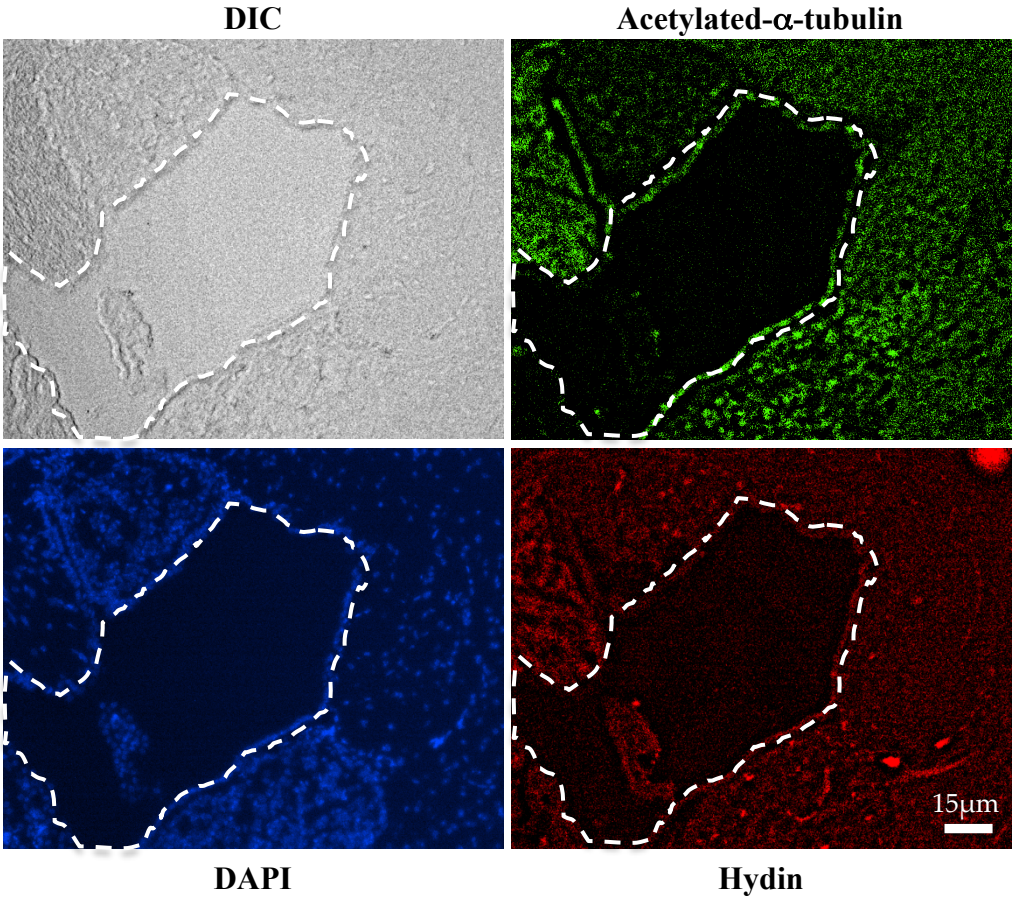

Figure 5b. Stained third ventricle section of the mouse brain

A section of the third ventricle of mouse brain marked with white dashed line. The section is presented in DIC and stained with anti-acetylated-α-tubulin (green), anti-*hydin* (red), and counter-stained with DAPI, the nuclear marker (blue) at low magnification (10x)

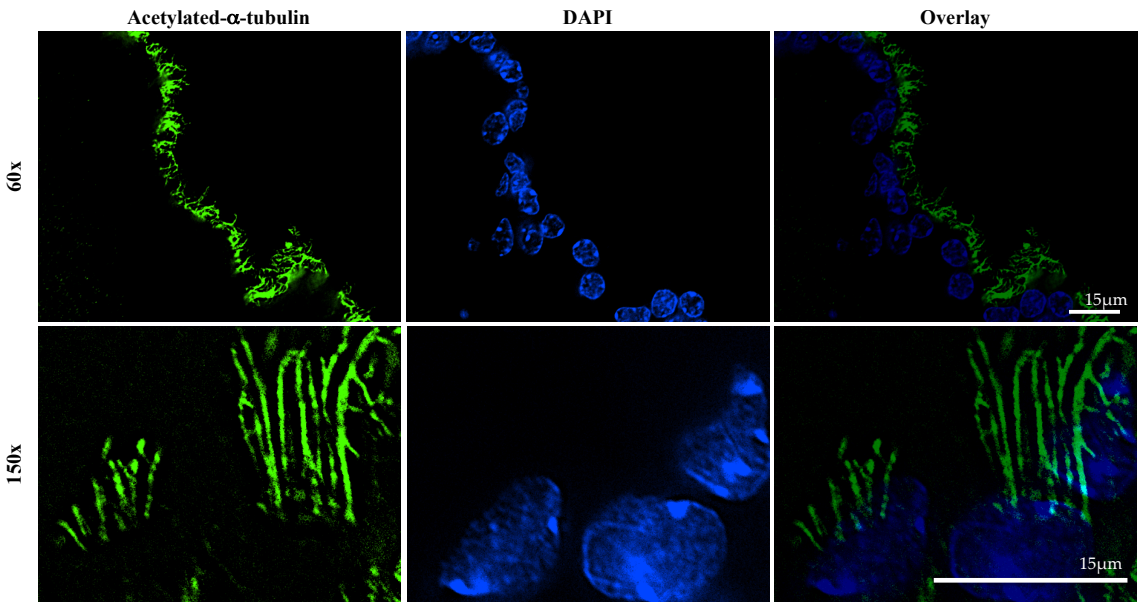

Figure 6. Immunohistological section of the brain ependymal cilia

Ependymal cells of mouse brain lining the lateral ventricle are stained with anti-acetylated-α-tubulin (green) and counter-stained with DAPI, the nuclear marker (blue) at high magnifications.

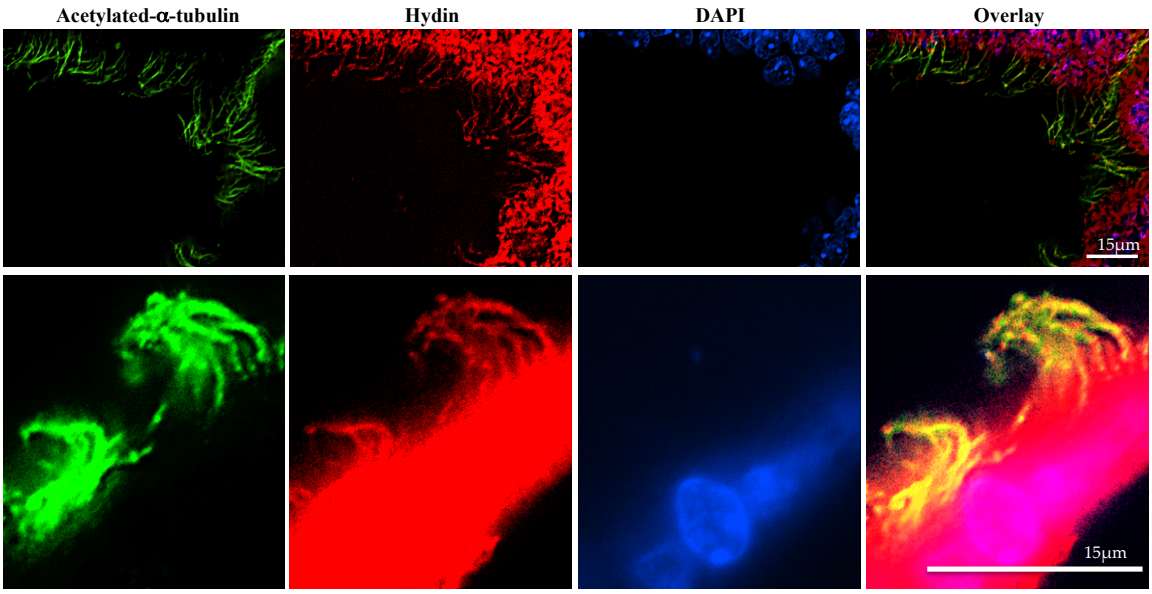

Figure 7. Immunohistochemical detection of hydin in the ependymal cilia of mouse brain

This figure shows the ciliary co-localization of hydin (red) with the cilia marker acetylated-α-tubulin (green); both extend throughout the cilia body. The section is counterstained with the nuclear marker, DAPI (blue).

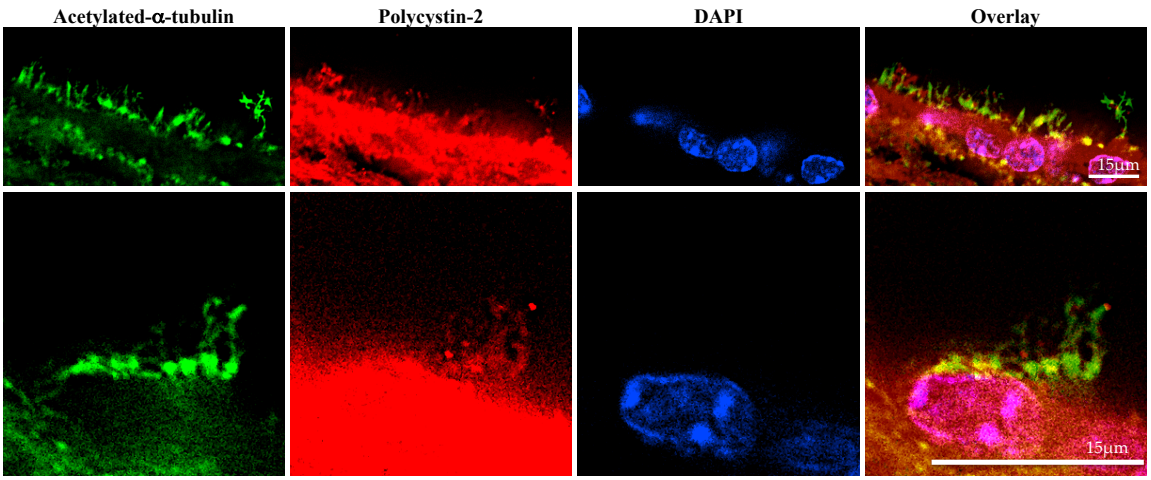

Figure 8. Immunohistochemical detection of Polycystin-2 in the ependymal cilia of mouse brain

Co-localization of Polycystin-2 (red) with the ciliary marker, acetylated-α-tubulin (green) in the ependymal cilia of the brain lateral ventricle. The ependymal cells were also counterstained with the nuclear marker, DAPI (blue)

3.4. The *ex-vivo* effect of ethanol on the ependymal cilia beating

The *ex-vivo* alcohol experiment was performed to examine the accuracy of the ependymal cells classification, as well as to investigate whether the effect of ethanol is consistent within all three types. In addition, it enabled close observation of the effect of alcohol consumption on the ependymal cilia in controlled environment settings. Our data from the *ex-vivo* experiments indicate that there is a significant decline in cilia motility after incubating the brain section in 0.25% ethanol for 5 minuets. By analyzing and comparing the movement of the three types of cilia before and after the ethanol treatment, we found a reduction in cilia movement occurring in all three types **(Figure 9) (Movies 1-6).**

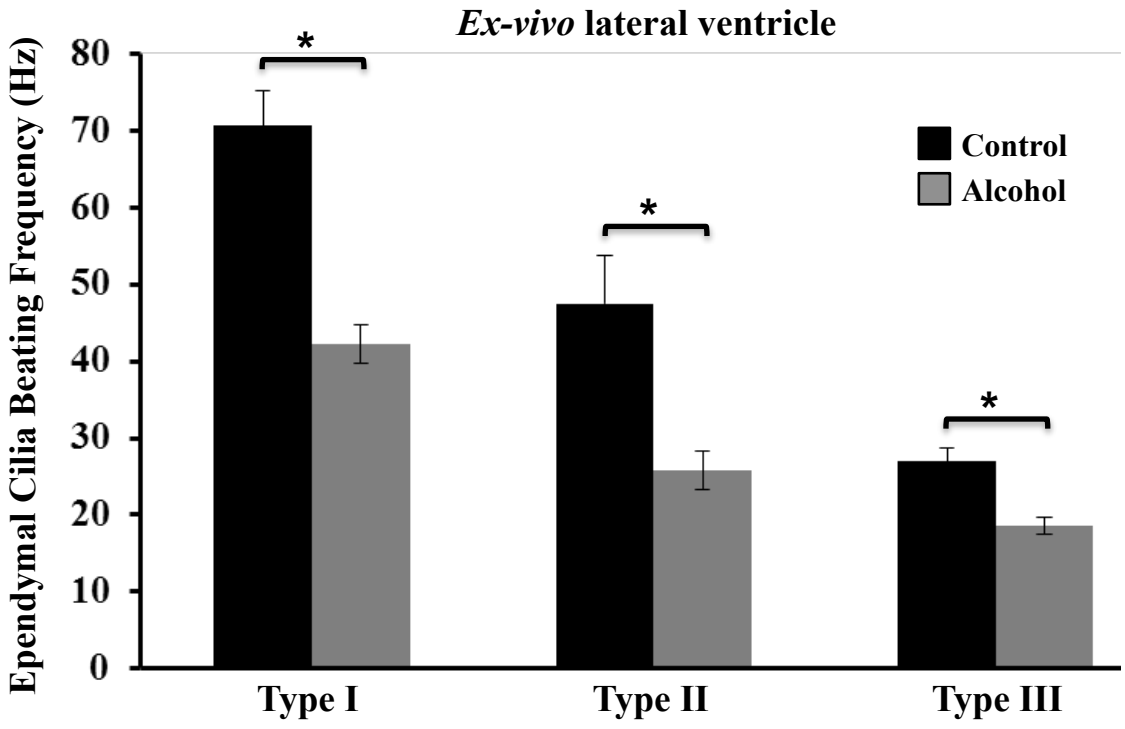

Figure 9. The effect of ethanol on the three types of ependymal cilia

Alcohol treatment in mice with 0.25% shows a significant reduction in the beating frequency of all three types of ependymal cilia. Up to 22 independent preparations were used in this data and an asterisk (*) was placed to denote significant difference at $p<0.05$.

3.5. The *in-vivo* effect of ethanol on the ependymal cilia beating

After our observation of the reduction in ependymal cilia beating frequency in the *ex-vivo* alcohol treatments in mice, the effects were then examined *in-vivo* to confirm that the observations made in the previous studies are physiologically relevant. Our *in-vivo* data clearly demonstrated a significant decrease in the ependymal cilia dynamics after chronic treatment with ethanol in rats, which is supported by our previous findings **(Figures 10a**

and 10b). Both, the lateral and the third ventricles were studied confirming that cilia motility within both ventricles was affected by alcohol drinking in the same manner **(Figure 10c and Movies 7-12).**

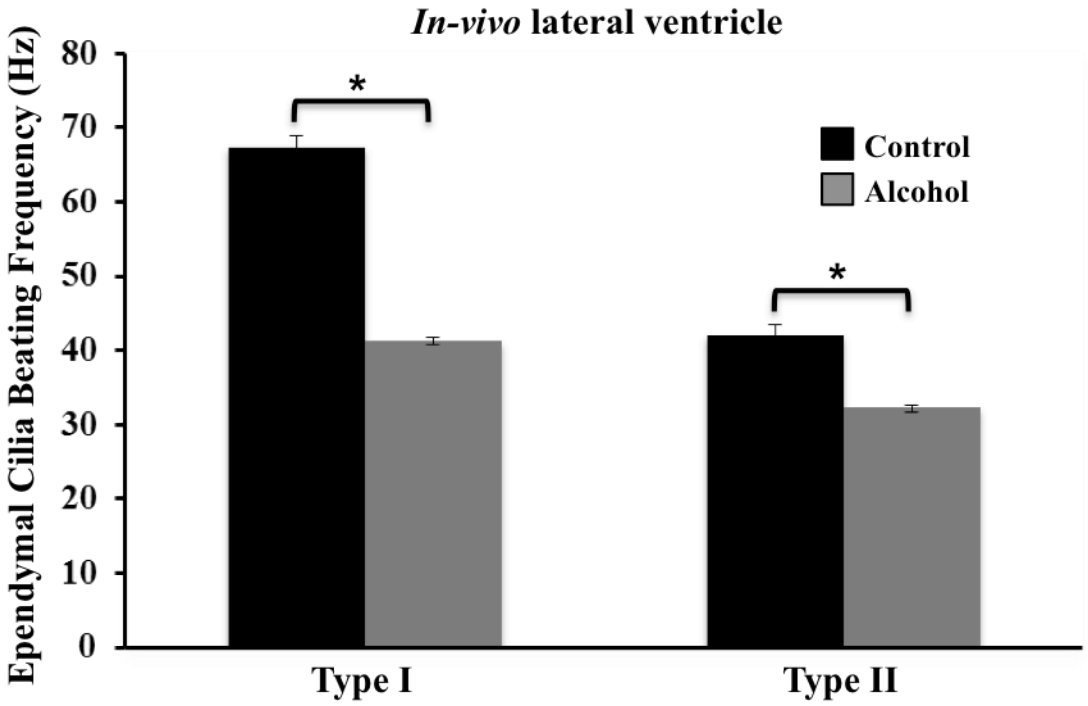

Figure 10a. The effect of ethanol on the lateral ventricle ependymal cilia

After oral acute treatment with alcohol, the rat's brain was dissected to examine the dynamics of ependymal cilia. The results showed a significant decrease in the beating of the cilia of the lateral ventricle in alcohol-treated rats compared to the control group.

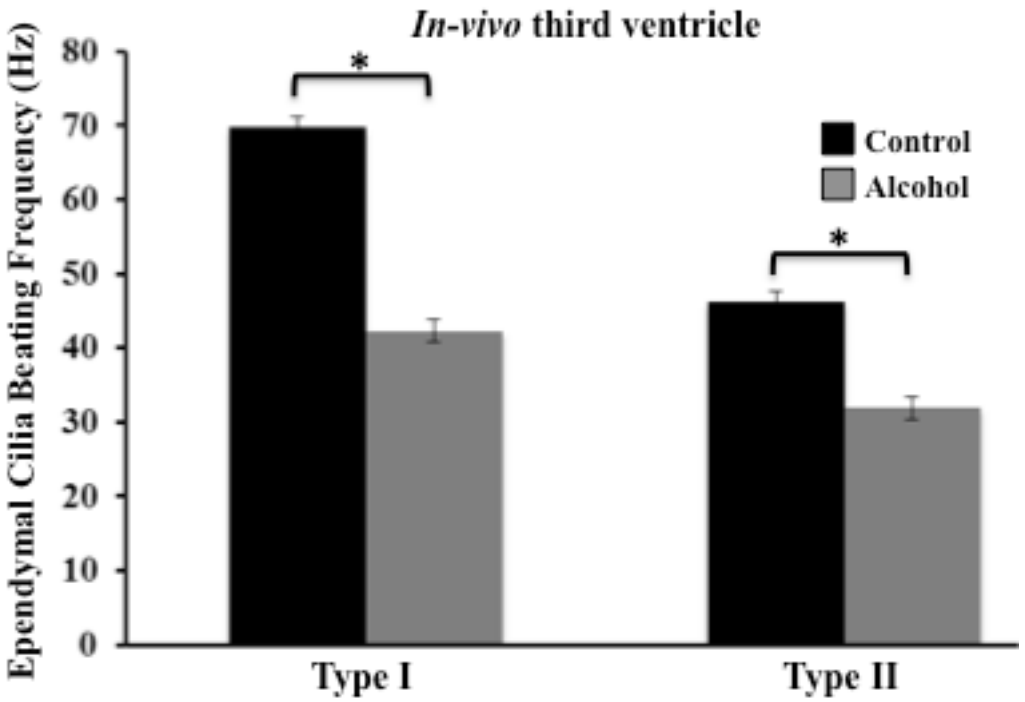

Figure 10b. The effect of ethanol on the third ventricle ependymal cilia

After acute oral treatment with alcohol, the rat's brain was dissected to examine the dynamics of the ependymal cilia. The results showed a significant decrease in the beating of the cilia of the third ventricle in alcohol-treated rats compared to the control group.

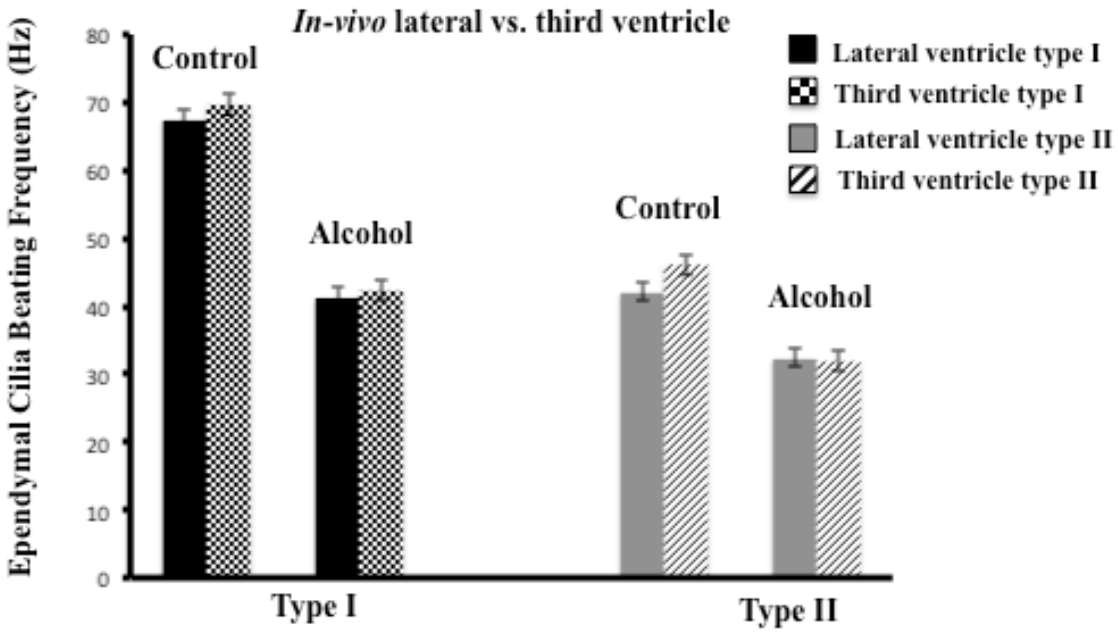

Figure 10c. Comparison between the dynamics of the ependymal cilia in the lateral ventricle and the third ventricle

We compared the difference in the effect of ethanol in rats on the beating frequency of the ependymal cilia in the lateral ventricle and third ventricle. No significant difference was found between the ciliary beating frequency in the lateral and third ventricle within the control group and the ethanol-treated group.

Chapter 4

Discussion

We report, for the first time, the classification of the ependymal cilia in the mouse brain lateral ventricle confirming a previous study from our laboratory on the classification of the ependymal cilia in the third ventricle (Liu, Jin et al. 2014). The types of cilia are distributed equally throughout the lateral ventricle to maintain efficient circulation of the CSF. The ependymal cilia is divided into three distinct types in regards to their beating frequency and beating angle; type I has a beating frequency greater than 60Hz and a movement angle domain of less than 90°, type II has a beating frequency between 30-60 Hz and an angle between 90°-135°, and type III has a beating frequency less than 30 Hz and an angle greater than 135°. Moreover, the three types vary in the velocity and volume of the fluid movement due to the differences in the beating pattern and speed.

As reported in this study, our classification of ependymal cilia is based on differences within the pattern of movement and velocity of beating. We attempted to explain the differences in the beating frequencies and angles with regards to the ultrastructure of the

three types by labeling structural proteins that may vary in expression between the individual cilia types. We were able to localize *hydin* for the first time throughout the entire axoneme of the cilia. Localization of *hydin* in the brain ventricle could in the diagnosis and the treatment of hydrocephalus, which is considered one of the most common birth defects (Clewell 1988). Furthermore, we were able to localize Polycystin-2 in the ependymal, which were detected in all of three cilia types.

There are several studies that introduce advanced methods in performing and analyzing high speed digital imaging of ependymal cilia (Lechtreck, Sanderson et al. 2009, O'Callaghan, Sikand et al. 2012) however, classification of ependymal cells has not been identified before. Several chemicals such as ethanol and its metabolite, acetaldehyde, were reported to either slightly increase the beating frequency or have no effect on motile cilia in concentrations between 0.1% and 1% (Sisson 1995, Smith, Radhakrishnan et al. 2013). Our study of *ex-vivo* treated ependymal cilia tissue with a minimum concentration of 0.25% ethanol reveled that alcohol causes a significant decrease in the ciliary beating frequency. The classification of cilia enabled the detection of the effect of ethanol on ciliary beating frequency, which was not possible before each type was analyzed separately.

As previously mentioned, the disruption of ependymal cilia is one of the main reasons for ventricle enlargement in hydrocephalous. Chronic alcohol consumption leads to numerous destructive effects on the brain, it could cause an increase in size of the brain ventricle by 31%-71%, the hallmark of hydrocephalus, (de la Monte 1988, Zahr, Mayer

et al. 2014). Several studies have been conducted to investigate the effects of alcohol on the brain in cases of chronic alcoholism; however, none have focused on the ependymal cilia in particular. In our study, we confirmed our findings of the reduction in ciliary beating frequency from the *ex-vivo* ethanol treatment. We found, for the first time, that ciliary beating frequency *in-vivo* showed a significant decrease, revealing a decrease in ciliary performance. This could potentially provide an explanation for the occurrence of hydrocephalus in patients suffering from acute alcoholism.

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Appendix A

"Published in The Journal of Visualized Experiments"

Live imaging of the ependymal cilia in the lateral ventricles of the mouse brain

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KEYWORDS
Motile cilia, lateral ventricle, cerebrospinal fluid, live imaging, hydrocephalus.

SHORT ABSTRACT
Using high-resolution differential interference contrast (DIC) microscopy, an *ex-vivo* observation of the beating of motile ependymal cilia located within the mouse brain ventricles is demonstrated by live-imaging. The technique allows a recording of the unique ciliary beating frequency and beating angle as well as their intracellular calcium oscillation pacing properties.

LONG ABSTRACT
Multiciliated ependymal cells line the ventricles in the adult brain. Abnormal function or structure of ependymal cilia is associated with various neurological deficits. The current *ex vivo* live imaging of motile ependymal cilia technique allows for a detailed study of ciliary dynamics following several steps. These steps include: mice euthanasia with carbon dioxide according to protocols of The University of Toledo's Institutional Animal Care and Use Committee (IACUC); craniectomy followed by brain removal and sagittal brain dissection with a vibratome to obtain very thin sections through the brain lateral ventricles, where the ependymal cilia can be visualized. Incubation of the brain's slices in a customized glass-bottom plate containing Dulbecco's Modified Eagle's Medium

(DMEM) /High-Glucose at 37 °C in the presence of 95%/5% O_2/CO_2 mixture is essential to keep the tissue alive during the experiment. A video of the cilia beating is then recorded using a high-resolution differential interference contrast microscope. The video is then analyzed frame by frame to calculate the ciliary beating frequency. This allows distinct classification of the ependymal cells into three categories or types based on their ciliary beating frequency and angle. Furthermore, this technique allows the use of high-speed fluorescence imaging analysis to characterize the unique intracellular calcium oscillation properties of ependymal cells as well as the effect of pharmacological agents on the calcium oscillations and the ciliary beating frequency. In addition, this technique is suitable for immunofluorescence imaging for ciliary structure and ciliary protein localization studies. This is particularly important in disease diagnosis and phenotype studies. The main limitation of the technique is attributed to the decrease in live motile cilia movement as the brain tissue starts to die.

INTRODUCTION

Cilia are sensory microtubule-based organelles that extend from the cell surface to the extracellular environment. Depending on their microtubule organization, cilia can be categorized into two types - “9+0” or “9+2”. Functionally, based on their motility, these can be classed as motile or non-motile cilia (Abou Alaiwi, Lo et al. 2009). Primary cilia is a term commonly used to denote “9+0” non-motile cilia. These have nine parallel doublet microtubules (denoted by ‘9’) and a central pair of microtubules is absent within the central sheath (denoted by ‘0’). However, some “9+0” cilia, such as nodal cilia, which regulate embryo laterality are motile (Nonaka, Tanaka et al. 1998). On the other hand, motile cilia are characterized, in addition to the nine parallel microtubule doublets, by an additional central pair of microtubule doublets and associated with dynein motor proteins to facilitate motility. In addition, some “9+2” cilia such as olfactory cilia are non-motile (Satir and Christensen 2007). Ependymal cells lining the brain ventricles and the central canal of the spinal cord are characterized by motile cilia that propel the cerebrospinal fluid (CSF) along the brain ventricles (Delbigio 1995).

The overall goal of this method is to facilitate studying the motile cilia dynamics and structural abnormalities. The brain’s health and development heavily depend on efficient circulation of CSF within the brain ventricles. For instance, normal CSF flow and fluid balance require normal beating and functional ependymal cilia (Genzen, Platel et al. 2009, Appelbe, Bollman et al. 2013), which in turn play critical roles in regulating the directional movement of neuronal cells and stem cell migration _ENREF_7(Sawamoto, Wichterle et al. 2006) _ENREF_7. As such, abnormal ependymal cilia function or structure can lead to abnormal CSF flow, which is associated with hydrocephalus, a medical condition in which there is an abnormal accumulation of CSF in the ventricles of the brain. This may consequently cause increased intracranial pressure and progressive

enlargement of the head, convulsion, tunnel vision, and mental disability (Banizs, Pike et al. 2005).

The advantages of this technique over existing methods is that it allowed for the first time to report three distinct ependymal cell types: I, II, and III, based on their unique ciliary beating frequency and beating angle. These ependymal cells are localized within certain regions in the brain ventricles. Furthermore, the effects of age and pharmacological agents such as alcohol and cilostazol on altering the ependymal cell types or their localizations can be demonstrated, which was not possible before this classification of ependymal cells. Cilostazol is an inhibitor of phosphodiesterase-3, an enzyme that metabolizes cAMP to AMP and it also regulates intracellular calcium (Kawanabe, Takahashi et al. 2012). Using high-speed fluorescence imaging analysis allows imaging and quantifying of the unique intracellular calcium oscillation properties of the ependymal cells. For instance, both alcohol and cilostazol significantly altered the ependymal ciliary beating frequency as well as the intracellular calcium oscillations properties, which in turn, could lead to a change in the cerebrospinal fluid volume replacement by ependymal cilia (Liu, Jin et al. 2014) ENREF_10. In summary, this technique was key to provide the first evidence of three distinct types of ependymal cells with different calcium oscillation properties.

In the following section, a detailed step-by-step overview of the procedure is provided, paying close attention to tissue preparation and handling.

PROTOCOL TEXT

The procedures for animal use were approved by the Institutional Animal Care and Use Committee (IACUC) of The University of Toledo in accordance with the guidelines of the Institutional Animal Care and Use Committee at the National Institutes of Health and the Guide for the Care and Use of Laboratory Animals.

1.) Brain extraction, sectioning and tissue preparation

1.1) Sacrifice wild-type mouse strain C57BL/6 by deeply euthanizing with CO_2 asphyxiation for 5 min. Assure death by cervical dislocation.

1.2) Clean the mouse head with 70% ethanol.

1.3) Perform craniectomy using sterile scissors and forceps by first pulling the skin off, starting with the top of the head to expose the skull.

1.4) Then, when the skull is exposed, remove the skull by peeling the bone piece-by-piece, starting from the posterior side and moving toward the anterior side. Be cautious not to destroy the brain ventricles.

1.5) Collect the whole brain.

1.6) Place the brain in a glass-bottom Petri-dish containing DMEM/High-Glucose supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin solution containing 10,000 units/mL of penicillin and 10,000 µg/mL of streptomycin and pre-warmed to 37 °C.

1.7) Slice the brain on the median sagittal plane by hand with a sharp blade, and obtain the first 100-200 µm section from each half using a vibratome.

1.8) Rinse the brain tissue with pre-warmed 37 °C phosphate buffered saline (1XPBS) solution.

1.9) Immediately place the brain section in DMEM/High-Glucose media pre-warmed to 37 °C.

2.) Live imaging configuration and setup

2.1) Place the brain tissue sections in 30 mm glass culture dishes containing 1 ml of DMEM/High-Glucose media. Adjust the microscope's enclosed chamber environment to 37 °C, 95%/5% O_2/CO_2 content (**Figure 1**).

2.2) Using a 60X objective oil immersion lens, collect the ependymal cells/cilia images by first placing an oil drop on the 60X objective lens and focusing on the cells with regular DIC transmitted light.

2.3) Then, follow the direction of the DMEM bubble movement as a guide to the location of the motile ependymal cilia as ciliary beatings create a kind of bubble movement in the area. Choose an area containing healthy cells with motile cilia in the brain's lateral ventricle using the DIC filter. Once the ependymal cilia are found, adjust the light and focus to obtain a satisfactory image.

2.4) Set the live imaging parameters according to a specific purpose using Metamorph imaging software. In the present demonstration, acquire twenty four-bit images with the camera binning set to 1x1 combined with 60X objective and 5-10 msec exposure time.

2.5) Collect the DIC images by opening the microscope aperture to an optimal level in order to have a minimum exposure time. Observe live images stream to the camera to provide fast and immediate image acquisition without delay. Calculate the speed of cilia beating based on the requirement of the minimal exposure times to obtain sufficient image contrast.

3.) Data visualization and analysis

3.1) Calculate the number of cilia beatings by counting the number of beatings in one min. Do this by decreasing the speed of the video and counting the number of beats using a cell counter or similar tool.

3.2) To calculate the frequency of beatings, multiply the exposure time at which the video is recorded by the number of the frames or time-lapse images acquired to get the number of seconds. (Example: exposure time 5 msec × 200 frames = 1000 msec or 1 sec).

3.3) Calculate the number of beatings in one second to obtain the frequency which is expressed as the number of beating per one sec. Do this by dividing the number of cilia beatings over a one-second time interval (Example: cilia beats 50 times in a 200-frames video recorded at exposure time of 5 msec i.e 5 msec x 200 frames = 1 sec; now divide 50 beats by one sec = 50 Hz).

3.4) Calculate the ciliary beating angle by evaluating the path taken by the ependymal cilia during both the power and recovery strokes. Perform this according to a previously described method, with minor modifications (Chilvers and O'Callaghan 2000). The precise movement of individual cilia is observed during the complete beat cycle.

3.5) On an acetate sheet placed over the monitor, draw a horizontal line along the ependymal edge and a vertical line through the midline position of the cilia at the start of the power stroke.

3.6) Plot the precise position of the cilium frame by frame as it moves forward during the power stroke. In a similar manner, plot the movement of the cilia during the recovery stroke.

3.7) Calculate the ciliary beating angle from the maximum deviation of the cilium from the midline during the power stroke as well as the recovery stroke.

4.) Calcium Signal Recording

4.1) After slicing the brain, briefly rinse the brain slice with 1XPBS or Dulbecco's PBS (pH 7.0). Prepare fresh Fluo-2 to avoid fluorescence quenching and to obtain good signal-to-noise ratio.

4.2) Prepare 1 mM stock solution of Fluo-2 solution in dimethylsulfoxide (DMSO), mix and vortex the solution for at least 5 min to ensure that Fluo-2 is homogenously dissolved in DMSO.

4.3) Dilute the Fluo-2 stock solution in 500 μl of DMEM/High-Glucose supplemented with 2% B27 pre-warmed to 37 °C to a final concentration of 20 μg/ml.

NOTE: B-27 is an optimized serum-free supplement containing vitamin A, antioxidant cocktail and insulin used to support short- or long-term viability of hippocampal and other CNS neurons.

4.4) Immediately incubate the brain slice with 20 μg/ml Fluo-2 for 30 min at 37 °C in a glass-bottom plate.

4.5) To determine the optimal loading concentration of calcium fluorophore Fluo-2 and to avoid calcium dye cell toxicity, challenge cells with ATP, and check the cell viability by determining the time course and peak magnitude of calcium signals in response to ATP.

4.6) Record the video for calcium oscillation at a capture rate of 5 msec for a minimum of 1 sec (200 frames per sec), with excitation and emission wavelengths of 488 nm and 515 nm, respectively (**Movie 2**).

4.7) To distinguish the Fluo-2 calcium signal from autofluorescence or movement artifacts, ensure that the intensities emitted at 515 nm is monitored separately.

NOTE: Fluo-2 is not the calcium fluoremetric suitable to quantify intracellular calcium. However, it is an excellent dynamic calcium dye to detect fast calcium changes, such as calcium oscillations. For more accurate calcium quantification, Fura-2 is recommended. However, the dynamic changes of Fura-2 are limited by its ratiometric nature of the dye.

4.8) Follow the formulas provided by the manufacturer to calculate the exact free intracellular calcium values. Example $[Ca^{2+}] = K_d \times (R–R_{min})/(R_{max} – R)$. Where K_d is the dissociation constant of the dye from the released calcium, R is the measured fluorescence at 488, and R_{min} and R_{max} are the fluorescence ratios at minimum and maximum ion concentration (Nauli, Jin et al. 2013).

5.) Immunofluorescence Microscopy

5.1) Fix the brain sections with phosphate buffered saline solution containing 4% paraformaldehyde (PFA) and 2% sucrose for 10 min. Alternatively, fix the whole brain with 4% PFA and then section into 50 μm sections using a cryostat.

5.2) Wash the tissue three times with 1XPBS for 5 min each time.

5.3) Incubate the brain slice with a solution of 0.1% Triton-X in 1XPBS for 5 min, then rinse three times with 1XPBS for 5 min each time.

5.4) Incubate the brain slice with mouse primary antibody, antiacetylated α-tubulin, used at a dilution of 1:5,000 in 10% FBS in 1XPBS for one hour at room temperature (RT) or overnight at 4 °C.

5.5) Wash the tissue three times with 1XPBS for 5 min each time.

5.6) Incubate the brain slice in secondary antibody, fluorescein anti-mouse IgG at a dilution of 1:500 in 10% FBS in 1XPBS solution for 1 hr at RT.

5.7) Before observation under a fluorescent microscope, counterstain the section with 4',6-diamidino-2-phenylindole (DAPI) for 5 min to stain the nucleus (or DNA)

(Aboualaiwi, Muntean et al. 2014). To minimize photobleaching, image the sections immediately with minimum exposure time possible.

REPRESENTATIVE RESULTS

Measuring ependymal cilia function in live mouse brain

The method described in this protocol is used to monitor ependymal cilia function and structure in the fresh tissue dissected from the mouse brain as well as to monitor and study cilia beating frequency. The steps followed to accomplish a complete experiment are depicted in a schematic flowchart **(Figure 1)**. It is highly recommended that the experiment is conducted within a short time frame in order to keep the motile cilia as active as possible. A representative time-lapse movie and images of the ependymal cells and their motile cilia are also shown **(Movie 1 and Figure 2a)**. Data analysis in the obtained stream is accomplished by counting the beating and angle pattern of the moving cilia. The criteria for dividing the cilia into three types are presented in **Table 1.** The presence of ependymal cilia is confirmed with a ciliary marker, acetylated-α-tubulin, and the ependymal cells are counterstained with DAPI (DNA marker) to show the nucleus **(Figure 2b)**. Based on our observations of ependymal cells in the brain lateral ventricle from at least 22 independent experiments, we were able to classify ependymal cells into three types based on their ciliary beating frequency. Moreover, we demonstrated that Ethanol at 0.25% concentration significantly repressed cilia beating frequency irrespective of their type (**Figure 3**). More importantly, these data are in consistence with our previous findings (Liu, Jin et al. 2014) ENREF_14 ENREF_14 ENREF_14.

Measuring calcium signaling by ependymal cilia

It has been previously shown that bending of cilia can trigger a cilium-dependent intracellular calcium signaling (Praetorius and Spring 2001, AbouAlaiwi, Takahashi et al. 2009, Jin, Mohieldin et al. 2014). This technique allows researchers to examine and measure the intracellular calcium signal within the brain ventricles **(Movie 2)**. One can apply a similar methodology to record cytosolic calcium oscillations in response to ependymal cilia activation or in response to treatment with pharmacological agents. To examine cytosolic calcium, the tissue is incubated for 30 min at 37 °C with the calcium indicator, Fluo-2. Live images of cytosolic calcium oscillation/level are streamed at the excitation and emission wavelengths of 488 and 515 nm, respectively.

FIGURES AND TABLES

Figure 1. Ependymal cilia imaging protocol flowchart. Ependymal cilia imaging protocol illustrates steps to complete an experiment starting from mouse brain extraction, sectioning and tissue preparation to image acquisition and analysis. An approximate one hour timeline is presented with step-by-step procedure.

Figure 2. Ependymal cilia localization in the brain ventricles. Shown here are ependymal cells from the lateral ventricle of a mouse brain. (**a**) DIC images of individual ependymal cells (bottom arrows) and cilia (top arrows) are shown. (**b**) An overlay image of a brain section is stained with antibody against a ciliary marker, acetylated α-tubulin, shown in green (top arrows), and counterstained with a nuclear/DNA marker, DAPI, shown in blue (bottom arrows). Please note that panels a and b represent different brain sections.

Figure 3. Alcohol and differences in cilia beating frequencies among types of ependymal cells of the mouse brain lateral ventricle. The *ex vivo* brain slice was incubated without (Control) or with (Ethanol) 0.25 % alcohol for 5 min. Compared to control, alcohol treatment significantly decreased cilia beating frequency, as indicated by an asterisk. At least 5 -10 independent preparations were used for each ependymal cell type and treatment group.

Movie 1. Recording of ependymal cilia in type III ependymal cells. Shown here are recordings of ependymal cilia, characterized by beating frequency and angle unique to type III ependymal cells from the brain's third ventricle. This figure has been previously reported (Liu, Jin et al. 2014) ENREF_17 and was reused with permission.

Movie 2. Intracellular calcium oscillation in ependymal cells. Shown here are recordings of calcium oscillations of ependymal cells through a section of the brain's lateral ventricle after incubating the brain slice with 20 μg/ml calcium indicator, Fluo-2, for 30 min at 37 °C. The brain section's calcium level was studied and pseudo-colored. The color bar indicates the ependymal cells' calcium level; where black-purple and red-yellow represent low and high calcium levels, respectively. For intracellular calcium quantification, ependymal cell calcium will be calculated from several individual ependymal cells, as mentioned in the protocol text and averaged between control and treatment groups. The video of calcium oscillation was recorded at 200 frames per sec with excitation and emission wavelengths of 488 nm and 515 nm, respectively. This figure has been previously reported (Liu, Jin et al. 2014) ENREF_17 and was reused with permission. _ENREF_3

Table 1. Types of ependymal cilia. The classification of ependymal cilia into type I, II or III was based primarily on the beating frequency and beating angle of ependymal cilia located within distinct regions of the brain's third ventricle. Parts of this data have been previously reported _ENREF_17 and were reused here with permission.

DISCUSSION

Described here is a protocol for the preparation of mouse brain tissue for both live-imaging and fluorescence microscopy that provides a rapid and sensitive close observation of the ependymal cilia within the brain ventricles. This technique is not restricted to the lateral ventricle only; it could be utilized to

observe the cilia in other brain ventricles. This imaging technique provides a live stream that resembles the movement of the CSF by ciliary beating in an *ex vivo* setting. Moreover, it enables analysis of the directional movement of the cilia. This is mainly facilitated by the use of a high resolution DIC and fluorescence imaging system. An advantage of this system is that the microscope is enclosed in an environmental chamber, which allows for a fine regulation of temperature, humidity and CO_2 levels. These are very critical parameters that should be considered for the survival of cells and tissues when performing live imaging experiments. The system is also equipped with automatic XY and Z modules as well as a digital camera and wavelength filter switcher, all of which are important features to facilitate imaging of dynamic cellular behaviors such as cilia movements. The microscope is connected to a computer and the acquisition of high quality images is facilitated by imaging software.
_ENREF_17
Using this technique to classify ependymal cells into distinct types offers a significant advancement over existing/previous methods used to study ependymal cilia. For example, the effect of certain pharmacological or toxic agents such as alcohol could be otherwise minimized or nullified if ependymal cells are considered as one population (Smith, Radhakrishnan et al. 2013). It is important to keep in mind that, despite the fact that the characteristics of cilia beating frequencies and angles are very different among these three types of ependymal cilia, we have just started to understand the physiology of these cells. Hence, according to our previous work, our classification is robust enough if the procedure is carried properly as described in this protocol.

Identifying distinct ependymal cilia is fundamentally important to gain a basic understanding of ependymal physiology. This method makes it possible to distinguish between three distinct types of ependymal cells uniquely and specifically positioned within the third ventricle in regards to the beating frequency and angle, leading to the classification into three different types (**Table 1**). In order to confirm that the differences in cilia beating frequencies are not due to differences in viability of the ependymal cells or to differences in the animal age, it is recommended that only intact and undetached ependymal strips or slices that are attached to neuronal tissue and at least 100 μm thick are used. Moreover, ciliary beating frequency should be measured at different locations along each ependymal section. We have previously demonstrated that ciliary beat frequency data generated using this technique has been consistently reproducible among 87 experimental observations (Liu, Jin et al. 2014)_ENREF_18_ENREF_18. Hence, ependymal cells are accurately classified depending on their ciliary beating. Moreover, when using pharmacological agents which are known to decrease the ciliary beating frequency, the cilia beating frequency should theoretically return to normal beating frequency following the removal of those pharmacological agents.

A critical step in this protocol is mainly related to handling the brain tissue and the time needed to complete the experiment. It is imperative to handle the brain

tissue gently in order to preserve the structure and function of ependymal cilia as well as to reduce trauma to the fragile tissue. More important, and to avoid deterioration and death of the brain tissue, which could be a limiting factor for the success of this technique, it is highly recommended that the steps related to this technique are performed in as close to one hour as possible. However, this limitation could be overcome in the future with the advances in culturing and growing ependymal or similar cell types with motile cilia *in vitro* or with the use of alternative nutritive media. The use of alternative nutritive media such as aCSF and Earle's salts for the incubation of the brain slices has been demonstrated in the literature (Genzen, Platel et al. 2009). However, in our hands, the use of DMEM/High-Glucose was more beneficial than aCSF possibly due to the presence of high amount of glucose (4500 mg/ml) in the medium as a potential energy source. Thus, the main criterion used to assess the viability of the tissue and hence the validity of the approach is assessing cilia beating, as this is a representative sign of live cells.

_ENREF_3 ENREF_13 ENREF_3

Live imaging of ependymal cilia offers a powerful tool to analyze downstream signaling pathways of cilia activation such as calcium signaling and oscillations. _ENREF_16For example, using live fluorescence imaging, it has been demonstrated that irrespective of the ependymal cell/cilia type, ependymal cells are characterized by unique calcium oscillation properties (Liu, Jin et al. 2014)_ENREF_17. All in all, this protocol is relevant to both basic scientific knowledge and clinical practice. From the basic science perspective, this technique offers an assessment of the functional and physiological roles of ependymal cilia structure, function and downstream mechanistic signaling pathways. From the clinical practice perspective, this method is highly relevant to the search for drugs that target ependymal cilia as a new therapeutic target for neurological disorders such as hydrocephalus and alcohol abuse.

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DISCLOSURES

No conflicts of interest declared.

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