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A dissertation

entitled

Ethanol Effect on Three Distinct Types of Ependymal Cells and the Intracellular Calcium Oscillation Property

by

Tongyu Liu

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

Master of Science Degree in

Pharmaceutical Sciences

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May 2014

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An Abstract of

Ethanol Effect on Three Distinct Types of Ependymal Cells and the Intracellular Calcium Oscillation Property

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Ependymal cells are multi-ciliated epithelial cells that line the ventricles in the adult brain. Although abnormal function or structure of ependymal cilia has been associated with various neurological deficits, the different types of ependymal cilia are unknown. For the first time, we report three distinct ependymal cell types, based on their ciliary beating frequency. These ependymal cells have specific localizations within the lateral ventricles of the mouse brain and are characterized by a unique ciliary beating frequency and beating angle. Furthermore, neither ependymal cell types nor their localizations are altered by aging range from 3 weeks to 54 weeks. Our high-speed fluorescence imaging analysis surprisingly reveals that these ependymal cells have an intracellular pacing calcium oscillation property that was previously found only in cardiac myocytes in the mammalian organ. Our studies further show that alcohol can significantly repress the amplitude of calcium oscillation and the frequency of ciliary beating, resulting in an overall decrease in volume replacement by the cilia. In summary, we provide the first evidence of three distinct types of ependymal cells with calcium oscillation property.

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Chapter 1

Introduction

1.1. Cilia

Cilia are generally classified as solitary non-motile and bundled motile organelles (Abou Alaiwi et al., 2009; Nauli et al., 2011). Motile and non-motile cilia have been implicated in fundamental processes of development and disease. The classification of cilia is based on the different structures of microtubules. Motile cilia (9+2) express an additional pair of microtubule singlet in the center of the 9-doublet microtubules, compared to non-motile (9+0), which are absent with this central pair of microtubules. Non-motile cilia are also named as primary cilia.

Primary cilia exist in every organ of human body acting as a sensory organelle, capable of detecting and transmitting mechanical and chemical information from the extra cell space into the interior. The dysfunctional primary cilia will result in the polycystic kidney disease.

Motile cilia can be found in the airways. They play a crucial role in clearing debris from

airways in general conditions. In addition, motile cilia are known as ependymal cells, which present in ventricle in brain and central canal of the spinal cord. They are important in circulating spinal fluid in the ventricles of the brain.

1.2. Ependymal cells

Ependymal cells are ciliated simple cuboidal epithelium-like glial cells that move cerebrospinal fluid (CSF) along the ventricles (Del Bigio, 1995). Abnormal ependymal cilia result in hydrocephalus induced by mechanical obstruction of CSF flow (Banizs et al., 2005; Baas et al., 2006; Wodarczyk et al., 2009; Tissir et al., 2010).

Ependymal cells also play an important role in regulating pluripotent neural stem cell (Rietze et al., 2001). Beating of ependymal cilia is required for normal CSF flow, which functions as a guide for specific directional migration of new neurons (Sawamoto et al., 2006). The coupling between ependymal cilia beating and hydrodynamic forces has been proposed to regulate planar cell polarity during development or stroke (Guirao et al., 2010; Mirzadeh et al., 2010; Devaraju et al., 2013). Planar cell polarity refers to the polarity of a cell in a plane of an epithelium (Nübler-Jung et al., 1987).

In addition, ependymal cilia play major roles in cerebrospinal fluid dynamics, cerebral fluid balance, secretion, toxin metabolism, and many other functions (Genzen et al., 2009; Appelbe et al., 2013). Although ependymal cells regulate CSF flow, which regulates many neuronal processes, the identity of the ependymal cells has not been reported.

1.3. Alcohol in cerebrospinal fluid

Ethanol is a general CNS depressant. Acute alcohol effect can cause anti-anxiety actions and produce behavioral disinhibition at a wide range of dosages. Uncontrolled mood swings and emotional outbursts that may have violent components. With more severe intoxication, CNS function generally is impaired, and a condition of general anesthesia ultimately prevails.

About 10% of alcohol drinkers progress to levels of consumption that are physically and socially detrimental. Chronic abuse is accompanied by tolerance, dependence, and craving for the drug. Chronic abuse results in shrinkage of brain owing to loss of both white and gray matter. The frontal lobes are particularly sensitive to damage by alcohol, with older alcoholics being more vulnerable than younger ones. In addition to loss of brain tissue, alcohol abuse will reduce brain metabolism.

Because the ethanol can pass the blood-cerebrospinal fluid-barrier, the acute consumption of ethanol via oral will cause the change of CSF composition. The final concentration of ethanol in CSF will be eventually equal to the concentration of ethanol in blood. One report indicated that after the administration of 3g ethanol/kg body weight, the concentration of ethanol in CSF was significantly higher than in peripheral blood a during the first 120 min of the experiment. The highest concentration of ethanol in this report was 45mM, which was about 0.25%.

The hydrocephalus ex vacuo is developed among the patients who have the alcohol abuse history. The hydrocephalus ex vacuo is one kind of hydrocephalus. It usually presents the increase of ventricle size in brain and is associated with the accumulation of CSF, which replaces the loss space of tissue of brain. While the dysfunctional ependymal cells cause the non-communicate hydrocephalus, we assume that the ethanol may have effect on ependymal cells.

1.4. Intracellular calcium signaling

Previous study in our lab indicates that the intracellular calcium signaling could be caused by the fluid stress above a layer of cells. In this study, the cell type we use is LLCPK, which is the cell from pig kidney with primary cilia. The induced calcium signal is due to the sensory function of primary cilia. Although the relationship between the primary cilia and calcium signal is well studied, the intracellular calcium in ependymal cells is still unknown.

In addition, the mammalian cilia motility is regulated by many factors, such as cyclic AMP, cyclic GMP, protein kinase A, protein kinase C, intracellular pH and calcium. Among these factors, although most of them have been already studied well, the calcium mechanism is still controversial. Thus, to study the intracellular calcium in ependymal cells will be an excited aspect.

Based on the information and previous study indicated above, we designed our project as three parts. First part is morphology of ependymal cells and ependymal cilia. Second is

the chemical testing, which is using ethanol as a treatment. Accompanied with the observation and chemical treatment, we measured the intracellular calcium in ependymal cells. We show here that ependymal cells can be categorized into three types. Furthermore, each type of ependymal cells is uniquely localized within the ventricle.

Chapter 2

Materials and Methods

All animal experiments were approved by The University of Toledo's Institutional Animal Care and Use Committee (IACUC). The wild type mice were euthanized with carbon dioxide for 5 minutes. After craniotomy, the whole brain was removed. The sagittal slice was dissected with a thickness of about 100 μm and was immediately embedded in Dulbecco's Modified Eagle Medium (DMEM, *Cellgro*, Inc.) at 39°C in the presence of 95%/5% O_2/CO_2 mixture.

2.1. Immunofluorescence microscopy

The brain slice was fixed in phosphate buffer containing 3% paraformaldehyde and 2% sucrose for 10 minutes. Mouse primary antibody anti-acetylated a-tubulin was used at a dilution of 1:5,000 (*Sigma,* Inc.). The brain slice was incubated in primary antibody solution for overnight. Secondary antibody fluorescein anti-mouse IgG was used at a dilution of 1:500 (*VectorLabs*, Inc.), and the brain slice was incubated with secondary antibody solution for one hour. Before observation under a fluorescent microscopy (Nikon TiU), the section was dyed with DAPI for 5 minutes.

2.2. Measurement of cilia beating frequency

The prepared brain slice was kept in a customized glass-bottom plate covered with 500 μL DMEM containing 2% B27 at 39°C. In some cases, 0.25% ethanol was added to the media. The video of cilia beating was captured with high-resolution differentiation interference contrast (DIC) microscope (Nikon TiU). The capture rate of the video was 5 milliseconds for a minimum of one second (200 frames per second).

2.3. Measurement of the location of different types of ependymal cilia

To study the connection between the different types of cilia with their location in lateral ventricle, we observed the cilia and recorded them first. Then the cilia beating frequency was measured to certain the type of cilia. After we confirmed the type, the location of the observation point was aimed in the lateral ventricle and was recorded. With summarizing all the observations, we drew the picture to show the location relationship with these three types of ependymal cilia.

2.4. Measurement of fluid movement and volume replacement

Because of the transparency of the buffer solution, we used nanoparticles to help analyze speed in the solution movement. The velocity of fluid movement was calculated by tracing one single nanoparticle flowing across the lateral ventricle wall. The overall fluid volume moved by ependymal cilia was calculated with the following formula:

Volume replacement (μm^3/stroke) = fluid movement velocity (μm^3/second) ÷ cilia beating frequency (stroke/second)

2.5. Calcium signal recording

To record cytosolic calcium oscillation, the brain slice was incubated with 20 μg/mL fluo-2 (*TefLabs,* Inc.) for 30 minutes at 39°C. The tissue was then transferred to a glass-bottom plate covered with 500 μL DMEM containing 2% B27 (*Gibco, Inc.*) at 39°C. In some cases, 0.25% ethanol (190 proof) was added to the media. The video of calcium oscillation was recorded at a capture rate of 5 milliseconds for a minimum of one second (200 frames per second), with excitation and emission wavelengths of 488 nm and 515 nm, respectively.

2.6. Statistics

All images were analyzed using Metamorph software. All quantifiable data were reported as mean ± SEM. Comparisons between means were performed using ANOVA with post hoc comparisons via Dunnet, and statistical significance implies $p < 0.05$. All data analysis was done using GraphPad Prism v.5.

Chapter 3

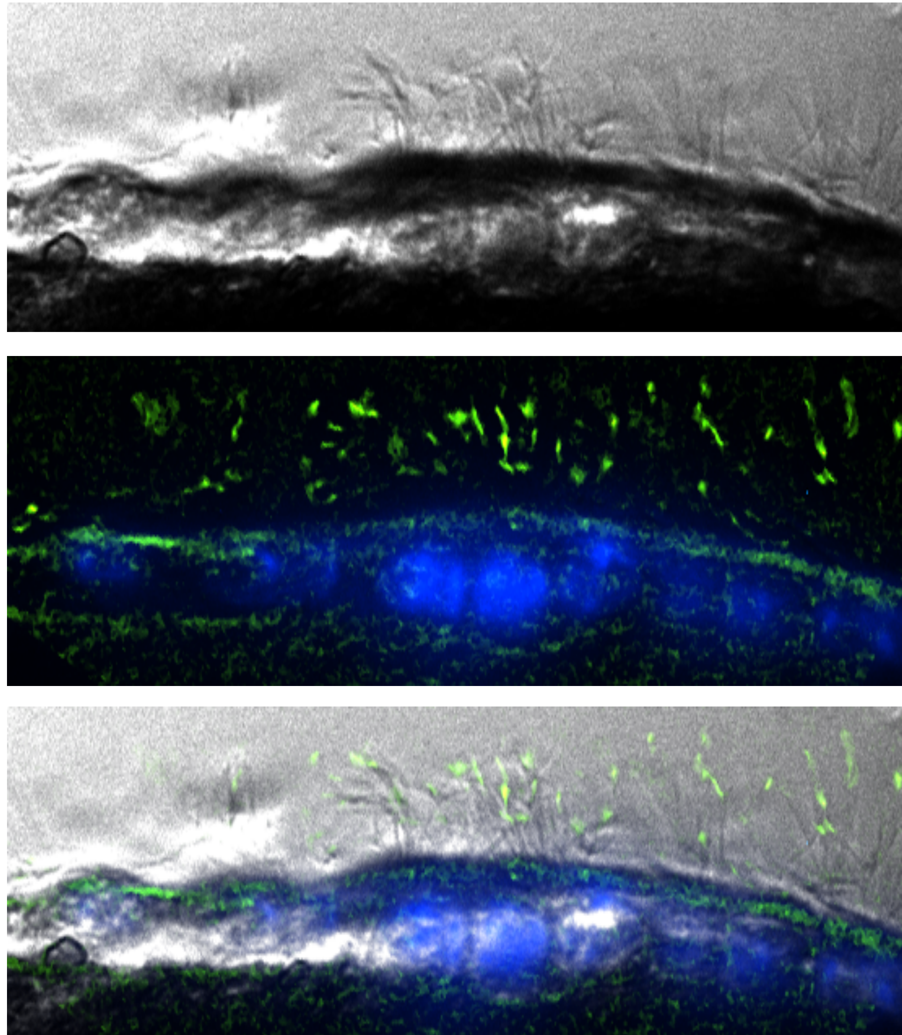
Results

3.1. Ependymal cells can be classified into three types based on their cilia beating frequency.

We cut the mouse brain in a sagittal plane to better observe the entire lateral ventricle. To verify our high-resolution differential interference contrast and fluorescence microscope systems, we examined the presence of ependymal cilia in the lateral ventricle (**Figure 1**). Ependymal cilia were confirmed with a ciliary marker, acetylated-α-tubulin. While in the control permeabilized brain no fluid movement was observed in non-beating ependymal cilia, we could observe the direction of fluid movement via oil ink in a freshly prepared brain *ex vivo*.

We next attempted to quantify the cilia beating frequency. Based on our observation of individual ependymal cells from over 100 independent experiments, we surprisingly noticed that there were wide variations in the beating frequencies of ependymal cilia (**Figure 2**). Depending on its ciliary beating, we could accurately assign each ependymal cell to one of three classifications: Type I ependymal cells had the highest beating

frequency (>60Hz) and had a beating angle of less than 90°; Type II ependymal cells had a medium beating frequency (30-60 Hz) and had a beating angle between 90° and 135°; and, Type III had the slowest beating frequency (<30 Hz) with a beating angle of more than 135°.



<u>**Figure 1.** The presence of ependymal cilia in mouse brain</u>
Shown here are ependymal cells from the lateral ventricle of a mouse brain. The brain section was stained with anti-acetylated-α-tubulin, a ciliary marker (green), and counter-stained with DAPI, a nucleus marker (blue). Individual differential interference contrast (**top panel**), fluorescence (**middle panel**), and merged (**lower panel**) images are shown.

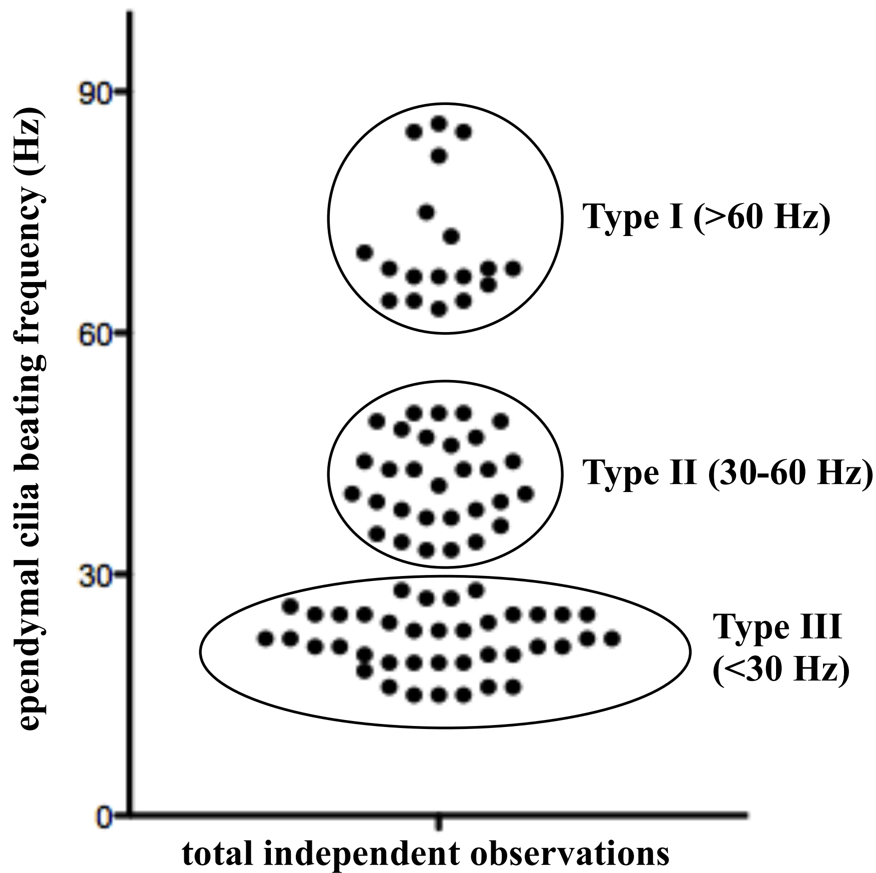


Figure 2. Categories of ependymal cells in mouse brain

Based on a total of 87 independent preparations, ependymal cells could be classified into three types. Type I ependymal cilia had the highest beating frequency (>60 Hz), with a ciliary beating angle of less than 90°. Type II ependymal cilia had a medium beating frequency (30-60 Hz), with the ciliary beating angle between 90°-135°. Type III ependymal cilia had the slowest beating frequency (<30Hz), with a ciliary beating angle of more than 135°. The cartoon on the right depicts the beating angles of ependymal cilia.

To understand the difference in localizations among these ependymal cells, we mapped the distributions of ependymal cell types within the lateral ventricle (**Figure 3**). Our mapping analysis indicated that Type I cells were widely distributed along the ventricle walls, but they were absent at both corners of the lateral ventricle. Type II cells were mainly observed on the upper wall of the ventricle, but they could also be found at the lower wall of the ventricle. Type III cells were distributed almost exclusively in the corners of the lateral ventricle. Of note is that we could find all three cell-types at the lower wall of the lateral ventricle.

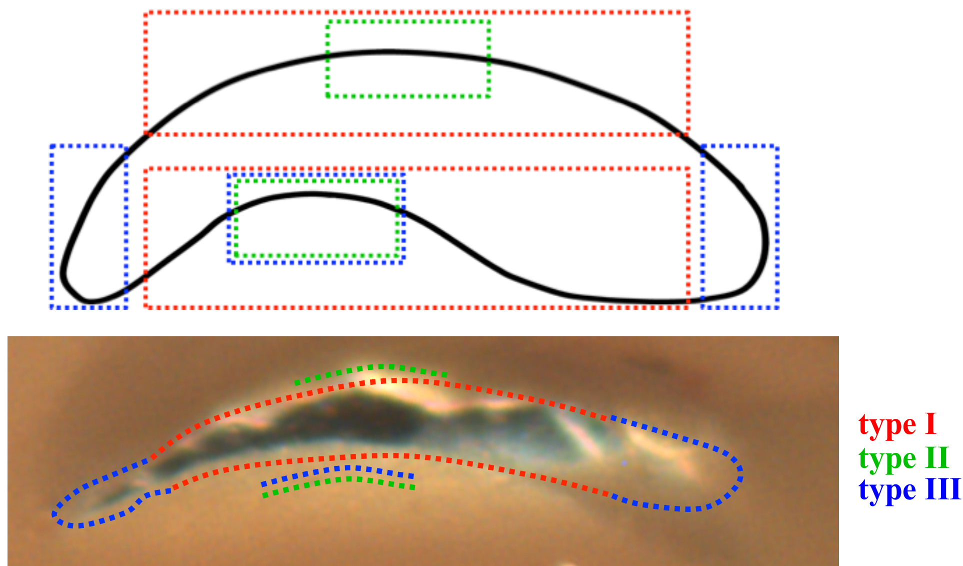


Figure 3. Differential of ependymal cell types localization
Based on the ependymal cilia beating frequency, different ependymal cell types were enriched at certain locations within the lateral ventricle. The cartoon (**top panel**) of and brain section (**bottom panel**) of the lateral ventricle depict localizations of different ependymal cells from a sagittal view. Bar=30 μm

3.2. Cilia beating frequency is age-independent in lateral ventricles.

Because ependymal cilia have been proposed to move cerebrospinal fluid (CSF) to support migration of various substances (Banizs et al., 2005; Sawamoto et al., 2006), we next aimed at understanding the role of aging in ependymal cilia beating. Mice were grouped according to age at 3-5 weeks, 9-11 weeks, or 37-54 weeks old. Our data indicate that we could distinctively classify the ependymal cilia beating into three types regardless of the age groups (**Figure 4**). More importantly, there was no evidence that age was a factor in regulating ependymal cilia beating.

3.3. Ependymal cilia beating can be repressed by ethanol.

To further confirm that our classification of ependymal cells was valid and consistent, we preformed chemical screenings on the cilia beating frequency. Ethanol at a concentration of 0.25% provided us with the most consistence changes in ependymal cilia beating. Our data indicate that ethanol repressed ependymal cilia beating regardless of the age group (**Figure 4**). Most importantly, ethanol repressed ependymal cilia beating (**Figure 5a**), resulting in a significant decrease in fluid movement velocity around ependymal cilia (**Figure 5b**). Due to the transparency of the fluid media, we used nanoparticles to guide us to measure the speed of the fluid movement. Given the fluid movement velocity, we estimated the volume replacement for each stroke of cilia beating efficiency. Interestingly, our calculation indicated that ethanol not only repressed cilia beating

frequency but also decreased the efficiency of ependymal cilia to move fluid per each stroke (**Figure 5c**).

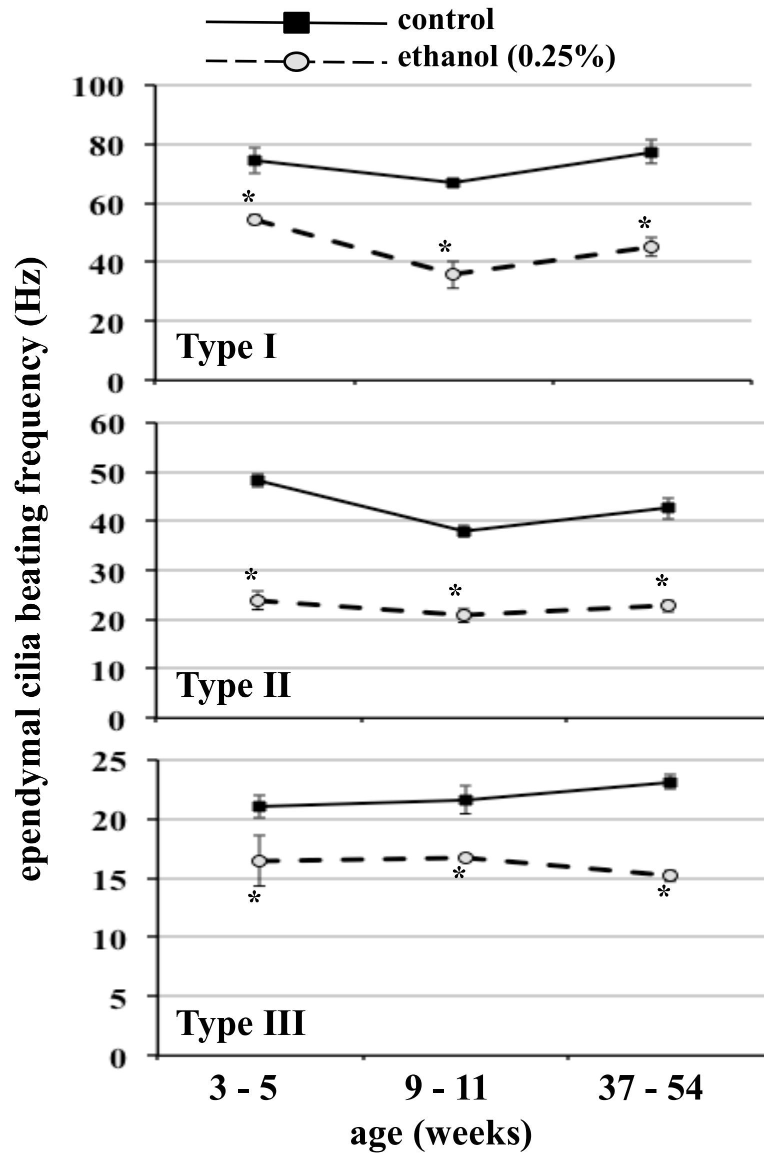
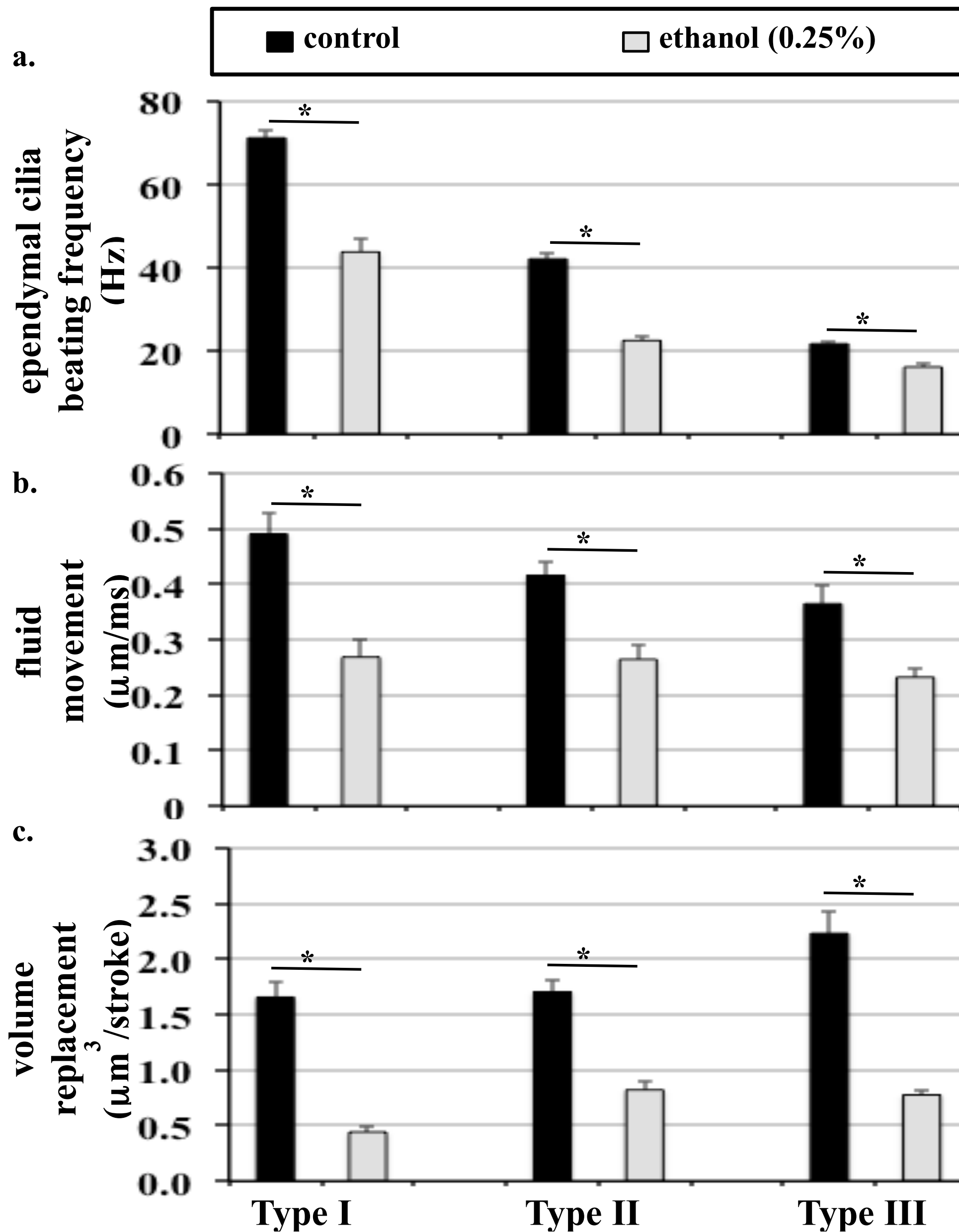


Figure 4. Ependymal cilia beating frequency in different mouse age groups

All types of ependymal cilia in control and alcohol-treated mouse lateral ventricles were studied at different ages. Although beat frequency of ependymal cilia was not affected by age (from 3 to 54 weeks), acute alcohol treatment sufficiently decreased cilia beating frequency in all types of ependymal cells. At least three independent preparations were used for each ependymal type and age group.

Figure 5



<u>**Figure 5.** Effects of alcohol on the dynamics of mouse lateral ventricle</u>

The *ex vivo* brain slice was incubated without (control) or with alcohol (ethanol) for 5 minutes. **a.** Compared to control, alcohol treatment significantly decreased cilia beating frequency. **b.** This resulted in a decrease in fluid movement, as indicated by speed of fluid movement surrounding the ependymal cilia. **c.** Further calculation of the volume replacement and cilia beating indicated that compared to control, alcohol significantly decreased volume replacement for each stroke of cilia beating. This indicates that alcohol not only decreases the frequency of ependymal cilia beating but also reduces the efficiency of each cilia stroke. At least ten independent preparations were used for each ependymal type and treatment group.

3.4. Calcium signaling by ependymal cilia can be altered by alcohol.

Fluid-shear stress resulting from fluid movement above a layer of cells can generate intracellular calcium signaling (AbouAlaiwi et al., 2009; Jin et al., 2013). We thus examined the calcium signaling within the lateral ventricle (**Figure 6a**). In the absence or presence of ethanol, calcium oscillation was observed on ependymal cells. Interestingly, the frequency of calcium oscillation was not changed either by cilia beating frequency or by ethanol treatment (**Figure 6b**). Although the frequency of calcium signal in each ependymal cell type was unchanged, the amplitude of calcium signal was significantly repressed in the ethanol group compared to the control groups of each of the corresponding ependymal cell types (**Figure 6c**).

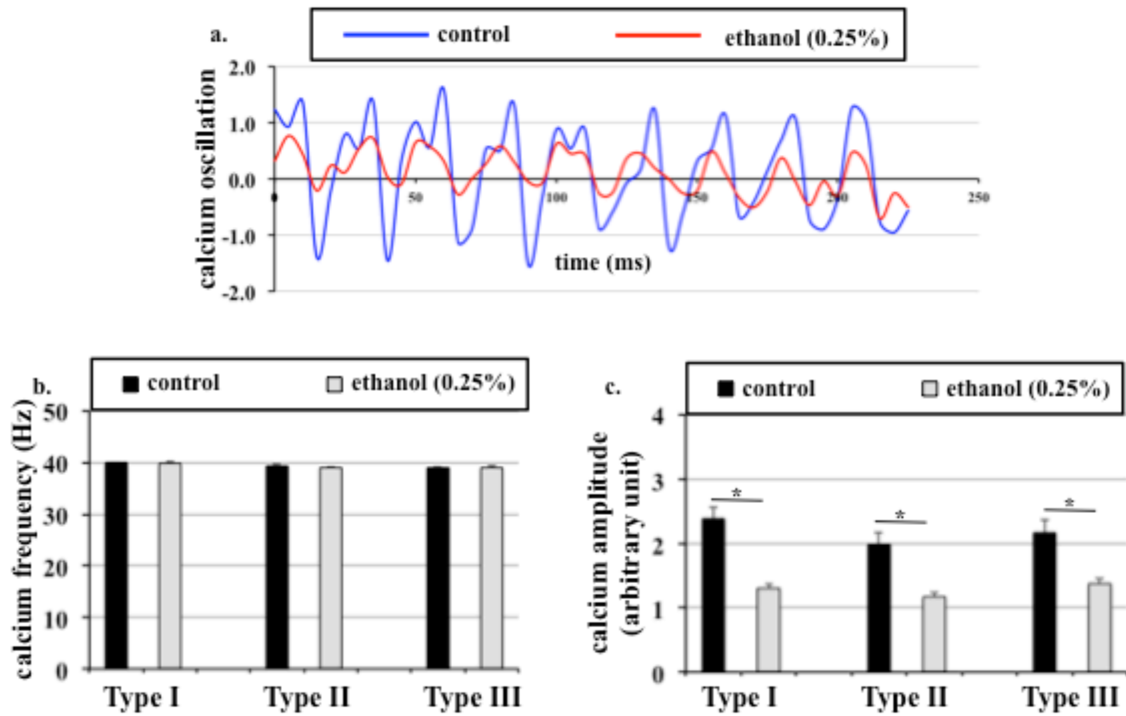


Figure 6. Effects of alcohol on calcium oscillation in mouse brain ependymal cells
After being loaded with calcium indicator fluo-2, the *ex vivo* brain slice was incubated without (control) or with alcohol (ethanol) for 5 minutes. **a.** Intracellular calcium of ependymal cells was measured every 5 milliseconds, as indicated by the representative blue and red lines. **b.** There was no difference in calcium oscillation frequency between control and alcohol-treated groups. **c.** However, the amplitude of calcium oscillation was significantly repressed in alcohol-treated groups compared to control groups in all types of ependymal cells. At least five independent preparations were used for each ependymal type and treatment group.

Chapter 4

Discussions

In the present studies, we report for the first time that there are three distinct types of ependymal cells uniquely and specifically localized within the lateral ventricle. Based on their cilia beating frequency, we classified them as type I (>60Hz), type II (30-60 Hz) and type III (<30 Hz). The beat frequency for each type of ependymal cilia is age-independent. We also report here for the first time that ependymal cells are characterized by calcium oscillations, the frequency and amplitude of which are the same in all ependymal cell types. Our chemical screening indicates that alcohol has a profound effect on the beating frequency of the ependymal cilia, resulting in a significant decrease in fluid movement and volume replacement. Although alcohol did not change the frequency of calcium oscillation in the ependymal cells, the amplitude of calcium oscillation was significantly repressed.

Even with the advancement in the technology of high-speed digital imaging (Lechtreck et al., 2009), there was no report on different types of ependymal cells. We thus believe that our study is the first to identify distinct ependymal cilia, which is fundamentally

important to gain basic understanding of ependymal physiology. For example, many substances that are known to alter cilia beating (Sisson et al., 1991; Sisson, 1995) may fail to show an effect in ependymal cells, especially when ependymal cells were randomly analyzed (Smith et al., 2013). To further validate our point, 1% ethanol was reported to have no effect on the beating frequency of ependymal cilia(Smith et al., 2013). After we classified the ependymal cilia into 3 types, however, our data clearly showed that ethanol as low as 0.25% had a definitive effect on ependymal cilia beating frequency.

Our studies also reveal a unique aspect of calcium signaling in ependymal cells. It was previously thought that cardiac myocytes were the only cells that naturally have a pacing calcium oscillation. We used a florescence high-speed digital imaging system to demonstrate for the first time that, like myocytes, ependymal cells also have an oscillating intracellular calcium pattern. The frequency of this calcium oscillation is the similar among all 3 types of ependymal cells, indicating that the frequencies of ciliary beating and calcium oscillation on ependymal cells might not be associated with one another. Consistent with this idea, an ethanol-induced decrease in ciliary beating frequency did not alter the frequency of calcium oscillation. However, the amplitude of calcium oscillation is decreased by ethanol. This decrease may be caused by the decrease of fluid movement in the lateral ventricle. In the case of primary cilia, the change of fluid movement will induce the calcium signal change due to the sensory function of primary cilia. Through our study, it also occurs in the ependymal cilia. This phenomenon may demonstrate the motile cilia have the sensory function as primary cilia.

It is worth mentioning that within the 3 types of ependymal cells, type III cells were the most efficient at moving fluid volume with each ciliary stroke. Although their frequency of ciliary beating was the slowest, type III cells had the largest angle of stroke. The angle of stroke might therefore contribute significantly to moving fluid volume. However, it is important to mention that ciliary beat frequency was also a critical contributing factor in moving fluid volume, as seen with the ethanol treatment. Treatment with ethanol decreased fluid replacement significantly, primarily due to a slowdown in ciliary beating.

Alcohol can produce a variety of detrimental effects in the central nervous system, leading to a wide range of impairments. Within minutes of alcohol consumption, the alcohol in the CSF reaches the same level as that in the blood (Kilanmaa and Virtanen, 1978; Agapejev et al., 1992; Huang and Huang, 2007). However, the effect of alcohol on ependymal cells had never been examined before, although abnormal ependymal cilia are associated with ventricle enlargement associated with hydrocephalus (Banizs et al., 2005; Baas et al., 2006; Wodarczyk et al., 2009; Tissir et al., 2010). Consistent with this notion, the brains of alcoholics are known to have an increase in the size of the ventricles, causing hydrocephalus ex vacuo (de la Monte, 1988). The use of 0.25% of ethanol in our study was within the range of alcohol levels seen in human models and various animal models. Thus, our study also reflects a serious clinical implication in alcohol abusive behavior with regard to ventricle-lining ependymal cells, in addition to providing fundamental basic understanding of ependymal cilia and calcium signaling.

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