Cilia in Glial Cells of *Gallus gallus* Embryos

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Introduction

The two main classes of cells in the nervous system are glial cells and neurons with glial cells outnumbering neurons in the brain three to one. In the central nervous system (CNS) there are three main types of glia: astrocytes, oligodendrocytes, and microglia (Kandel et al. 2013). The functions of astrocytes are to nourish neurons, regulate ion and neurotransmitter concentrations, stabilize new synapses, and prepare the surface of neurons for synapse formation. Oligodendrocytes form myelin sheaths by extending their cell bodies to wrap around neuronal axons to propagate action potentials. Finally, microglia provide immunological aid to the CNS. Next, there are glial cells of the peripheral nervous system (PNS) called Schwann cells. The function of these cells is to form myelin sheaths around the neuronal axons within the PNS, which helps to propagate action potentials (Kandel et al. 2013).

Cilia are hair-like organelles approximately one to ten micrometers in length and one micrometer in width extending from the cell. Cilia extend from the surface of many different cell types and are composed of nine doublets of microtubules forming a ring (Marshall 2008). Microtubules are composed of different forms of tubulin, depending on the location and type of microtubules. There are two main types of cilia: non-motile (primary) and motile (Lee 2011). Motile cilia have dynein arms and two central microtubules in the center of the nine doublets. Non-motile cilia do not have central microtubules or dynein arms (Lee 2011). Motile cilia move mucus, dirt, and other particles and help the flow of cerebrospinal fluid (Lee 2011, Structure and Function of Cilia 2008) and are often found in the lungs, respiratory tract, and spinal column. While non-motile cilia regulate signal transduction and can be found in neural stem cells, neurons, and astrocytes (Lee 2011). When cilia are not functioning properly it can lead to the following conditions: cognitive impairment, fibrosis, cysts, sterility, polydactyly, congenital heart defects, and retinal degeneration.

Neurological deficits have been found in the following ciliopathies: Joubert syndrome (JBTS), Bardet-Biedl syndrome (BBS), Meckel-Gruber syndrome (MKS) and nephronophthis (NPHP) (Lee 2011). The neurological deficits present in these ciliopathies indicate that cilia may impact neurological development. If this is the case cilia must be present in cells within the nervous system, of which glia are. Therefore, cilia in glial cells could potentially be the basis for any of these diseases. The present study tested the hypothesis that glial cells of *Gallus gallus* in primary cell culture have cilia. If this hypothesis were proven true it would lead to future studies on how cilia impact neurological development and eventually lead to a treatment of these ciliopathies as well as potentially other neurological diseases.

The hypothesis of cilia in glial cells was tested using immunofluorescence of sympathetic neurons of *Gallus gallus*. *Gallus gallus* was chosen for the experimental model because it has not been used to study cilia in glial cells. Due to the lack of previous evidence of cilia in glial cells, *Lytechinus pictus* was used as a positive control; *Lytechinus pictus* are known to have cilia. Immunofluorescence was used because it can indicate the presence of certain antigens in cells. In this study the antigens of IFT88 and α-acetylated tubulin were chosen because they are commonly present in the cilia (Grainger 2014, McIntyre 2012). Labeling these antigens indicated the presence of tubulin commonly associated with cilia.

Materials and Methods

Materials

Tetramethyl Rhodamine goat anti-rabbit IgG, Cat No. T-2788 purchased from Molecular Probes.
Alexafluor 555 goat anti-mouse IgG, Cat No. A21422 purchased from Molecular Probes.
Mouse anti-acetylated tubulin antibody, Cat No. T-6793-0.2mL purchased from Sigma.
Rabbit IFT88—N-terminal region pAb, Cat No. ARP53657_P050 purchased from Aviva.
Images were taken using a Nikon Eclipse E200 at 40x magnification, SPOT InSight Firewire 2 Megasample camera, and the SPOT program, version 4.5.9.9, on a Macintosh computer.
Images were edited on a Macintosh computer using Adobe Photoshop CS5.1 version 12.1 x64.

Experimental Set Up and Dissection

A protocol outlined by Dr. Robert L. Morris was followed for the preparation and dissection of neurons of ten day *Gallus gallus* (Morris 2014a). However, the flame constriction of Pasteur pipettes was not performed by the students but by the lab assistant, Elena Llabovitiadhi and Dr. Morris. DMEM (Dulbecco's Modified Eagle Medium) was used in place of HBSS (Hank's Balanced Salt Solution).

Cell Culture

After dissection, dissociation, and plating of ganglia and sympathetic nerve chains, with a density of approximately 1/3 DRG per coverslip, from ten day *Gallus gallus*, coverslips were incubated at 37°C in F+ medium (Leibovitz L-15, 10% fetal calf serum, 0.6% glucose, 2 mM L-glutamine, 100 ug/mL streptomycin, 100 U/mL penicillin, 50 ng/mL NGF) (Morris 2014a). Shelves of incubator were lined with rubber to absorb vibrations that would disrupt neuron growth.

Fixation/Rehydration of Cells

Coverslips with chick neurons were fixed in Methanol (MeOH) or Formaldehyde/Gluteraldehyde; three coverslips in MeOH and two

coverslips in Formaldehyde/Gluteraldehyde (Morris 2014b). This research was conducted on neurons fixed with formaldehyde/gluteraldehyde. *Lytechinus pictus* embryos were fixed in MeOH as well to be used as positive control samples (Morris 2013). MeOH fixed samples were rehydrated prior to staining (Morris 2013).

Immunofluorescent Labeling

The procedure for antibody labeling was outlined by Dr. Morris and followed for this experiment (Morris 2013). Experimental condition one had concentrations of antibodies as follows: mouse α-acetylated tublin (1:2000 µL), rabbit IFT88 (1µg/750 µL), Alexafluor 555 goat anti-mouse (5µg/mL) and tetramethyl rhodamine goat anti-mouse (5µg/mL) and Hoechst (0.01 mg/mL). Antibodies were added in the order listed above. Experimental condition two, only tested in MeOH fixed cells, varied in concentrations of α-acetylated tublin and rabbit IFT88 antibodies, 1:1000 µL and 1µg/75µL, respectively, then treated with secondary antibodies and Hoechst stain. Negative controls were used to test for autofluorescence and only used the secondary antibodies. The positive control was used to test if the labeling conditions were working and used mouse anti-alpha tubulin (FITC-DM1A), rabbit IFT88 antibody (1µg/750 µL), and Tetramethyl Rhodamine goat anti-mouse antibody (1:200) (Conn et al. 2014).

Data Collection and Analysis

Images of the glia were taken using a Nikon Eclipse E200 at 40x magnification, SPOT InSight Firewire 2 Megasample camera, and the SPOT program, version 4.5.9.9, on a Macintosh computer. The exposure time to fluorescence light was approximately 500 milliseconds. Images of the rhodamine-labeled IFT88, alexafluor 555-labeled α-acetylated tublin, and the Hoechst stain were maximized for contrast then overlaid for RGB in Adobe Photoshop CS5.1 version 12.1 x64.

The number of glial cells in each image were counted then analyzed for the presence/absence of a cilium. A cilium would appear as a small, short, bright region that is relatively straight near the nucleus of the glial cell (Grainger 2014). Due to the different fixations of the control and treatment, the background brightness had to be made equivalent in order to compensate for any differences in brightness the fixation may have caused. In order to determine the brightness of the cilium relative to the background staining, the background mean brightness was determined using the measure function of ImageJ 1.40g. This was done for the unedited images of the Rhodamine stain of both the control and experimental slides. The experimental image brightness was then divided by 2.298 using the math function in ImageJ to have the same brightness as the control image to allow for comparing of the two images. Next, using the measure function, the brightness of the cilia was found to compare to the brightness of another stained region. This allows for the determination if the believed ciliary region does contain a cilium.

Results

The results of this experiment depict that one in seven glial cells had identifiable cilia in *Gallus gallus* (figure 3), indicated with a circle. In *Lytechinus pictus* there are many cilia along the curved edge of the cell (figure 2). After the brightness of the cilium in the glial cell was found and the brightness of the control and experimental images adjusted to be equivalent they were compared to show the brightness of the cilia compared to the brightness of the rest of the cell. To compare the brightness of the cilia with the brightness of the cell the means were found using the measure function and then subtracted to find how much brighter the cilia was than the cell.

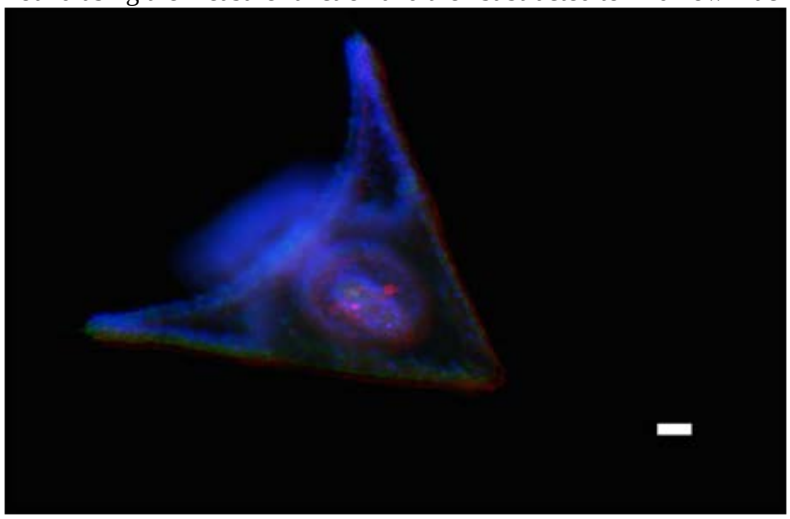

Figure 1 (Conn et al. 2014). Image of MeOH fixed *Lytechinus pictus* labeled for anti-alpha tubulin (green) and IFT88 (red) antibodies, and Hoechst stain taken using fluorescent microscopy. Cilia are present on the curved edge of this embryo, indicated by the brightness of the stain relative to rest of the embryo. Bar: 7.7 µm.

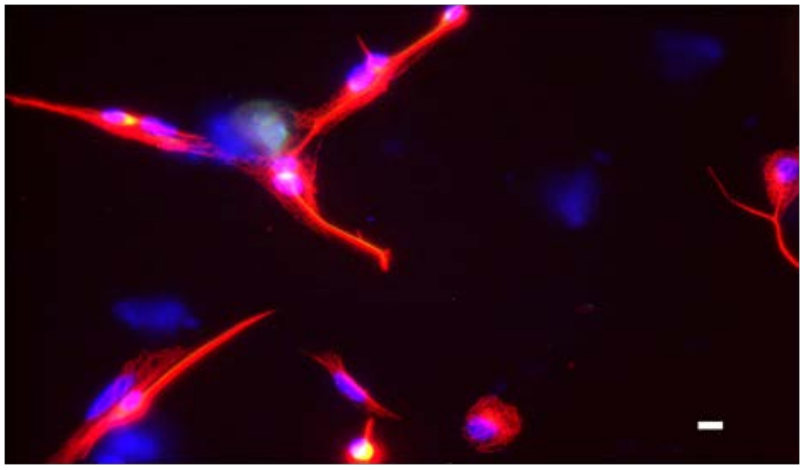

Figure 2. Glial cells of *Gallus gallus* fixed in formaldehyde/gluteraldehyde, labeled with IFT88 (red), and anti-acetylated tubulin (green) antibodies and Hoechst (blue) stain taken using fluorescent microscopy. A cilium like structure is seen inside the white circle. Bar: 7.7 μm.

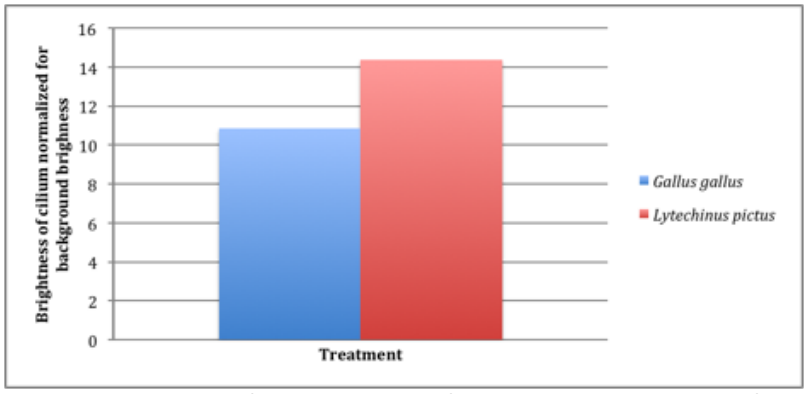

Figure 3. Brightness of cilia normalized for background brightness of *Gallus gallus* and *Lytechinus pictus* in the Rhodamine stain indicating the presence of IFT88. The brightness of the cilia in the *Lytechinus pictus* is close (3.527) to that of *Gallus gallus* indicating that the region found is potentially a cilium. IFT88 is a key protein in cilia and the presence of more in one region is indicative of a cilium.

Discussion

Figures 2 and 3 support the hypothesis that glial cells have cilia. This is due to the presence of a small, bright spot near the nucleus of the experimental image with brightness comparable to that of the known cilia in the control image. This supports previous research suggesting that glial cells have cilia (Lee 2011). The observation of a small, singular, bright spot near the nucleus compared to the background suggested that this was a part of the cell with high concentrations of IFT88 and acetylated tubulin (figure 2). Due to the relatively high presence of these forms of tubulin and IFT protein in cilium it was assumed that these bright spots indicated the presence of cilia. The brightness of the cilia-like structure in the glial cell was compared to the brightness of the *Lytechinus pictus*, known to have cilia. It was difficult to identify the cilia because of the high background noise on the slides; therefore more washes should be performed between treatments with antibodies. To refine this experiment only one type of fixation should be used and kept constant between all experimental and control treatments. Because the *Lytechinus pictus* embryos were previously fixed in methanol, this is the fixation I would recommend for the negative controls and experimental treatments.

While the data did suggest that there are cilia in glial cells there was not enough to conclude that all glial cells have cilia. Therefore, this experiment should be repeated to find more glial cells stained for IFT88 and anti-acetylated tubulin and determining if there are cilia present. If the results from this experiment were found after numerous replications it would be concluded that there are cilium in glial cells. This would lead to the conclusion that cilia do play a role in neurological development. However more research would need to be conducted into the function of cilia in development of the nervous systems. With this information ciliopathies affecting neurological development could be understood and potentially treated.

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