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Expanding on the involvement of primary cilia in neurological function through observing EEG/EMG changes in different genotypic mice

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ABSTRACT

Primary cilia have been known to regulate neurological functions, as primary cilia exist on astrocytes and neurons in the mature brain.[1] More is being discovered about primary cilia and their role in the vertebrate nervous system; however, little is known about their functions and molecular pathways in the mature brain. There also lacks methods of diagnosis for primary cilia-related defects and diseases. The purpose of this project was to provide more information on the involvement of cilia in the neurological functions and offer a potential means for diagnosis of cilia-related disorders by comparing the EEG and EMG signals of wildtype mice with the signals of IFT88 knockout and Arl13b-mCherry transgenic mice. The study was successful in identifying several potential points of comparison in the EEG/EMG plots of wildtype, IFT88 knockout, and Arl13b-mCherry transgenic mice. More data must be collected and analyzed to confirm these findings; however, the comparisons found in this project could be crucial in furthering knowledge of how we understand and study primary cilia and how they affect neurological functions and patterns in humans.

INTRODUCTION

Primary cilia are centriole-derived sensory organelles which act as small microtubule-based signaling devices. Present in most mammalian cells, primary cilia regulate a variety of physiological functions such as metabolism and cell division. Primary cilia have also been known to regulate neurological functions, as primary cilia exist on astrocytes and neurons in the mature brain. Primary cilia are not directly involved in synaptic communication; however, their impacts on obesity and mental disorders are widely recognized.[1] Recent studies have shown evidence for primary cilia in the vertebrate nervous system, connecting primary cilia to crucial neurological pathways such as the Sonic Hedgehog pathway. The Sonic Hedgehog pathway is a signaling pathway that plays an essential role in vertebrate embryonic development and tumorigenesis. As a result of discovering primary cilia's impact on many important factors in human development, there is now a large group of genetic disorders that have been tied to defects in cilia structure or function, called ciliopathies. One example of a ciliopathy related to

neurological function is Joubert Syndrome.[2] Joubert Syndrome is a rare genetic disorder characterized by cerebellar and brainstem defect in infants and children. This neurological developmental disorder is caused by a mutation of genes encoding primary ciliary proteins, resulting in dysfunctional cilia in the nervous system, affecting embryonic and neurological development. [3] Joubert Syndrome displays how defects in primary cilia can be related to changes in neurological function. More is being discovered about primary cilia and their role in the vertebrate nervous system; however, little is known about their functions and molecular pathways in the mature brain. There also lacks methods of diagnosis for primary cilia-related defects and diseases. The purpose of this project is to study neurological patterns of mice with a cilia-related knockout or transgene to learn more information about cilia and its involvement in the brain.

Knockout mice and transgenic mice associated with different cilia presence and function were studied in this project to provide more information on primary cilia's effect on neurological patterns and function. One type of mice being observed in this study is a Intraflagellar Transport (IFT) knockout. IFT is an active event in which cargo is transported along microtubules by motor proteins. IFT proteins are required for the formation and maintenance of flagella and cilia. [4] IFT protein 88 (IFT88) has been discovered as a crucial protein required for the assembly of primary cilia. The deletion of IFT88 has been associated with loss of primary cilia, causing a variety of defects such as in neurological function.[5] The other type of mice studied in this project are Arl13b-mCherry transgenic mice. In the adult brain, astrocytic primary cilia are often marked by ADP-ribosylation factor-like protein 13b (Arl13b). This protein is involved in regulating ciliary protein trafficking, the Sonic Hedgehog pathway, and neural development. Arl13b-mCherry is a double transgenic mouse strain in which the mCherry fluorescence reporter labels Arl13b-positive primary cilia.[6] This transgenic mouse was representative of the overexpression of primary cilia in mice. By studying mice with the IFT knockout gene and the Arl13b transgene, more insight can be achieved on cilia's effect on neurological patterns and its involvement in different neurological defects.

This project attempted to achieve more insight on the effects of cilia in the brain by using Electroencephalogram (EEG). EEG is a technique used to record and monitor neurological patterns. This technique is commonly used in research with rodents to help better understand states of sleep and wakefulness in animals and humans. Additionally, EEG is used to help study differences in physiological and disease states. Electromyography (EMG) is also used in this study. EMG measures the muscle response and electrical activity in response to a nerve's stimulation of the muscle. This technique can be used to help detect neuromuscular changes or abnormalities.[7] EEG and EMG techniques are utilized in this study in conjunction with anesthesia. The use of anesthesia in this project allowed EEG and EMG to be used more effectively to monitor changes in EEG waveform pattern in the mouse neocortex. In this project, the EEG and EMG signals of wildtype mice will be compared with the signals of IFT knockout and Arl13b transgenic mice. This comparison will not only provide more information on the involvement of cilia in the neurological functions, but it will also offer a potential means for diagnosis of cilia-related disorders.

MATERIALS & METHODS

Mouse Conditions

Mice were used in this project to evaluate differences in EEG signals upon anesthesia treatment. All procedures involving mice were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of New Hampshire and performed in accordance with their guidelines. Mice for experimentation were bred and genotyped. Data from 7 male and 7 female mice at ages between 4 and 5 months was recorded and analyzed. Mice were maintained in standard housing cages with controlled temperature at 22°C, where they had access to food and water ad libitum. A 12:12 hour light-dark cycle was used, as a consistent photoperiod is critical for regulating mice behavior and health.

EEG Headmount Implantation Surgery

Headmounts were implanted into the skulls of each mouse for the purpose of recording EEG and EMG signals in experimentation. Pinnacle's standard SL/SE headmounts were used for the 2 EEG/ 1 EMG mouse surgeries. All instruments were sterilized before the start of the operation, the surgical area was disinfected, and the experimenter was wearing proper PPE (clean lab coat, sterile gloves, covering mask, and hair net). The following procedure was followed for EEG headplate implantation surgeries.

Anesthetics and Pre-surgical Preparation

The mouse was sedated with isoflurane before transferring to the surgical apparatus. The mouse was treated with 3% isoflurane at 1000mL O_2 for approximately 5 minutes. The mouse was then transferred to a stereotaxic frame to fix the mouse's head in an appropriate position for the EEG implantation. The apparatus contained a heating pad to help minimize heat loss from the anesthetics. The surgical area was illuminated with a high-intensity light source.

The teeth of the mouse were placed in the tooth holder, with the tongue of the mouth below to avoid suffocation. The mussel piece was then screwed into place, securing the mouse's position, while also providing the anesthetic gas throughout the surgery. At this point, the anesthetics were reduced to 2% isoflurane at 500mL O_2. The ear bars were used to fix the head by positioning them below the mouse's ears. A protective gel was added to the eyes of the mouse to prevent from drying out.

The scalp of the mouse was disinfected with ethanol, and scissors were used to cut the hair on the scalp to prepare for incision. Further anesthetics were injected to prevent and treat pain from the procedure. A bupivacaine/ lidocaine mixture (1 mg/kg, 2 mg/kg) was administered to numb the skin and the periosteum at the incision site prior to starting surgery for local anesthesia. Butorphanol (1mg/kg) and Ketoprofen (5mg/kg) or Carprofen (5mg/kg) were administered by

subcutaneous injection. The back of the mouse was then covered with saranwrap to help avoid contamination. The scalp was cleaned 3 times with disinfectant solution.

EEG Headmount Implantation

Using sterilized scissors and tweezers, an approximate 2 cm longitudinal incision was made in the middle of the mouse's head, spanning from the eyes to ears. The skull periosteum was completely removed, and the area was dried thoroughly with a sterile Q-tip. The headmount was secured with cyanoacrylate and placed symmetrically so that the holes in the headmounts were in the frontal contralateral, frontal ipsilateral, parietal contralateral, and parietal ipsilateral locations on the skull. The holes were positioned bilaterally over the frontal and parietal cortex, so that the screw or electrode placement is optimal for recording EEG signals. Figure 1 shows the locations on a mouse skull to be used for reference for headmount placement. The figure also shows the purpose of each screw to be inserted in the headmount, including for grounding, reference, EEG 1 signal, or EEG 2 signal. The headmount should be left alone to dry for at least 5 minutes before continuing.

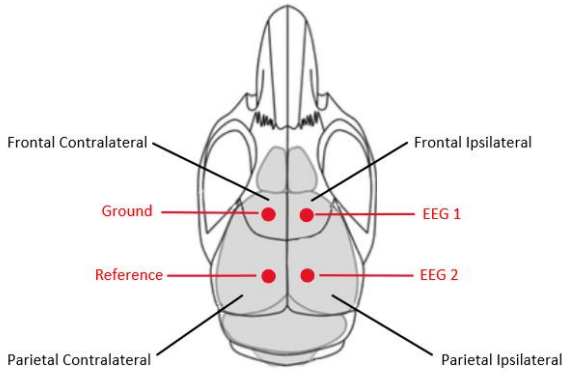

Figure 1. Reference figure for headmount placement. The headmount should be placed so that each section of the skull (frontal contralateral, frontal ipsilateral, parietal contralateral, and parietal ipsilateral) has a hole opening to be used for screws (shown in red). Frontal ipsilateral screw is used for the EEG 1 signal, the parietal ipsilateral screw is for the EEG 2 signal, the frontal contralateral screw is for the ground, and the parietal contralateral screw is a control for reference.

Holes in the skull were created with a 23-gauge needle by carefully rotating the needle and applying minimal pressure. Deep penetration of the needle into the skull was avoided to prevent unwanted damage to the skull and brain. If bleeding occurred, a sterile Q-tip was used to absorb excess liquid until bleeding ceased.

A screwdriver was used to advance 0.10 inch stainless steel screws halfway into the holes of the headmount and into the skull. The screws served as electrodes for EEG signals but also had the purpose of securing the headmount. Screws were added diagonally to allow the headmount to sit as evenly as possible. A minimal amount of two-part epoxy was placed between the screw head and the headmount holes to ensure there is a solid electrical contact between the screws and the headmount board. Following epoxy application, all screws were tightened.

EMG wires were inserted by making a small pocket in the nuchal muscles and using forceps to carefully bend the wire and insert into the opening. The wire was positioned straight in the cavity, not requiring any additional anchoring.

Sealing of Headmount and Suturing

Two-part dental sealant coating was used to coat all screws on the headmount to insulate and protect the EEG leads. The sealant was applied around the entire base of the headmount. Upon the drying of the sealant, a 19nm nylon surgical suture was used to close the incision around the headplate. Each suture was secured with two knots.

Following the suturing, the surgery was complete. The mouse was returned to the home cage for recovery. A heating pad was placed under the home cage until the mouse recovered from the anesthesia. The mouse was monitored over the next few hours and few days for signs of neurological damage or infection. All mice were given at least a week of recovery time before any experimentation was performed.

EEG Recording of Mice under Anesthesia

This project involved observing and comparing the EEG/EMG signals of wildtype mice and different transgenic and knockout mice to learn more information about how changes in primary cilia are related to neurological patterns. Since anesthesia slows brain activity, anesthesia can be a useful tool in observing changes in neurological activity recorded by EEG. The following procedure was performed for each mouse to obtain EEG and EMG data of mice under anesthesia.

A recording cable was prepared with a connector plug to connect the mouse to the recording device. The cable consists of a standard flat-ribbon cable with 4 insulated leads. The recording wires were connected to the mouses headmount through the 6-pin connector. The wires and headmount are very fragile and were handled with care to prevent damage. The mice were allowed to habituate to the cable and new house cage for at least 6 days before experimentation persisted.

The software used to record EEG and EMG signals was Sirenia Acquisition (1.0.3). The recordings were set to the following conditions: sample rate of 1000Hz, preamp Gain of 100, EEG gain of 1 and filter of 25,000, and EMG gain of 1 and filter of 100,000. For each anesthesia treatment, the EEG/EMG signal was recorded for 5 minutes while the mouse was still in the home cage. After the 5 minutes, the mouse was transferred to the anesthesia box at 2% isoflurane at 500L O_2. The EEG signal was recorded for 15 minutes under the isoflurane anesthetic. After the 15 minutes, the mouse was moved back to the home cage, where the EEG recording was continued for a minimum of 5 minutes. This isoflurane anesthesia treatment was repeated four times for each mouse. After the first test, the mouse was allowed to rest for 1 hour, followed by a

1 hour shock training test. After 30 minutes of rest, the second isoflurane treatment was performed. The mouse was allowed to rest for 1 hour, followed by another 1 hour shock training test. After 3 hours of rest, the third isoflurane treatment was performed. Lastly, the mouse was allowed 24 hours to rest before the fourth and final isoflurane treatment.

EEG Recording Annotation Analysis

Each EEG recording was analyzed using the Sirenia Sleep software (1.0.3). For each recording, annotations were placed at times which marked the start of the anesthesia treatment, each minute of the 15-minute anesthesia treatment, and the end of the anesthesia treatment. These annotations were used to observe and quantify the EEG/EMG signals throughout the anesthesia treatment, to help compare signal differences between each of the four anesthesia experiments, and to help compare signal differences between each mouse genotype.

The mouse genotypes compared in this study were wildtype, Intraflagellar Transport (IFT) protein 88 knockout, and Arl13b-mCherry transgenic mice. The purpose of using these mice was to see how EEG signals differ in mice with a cilia-related knockout or transgene versus the wildtype mouse. After annotating the plot of each of the 14 mice tested by EEG, one mouse from each genotype was chosen for the detailed comparison and analysis. Information about the three mice can be found in Table 1. These mice were chosen for comparison due to their strong EEG and EMG signals. For comparison of all EEG/EMG recordings, the plots were observed directly from the Sirenia Sleep Software. For the best resolution and visual for each signal type, each plot was observed with the EEG 1 and 2 signals on a scale of +/- 200 µV and the EMG signal on a scale of +/- 100 µV in Sirenia Sleep. Plots were compared to each other by observing changes in signal amplitude, frequency, and consistency. Differences from the first isoflurane treatment to the fourth treatment were also observed and compared between the different genotypes. The goal of the EEG analysis was to find distinct changes between the three genotypes to help provide more information about how ciliary-related defects affect neurological function and patterns.

Table 1. Information on the representative mice chosen for detailed comparison of EEG and EMG.

Genotype	Test #	Ear Tag	DOB	Surgery Date	EEG/EMG Test Date
Wildtype	14	950	5/21/2021	9/22/2021	10/12/2021
IFT88 KO	9	23	5/24/2021	9/15/2021	9/29/2021
Arl13b-mCherry	3	915	3/2021	7/9/2021	9/7/2021

RESULTS

Summary

The EEG and EMG signals from mice with a cilia-related knockout or transgene were compared to the signals of a wildtype mouse under anesthesia to help draw conclusions about ciliary

involvement in neurological function and patterns as well as provide potential means for diagnosis of cilia-related disorders. Three mice were chosen for EEG comparison. One mouse was a wildtype mouse, acting as a control representing normal ciliary function. The second mouse was a Intraflagellar Transport protein 88 (IFT 88) knockout. Since IFT 88 is crucial for the formation and maintenance of primary cilia, the knockout of this gene is associated with loss of primary cilia.[4,5] The third mouse studied in this project was an Arl13b-mCherry transgenic mice. This transgenic mouse refers to changes in the gene which encodes a member of the ADP-ribosylation factor-like family called Arl13b, which is involved in regulating ciliary protein trafficking, the Sonic Hedgehog pathway, and neural development. This double transgenic mouse type also has a mCherry fluorescence reporter which labels Arl13b-positive cilia, representing mice with an overexpression of primary cilia.[6] This transgenic mouse with ciliary defects was also compared to the wildtype mouse for changes in neurological patterns.

For comparison of all EEG/EMG recordings, the plots were observed directly from the Sirenia Sleep Software with the EEG 1 signal on the top (blue), the EEG 2 signal in the middle (orange), and the EMG signal on the bottom (green). The following analysis of each genotype's EEG plot was done by comparing changes from the first isoflurane treatment with the fourth treatment and observing changes in EEG/ EMG signal amplitude, frequency, and consistency. The goal of the EEG analysis was to find differences between the genotypes to lead to more information about how ciliary-related defects affect neurological patterns and how these defects are diagnosed.

Whole Plot Analysis

The entire range of the EEG/EMG plot from each mouse genotype was first analyzed to make distinctions in EEG/EMG signals before, during, and after isoflurane treatment. General differences were also observed between the first and fourth isoflurane treatments from each mouse. Figure 2 shows the whole plot of each genotype (A and D for Wildtype, B and E for IFT 88 Knockout, and C and F for Arl13b transgenic mice) from the first and fourth isoflurane treatments.

First, observations were made from the plots from the first isoflurane treatment. In general, all EEG and EMG signals can be seen with a relatively high amplitude directly before and after administration of isoflurane treatment. During the 15 minutes of the isoflurane treatment, EEG signal amplification decreases significantly for each genotype. All genotype plots appear to take a minimum of 5 minutes under isoflurane to decrease in amplitude and stabilize to consistent EEG signals. The Arl13b (C) EEG signal appears to take the longest to stabilize, taking around 7 or 8 minutes to produce a consistent signal. Some differences can be seen in amplitude of EEG signals during isoflurane treatment, such as Arl13b having the highest amplitude and IFT (B) having the lowest amplitude. Another interesting difference is that the wildtype (A) and Arl13b plots show large spikes in EEG activity throughout the isoflurane treatment. The IFT knockout plot appears to be most consistent, with no large spikes throughout isoflurane treatment.

Many changes in the plots can be seen from the fourth isoflurane treatment. All genotypes experienced higher amplitude EEG signals throughout the 15 minutes of the fourth isoflurane treatment compared to the first treatment. All genotypes also take a longer time to show an amplitude decrease and stabilization of EEG signals during the fourth treatment, with the stabilization time increasing to 6+ minutes for all genotypes. The IFT knockout (E) took the shortest amount of time (~6 minutes) for the EEG signal to stabilize, but the plot experienced large EEG signal spikes periodically throughout the isoflurane treatment. The wildtype (D) and Arl13b (F) EEG signals both never appear to fully stabilize during the isoflurane treatment, but rather their EEG signal amplitude appear to periodically decrease throughout the 15 minutes. The wildtype plot experienced the greatest increase in signal amplitude from the first treatment. The wildtype plot also displayed an extremely dense EEG 1 signal (blue), most likely resulting from a signal connection issue.

There can be several general conclusions drawn from these plots. First, the EEG signals during the 15 minutes of isoflurane administration increase in amplitude and experience less deviation from the normal EEG signals after multiple treatments have been performed. The IFT mouse displayed unique behavior compared to the other genotypes, as there was an apparent stabilization in EEG signals after 5 or 6 minutes, no matter what the treatment number. The wildtype and Arl13b EEG signals behaved very similarly to each other when observing the whole plots, as they both showed large signal spikes throughout the 15 minutes of the first isoflurane treatment. In addition, both wildtype and Arl13b EEG signals never seemed to stabilize in the fourth isoflurane treatment, but rather decreased gradually throughout the 15 minutes. Based on these plots, differences were observed that could potentially serve as a basis for distinguishing IFT knockouts from wildtype and Arl13b mice.

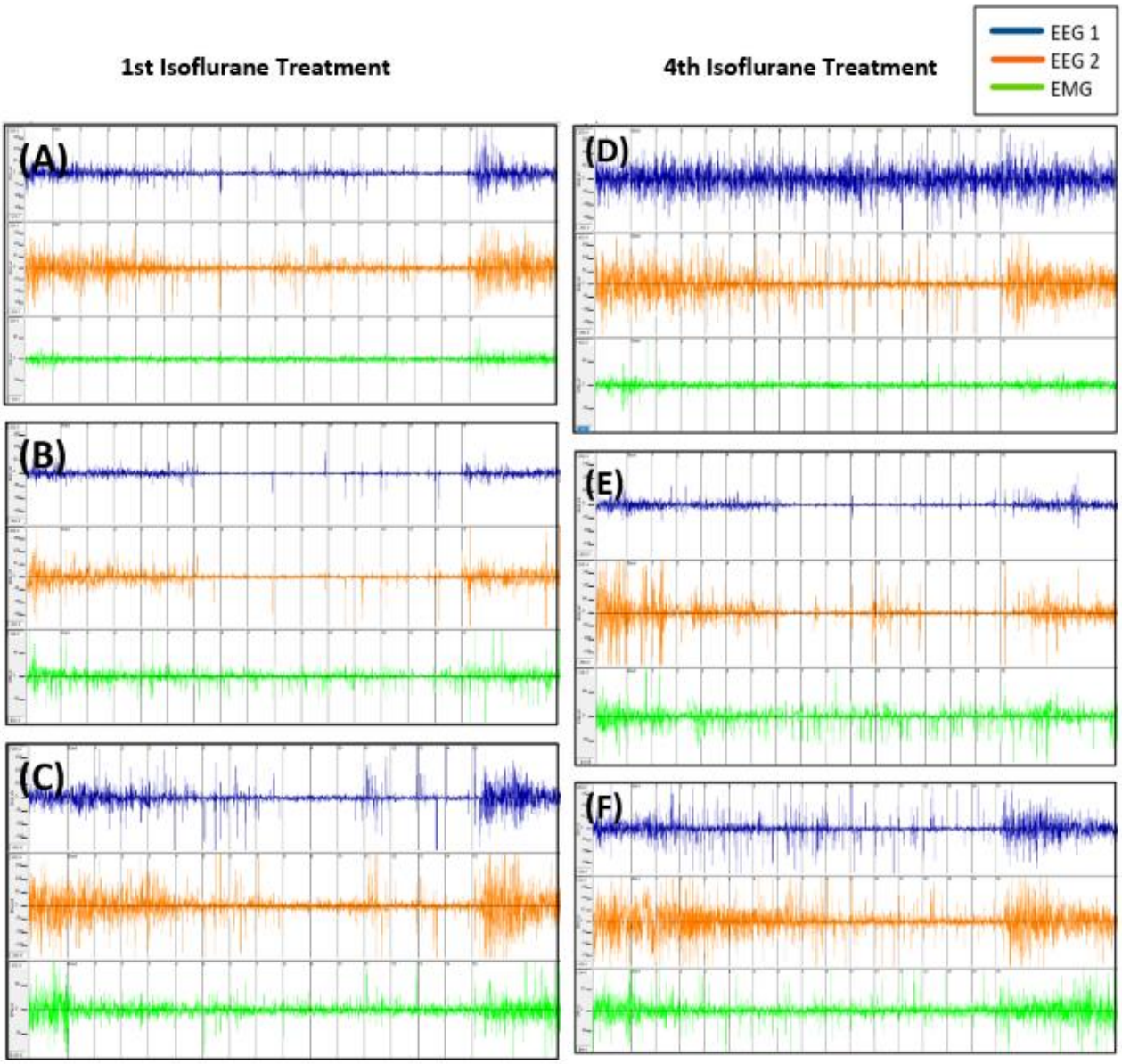

Figure 2. Whole EEG/EMG plot from each genotype (Wildtype, IFT88 Knockout, and Arl13b transgenic mice). This figure shows plots from the Wildtype mouse from the first isoflurane treatment (A), the IFT knockout mouse from the first isoflurane treatment (B), the Arl13b transgenic mouse from the first isoflurane treatment (C), Wildtype mouse from the fourth isoflurane treatment (D), the IFT knockout mouse from the fourth isoflurane treatment (E), and the Arl13b transgenic mouse from the fourth isoflurane treatment (F).

Detailed Plot Analysis

The last two minutes (minute 13 and 14) of the EEG/EMG spectra from each mouse genotype was analyzed to allow for more detailed observations to be made about signal amplitude, frequency, and consistency. Some more detailed distinctions were also made between the first and fourth isoflurane treatments from each mouse. Figure 3 shows the whole plots of each

genotype (A and D for Wildtype, B and E for IFT 88 Knockout, and C and F for Arl13b transgenic mice) from the first and fourth isoflurane treatments.

First, observations were made from the first isoflurane treatment plots. When observing the plots on a minute-to-minute scale, small and relatively consistent spikes in EEG signals can be seen. The wildtype (A) plot displayed the most frequent and consistent spikes, occurring around every 1-2 seconds. The Arl13b (B) displayed the second most frequent spikes, occurring around every 4-5 seconds. The IFT knockout (C) displayed the least frequent and least consistent spikes, ranging from every 2-3 seconds to every 10 seconds.

There are many similarities and differences in the plots from the fourth isoflurane treatment. First, it is important to point out that the wildtype plot (D) is displaying extremely frequent EEG 1 signals, most likely a result of a signal problem. However, the EEG 2 signal for the wildtype appears to be normal. In the fourth isoflurane treatment, the wildtype still has the most frequent EEG signal spikes, now occurring around every 2-3 seconds. The wildtype EEG signal spikes became slightly less frequent, less consistent, and with a higher amplitude than from the first isoflurane treatment. The Arl13b (F) plot still displays the second most frequent spikes, occurring around every 3 seconds. The Arl13b EEG signal spikes became more frequent and more consistent than from the first isoflurane treatment. The IFT knockout plot still has the least frequent EEG signal spikes, occurring around every 3-4 seconds. The IFT knockout signal spikes also became more consistent compared to the first isoflurane treatment.

Some conclusions can be drawn from these plots. First, the IFT knockout and Arlb both shows more consistency and frequency in their EEG signal spikes after four isoflurane treatments. The wildtype differed from the other genotypes in this aspect, as the wildtype EEG signal spikes became less consistent after the four isoflurane treatments. This information can be used to help distinguish between the Wildtype versus the IFT knockout and Arl13b transgenic mice.

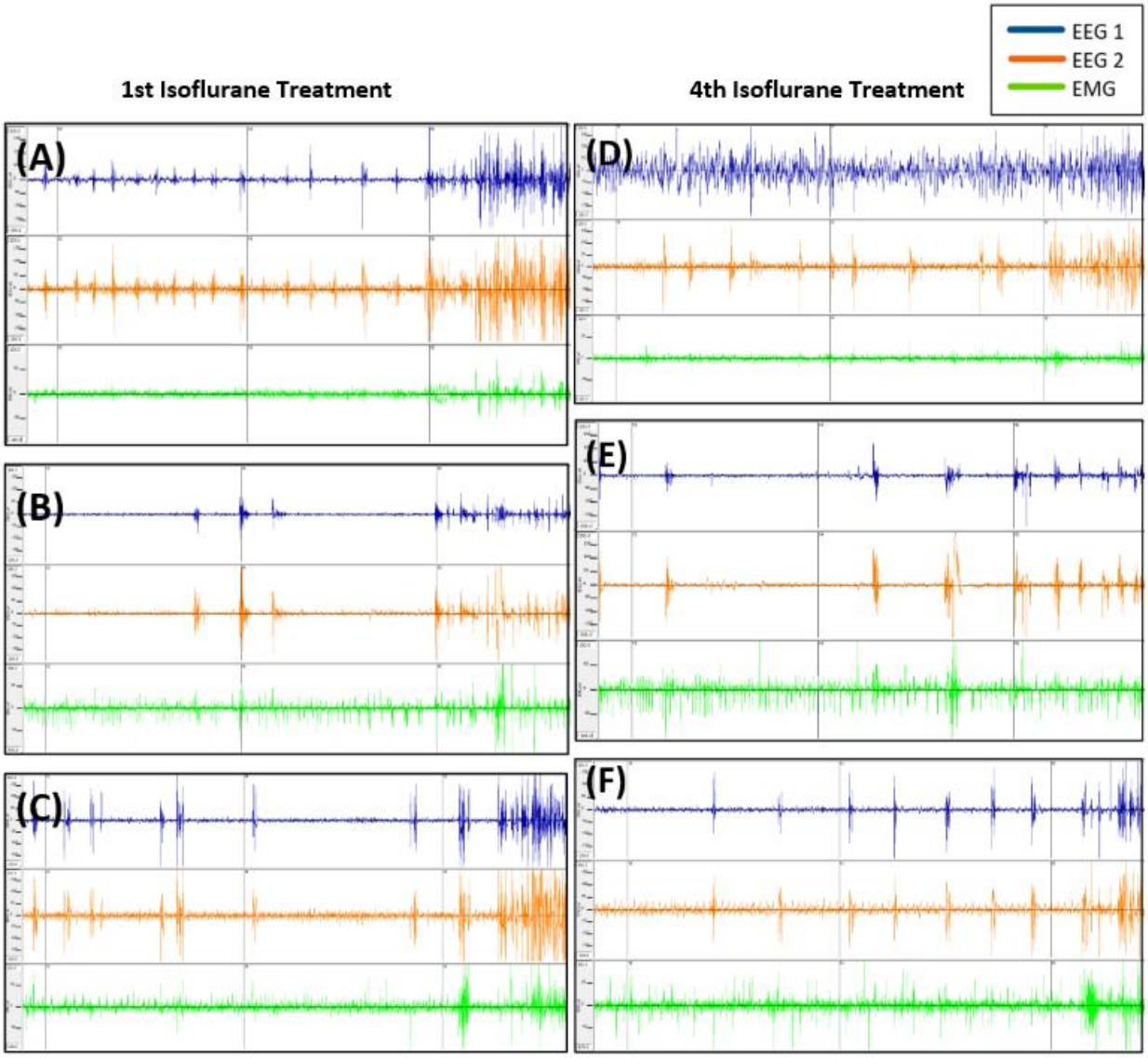

Figure 3. Detailed EEG/EMG plot from each genotype (Wildtype, IFT 88 Knockout, and Arl13b transgenic mice) from the last two minutes of isoflurane treatment. This figure shows plots from the Wildtype mouse from the first isoflurane treatment (A), the IFT knockout mouse from the first isoflurane treatment (B), the Arl13b transgenic mouse from the first isoflurane treatment (C), Wildtype mouse from the fourth isoflurane treatment (D), the IFT knockout mouse from the fourth isoflurane treatment (E), and the Arl13b transgenic mouse from the fourth isoflurane treatment (F).

Discussion

The purpose of this project was to provide more information on the involvement of cilia in the neurological functions and to offer a potential means for diagnosis of cilia-related disorders by comparing the EEG and EMG signals of wildtype mice will with the signals of IFT88 knockout and Arl13b-mCherry transgenic mice. The study was successful in identifying several potential

points of comparison in the EEG/EMG plots of wildtype, IFT88 knockout, and Arl13b-mCherry transgenic mice. First, when looking at the entirety of the EEG/ EMG plots, the IFT88 knockout mouse displayed a stabilization in EEG signals significantly sooner than the other mice under anesthesia, regardless of the anesthesia treatment number. In addition, the wildtype and Arl13b mice displayed EEG signals which never seemed to stabilize in the fourth anesthesia treatment, but rather decreased gradually throughout the 15 minutes. When looking at the detailed plot analysis of the last two minutes of the anesthesia treatments, the IFT knockout and Arl13b transgenic mice showed more consistency and a higher frequency in their EEG signal spikes after four anesthesia treatments, whereas the wildtype mice EEG signal peaks became less consistent after the four isoflurane treatments. These differences in the EEG/EMG plots can potentially be used to distinguish between the different genotypic mice.

This project had several limitations. One limitation was that changes or differences in EEG/EMG signals could have been a result of changes in the EEG/EMG signal being stronger or weaker in some mice versus others. This limitation can be reduced by continuing this study with more mice to confirm the comparisons made. When continuing the study, it will also be important to handle mice and the EEG/EMG recording cables with care to prevent damage which may affect the EEG or EMG signals. Another limitation was that there was a decent sample size of mice observed for this study, but many of the mice displayed poor EEG and EMG signals that could not be used for comparison and analysis in this study. To avoid this, consistency in surgical methods is very important.

Despite its limitations, this project can serve as a launching point for important neurological research regarding primary cilia function in neurological behavior. Primary cilia have been known to regulate neurological functions, as primary cilia exist on astrocytes and neurons in the mature brain.[1] More is being discovered about primary cilia and their role in the vertebrate nervous system; however, little is known about their functions and molecular pathways in the mature brain. There also lacks methods of diagnosis for primary cilia-related defects and diseases. The purpose of this project was to provide more information on the involvement of cilia in the neurological functions and offer a potential means for diagnosis of cilia-related disorders. Using wildtype mice, IFT88 knockout mice, and Arl13b-mCherry transgenic mice, this study compared the EEG and EMG signals of these mice under a 15 minute anesthesia treatment. By comparing these mice with genotypic differences in primary cilia function, some differences were discovered regarding EEG patterns under a series of anesthesia treatments. These differences can be informative of how changes in ciliary prescence and functions affects neurological functions. In addition, with more data and analysis, the changes in EEG patterns discovered in this project can potentially serve has a basis of diagnosis for primary cilia-related disorders and diseases. Much more data must be collected and analyzed, but the comparisons found in this project could be important in furthering knowledge of how we understand, study, and diagnose neurological patterns and abnormalities in humans.

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