PUPILLARY DILATION RESPONSES TO CHANGES IN SOUND STIMULI

by

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A THESIS

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To understand the world around us, the auditory system of our brains discriminates between different sounds to interpret our surroundings. Normally, simple sounds (like pure tones) are used to study the neural mechanisms for processing sounds by training animals. Training animals to discriminate between sounds is an arduous endeavor. Further, using simple sounds limits our understanding of how the brain interprets sounds of the complexity that is experienced every day. To address these problems, we developed a methodology to study sound discrimination in naïve mice without training the animals by using pupillometry.

Changes in pupil size is one of the many responses to stimuli an animal can have. A study performed by Montes-Lourido et al. found pupil diameter changes correlate with an increase in motivation, effort, and arousal in the brain in subjects (Montes-Lourido et al., 2021). Previous studies found changes in pupil sizes to sounds like pure tones and animal calls (Montes-Lourido et al., 2021). We hypothesized pupil responses would occur to changes in complex sounds that are found in nature, like water rushing or leaves crunching. To study natural complex sounds, we first had to establish if pupillary dilation responses occurred to changes in simpler sounds like

chords. We found that the pupils exhibited a pupillary dilation response to changes in frequency. Through this project, we determined pupillary dilation responses can be used as a method to study frequency discrimination in mice.

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Glossary

Chord: A sound with a combination of 3 or more sinusoidal waves combined

Head-Fixed Mice: Mice with a head-bar restrained using clamps so their heads remain in a fixed position

Pure Tones: A sound with one sinusoidal wave

Trial: One Stimulus Event

Session: A combination of all of the trials for one animal for one time period

Introduction

To understand the world around us and to communicate with one another, the auditory system of our brains works hard to discriminate between different sounds and interpret our surroundings. Exploring how the auditory system solves this problem is key for addressing diseases related to hearing and helps us improve our understanding of the fundamental mechanisms involved in sound interpretation. Normally, simple sounds (like **pure tones**[1]) are used in laboratory experiments to study the neural mechanisms for processing sounds. However, using simple sounds limits our understanding of how the brain interprets sounds of the complexity experienced every day. To address this problem, we wanted to study how neural circuits process complex sounds to enable behavior in a natural setting.

Normally, we would train the mice to discriminate between the sounds to study sound discrimination. Training mice to discriminate between sounds in previous experiments was an arduous endeavor, so we wanted to find a more efficient way to test sound discrimination without training animals. To accomplish this task, we decided to explore pupillary dilation as a method for determining whether the brains of the mice were able to distinguish between different sounds.

We used mice as a model because we can observe the function of specific neurons within the brain in more detail, as compared to human subjects. This is because there are many techniques for measuring neural activity of individual neurons and manipulating neural activity (Moulin et al. 2021). These techniques include electrophysiology, which can be used to study individual neural activity, and the

[1] Bolded words are defined in the glossary

mechanism of groups of neurons (Harris et al. 2017). It is very challenging to simultaneously record behavioral and electrophysiological data in a singular experiment in freely moving mice (Sattler & Wehr 2021). Using **head-fixed mice**[2] would allow us to study behavior and use other techniques more easily. Pupillometry is one way we can indirectly study how the brain discriminates between different sounds in head fixed mice.

[2] Bolded words are defined in the glossary (pg viii)

Background

Pupillometry in Previous Studies

Training mice in the previous experiments was an arduous endeavor, so we wanted to find a more efficient way to test whether mice were able to discriminate sounds. To accomplish this task, we decided to explore pupillary dilation as a method for determining whether the mice can distinguish between different sounds. The changes in a mouse's pupil size are one of the many responses an animal can have to stimuli (Montes-Lourido et al., 2021). These responses allow for the state of the brain to be understood (Montes-Lourido et al., 2021). Previously, researchers have demonstrated that pupil dilation responses can be used to estimate the state of the brain, which helps us understand if the animal is engaged with a task (Montes-Lourido et al., 2021). Further, researchers determined that the presentation of sound stimuli are correlated with changes in pupil size (Montes-Lourido et al., 2021).

A study performed by Montes-Lourido et al. observed that pupil diameter changes correlate with an increase in motivation, effort, and arousal in the brain in subjects (Montes-Lourido et al., 2021). Montes-Lourido et al.'s study provides support for the idea that auditory discrimination can be studied using pupillometry and that pupil responses correlate with what is occurring in the brain (Montes-Lourido et al., 2021). Further, this study shows that animal models can help provide an understanding of how humans categorize auditory stimuli (Montes-Lourido et al., 2021).

Furthermore, Winn et al. explained the benefits to using pupillometry as a method of scientific investigation (Winn et al., 2018). Winn focused on applying pupillometry to evaluate mental activity, arousal state and motivation to listen in

experiments (Winn et al., 2018). Zekveld et al. describes similar observations as Winn et al., specifically supporting the idea that pupillometry can be used to evaluate mental activity, arousal state and motivation in experiments(Zekveld et al, 2018).

Similar to these other researchers, Schwartz et al. explored using pupil size as a way to measure the activity state of the brain, specifically studying ferrets with simple tones and natural vocalizations (Schwartz et al., 2019). This study found changes in pupil size correlated to neural spikes in sound activated activities and random activity (Schwartz et al., 2019). Further, they discovered that in groups of neurons, the acoustic threshold decreased and correlated with the timing of the pupil changing size (Schwartz et al., 2019). The work of Schwartz et al. shows that changes in the pupil size correlated with changes in sounds, and further these changes also correlated with specific groups of neurons that could be studied (Schwartz et al., 2019). This means that not only are the arduous aspects of training cut out from these types of experimentation, but also that pupillometry can be built upon to monitor and study specific groups of neurons at the cellular level. Bala and Takahashi performed an experiment to determine whether pupillary dilation could be studied in owls to study sound stimuli without any training of the animals (Bala & Takahashi, 2000). This study showed that pupillary dilation responses occurred in response to sound stimuli without training the animals (Bala & Takahashi, 2000).

Pupillary responses to sounds are not isolated to animal models. Liao et al. studied changes in pupil sizes in response to sounds in humans (Liao et al., 2016). There were two tests, one in which sounds were being played and the pupil sizes were measured, and the other in which the participants were given a set of tasks to perform

and the sounds were played in the background and the pupil size were measured (Liao et al., 2016). In both cases, the pupils dilated to the sound stimuli (Liao et al., 2016). Even if the subject is focused on other things, a change in pupil size will still occur in response to the sound stimuli (Liao et al., 2016). This is crucial in supporting the idea that the changes in pupil size are due to the sound stimuli (Liao et al., 2016). The findings in this experiment determined that no training is needed for the discrimination and that even if the subject that is being tested is running or distracted, a change in the pupil will occur to a sound stimulus (Liao et al., 2016). This study also supports the claim that research related to pupil dilation in animals can inform our understanding of human responses to sound.

In past experiments, researchers used simple sounds, like **pure tones**[3] to test pupillometry and sound discrimination (Bala & Takahashi, 2000). However, our daily lives are not filled with simple sounds. Our environments are constantly filled with complex sounds. It is important to work towards developing an understanding of how the brain categorizes and understands complex sounds to understand how our brain interprets our surroundings.

To study pupillary dilation responses to natural complex sounds, we first had to establish if pupillary dilation responses occurred to changes in simple sounds. To accomplish this task, we had to develop a methodology to study the pupils of mice for our lab. Our project focused on using pupillary dilation to indirectly measure the discrimination of sounds accomplished by the brain.

[3] Bolded words are defined in the glossary (pg viii)

Purpose

. We wanted to develop a methodology to test sound discrimination without training the animals. Further, we wanted to develop a way to test sound discrimination in head-fixed mice so other experimental techniques can be used simultaneously to study the individual neurons and brain circuitry. In this project, we observed changes in pupil size at the onset of a chord and to changes in frequency of chords. This project acts as a foundation for using pupillometry as a methodology to study sound discrimination. Future studies can begin to explore complex sound discrimination using pupillary dilation responses.

Research Questions

- Is there a pupillary dilation response to the onset of a sound stimulus?
- Is there a pupillary dilation response to changes in frequency of a sound stimulus?
- Can pupillary dilation responses be used to study sound categorization in mice?

Hypothesis

We hypothesized pupillometry could be used to study sound discrimination in mice.

Methods

Animal Care:

Two different strains of mice were used in this experiment: C57BL/6 and CB57BL/6J. Three mice were C57BL/6 and six mice were CB57BL/6J. Animals were housed in the University of Oregon terrestrial animal care facility and were cared for by the Terrestrial Animal Care Services.

Surgeries:

Head bar implant surgeries were performed by Dr. Jenny Mohn on all mice. Animals were under post-operation veterinarian care for four days. Animals recovered fully and did not show any signs of health issues, so they were cleared for experimentation.

Rig Set Up:

For these experiments, the rig we used was a controlled acoustical environment produced by IAC acoustics. Inside of the rig, used for the experiment, we placed a 3-D printed mouse wheel. Above this wheel were two head-bar clamps spaced according to the length of the head-bar implant. These clamps secured both ends of the head bar so the animal's head was in a fixed position for the duration of the session. A speaker was mounted on the left side of the mouse wheel. We recorded the animal's left eye using a AILIPU Night Vision Webcam 1080p 100 fps USC camera with a 1/2,.7" 3.6 mm lens attached. This camera was positioned using a Thorlabs 10.5" post and placed as close as possible to the animal for the best view of the animal's face. A small, white light LED, which will be referred to as the visible LED, was mounted on top of TR4 9" Thorlabs post in front of the wheel to the left, to be visible by the left eye of the animal. An

infrared LED was mounted on two Thorlabs 6” posts and placed on the left side of the wheel. The infrared LED on the post was positioned in the view of the camera. An infrared LED was used because the mice are unable to see infrared, so the LED should not impact the pupil of the mouse (Ma et al. 2019). This infrared LED was turned on every time a trial was initiated and was used to signify when the trial occurred in the videos recorded. An AmScope LED-144A four zone microscope light was used as an environmental light and kept on for the entire session to ensure the pupil was small enough to change in size, either larger or smaller. When the light was not present, the pupil size was much too large, and would not allow changes to occur in pupil size.

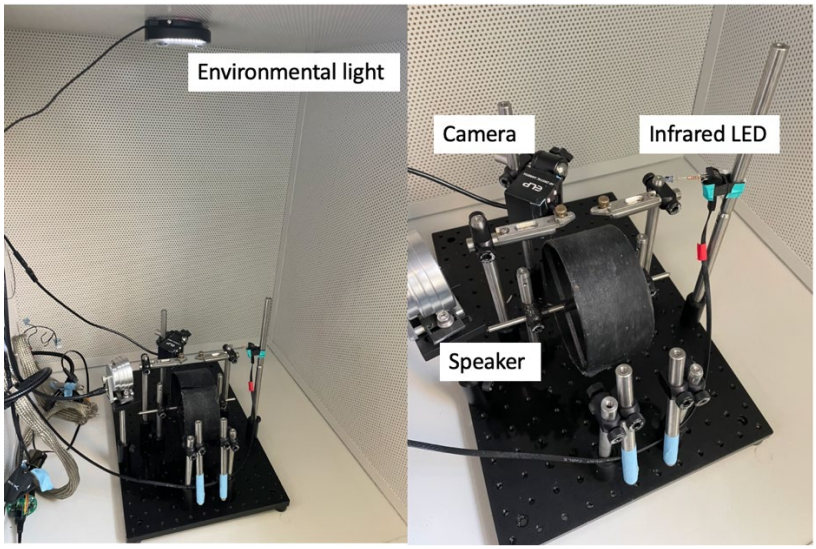

Figure 1: Set up of rig

The rig contained a visible LED, infrared LED, 1 speaker, a mouse wheel, camera, environmental light, and clamps for the head-bar implants.

Protocol for Each Session:

The mouse was placed on top of the wheel and the clamps were secured onto the head-bar. We opened the camera view window on our computer and test to ensure mouse’s pupil could be seen in focus and the infrared LED was in view. The door to the

rig would be closed and the environmental light was turned on. The mouse was left in the rig for 5 minutes to acclimate to the wheel and the lighting, then the session would start.

Control Stages:

To test pupillary changes to sounds and minimize the number of variables, we first came up with several control stages. The first control stage was the positive condition, which involved flashing the visible LED near the eye and the infrared LED. Mice have pupillary contraction responses to light, so we used this condition to determine if we could measure changes in the pupil (Bushnell et al., 2016). One **trial**[4] during the positive condition occurs when the visible light and the infrared LED are activated simultaneously. For one **session**, 100 trials were recorded. One trial occurred every 5 s +/- 2 s and lasted for 1s.

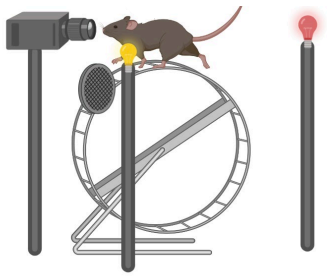

Figure 2: Positive condition

The positive condition includes the visible light and an infrared LED light. The mouse is running on a wheel as the video camera records each session. The visible light and the infrared LED simultaneously turn on. The light stimulus was on for 1s and occurred every 5 s +/- 2 s.

The next control stage, the negative condition, of the project involved the infrared LED. During these sessions, only the infrared LED would turn on during a

[4] Words in bold are defined in glossary

trial. Each session included 100 trials. One trial occurred when the infrared LED was on for 1 s. Trials occurred every 5 s +/- 2 s. The negative condition is crucial to establish if the infrared LED is changing the pupil size. If the infrared LED changed the pupil size, we would be unable to use the infrared LED to signify the onset of a trial during sound stimuli experiments because we would not know if the sound or the infrared LED was producing the changes in the pupil.

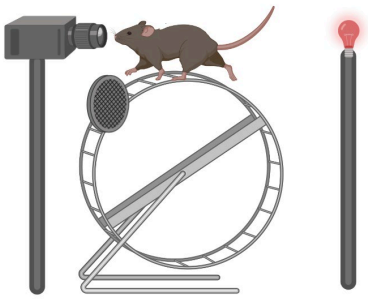

Figure 3: Negative Condition

The negative condition included just the infrared LED light. The mouse is running on a wheel as the video camera records each session. The infrared LED was on for 1 second and occurred every 5 seconds +/- 2 s.

Sound Stimuli Stages:

During the chordtrain stage, a **chord**[5] was played by the speakers and the infrared LED would simultaneously turn on with the onset of the sound. Initially, sounds were played for 1 s or 0.2 s every 5 s +/- 1 s for 100 trials. Then, various combinations of intensity(dB), intertrial interval time or ITI (s), and stimulus duration(s) were tested (Table 1).

[5] Words in bold are defined in glossary

Table 1: Configurations used for ChordTrain Experiments

Configuration	ITI (s)	ITI +/- (s)	Stimulus duration (s)	Stimulus Frequency (Hz)	Target Intensity (dB-SPL)
config1	5	1	0.2	6000	50
config2	5	1	0.5	6000	50
config3	10	1	0.5	6000	50
config4	10	1	0.2	6000	50
config5	5	1	0.5	6000	60
config6	20	1	0.2	6000	60
config7	30	1	0.2	6000	60
config8	5	1	0.2	6000	60
config9	13	1	0.2	6000	60
config10	20	1	0.2	6000	50
config11	20	1	0.2	6000	55
config12	20	1	0.2	6000	65
config13	5	1	0.2	6000	65
config14	10	1	0.2	6000	65
config15	15	1	0.2	6000	65
config16	15	1	0.5	13000	65
config17	10	1	2	2000	65
config18	10	1	2	3000	65

config19	10	1	2	5000	65
config20	10	1	0.5	17000	67
config21	10	1	0.5	17000	70
config22	10	1	0.5	17000	75

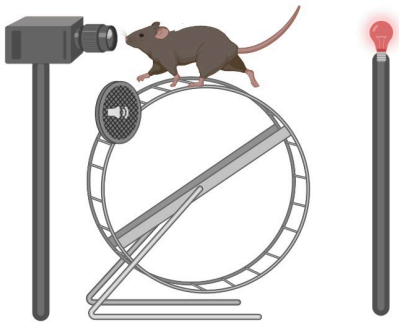

Figure 4: Chord train condition

A chord was played and the infrared LED simultaneously turned on.

After the Chord train condition, we began to test pupil responses to changes in frequency of chord sounds which is the frequency change condition. We specifically explored pupillary dilation responses to changes from one chord of one tone to another tone of a different frequency. Frequencies were chosen based on the hearing range of mice, which is 1 kHz to 100kHz (Reynolds et al., 2010). This experiment was preformed to indicate if the mouse's brain registers a change in the sounds and tested whether changes in simple sounds cause the animals pupils to dilate.

Many configurations were used to test the changes in frequency on the pupil (Table 2). These configurations were developed by adjusting the frequencies (kHz) used, the intertrial silence interval (ITI), the fade in of a sound (s), the sound intensity

(dB), the amount of environmental light and the length of time each of the sounds are played (figure 5). The fade in of the sound is the amount of time to slowly get louder until it reaches the target intensity. Each trial is followed by a period of silence called the intertrial interval(ITI) which is used to bring the pupil back to baseline.

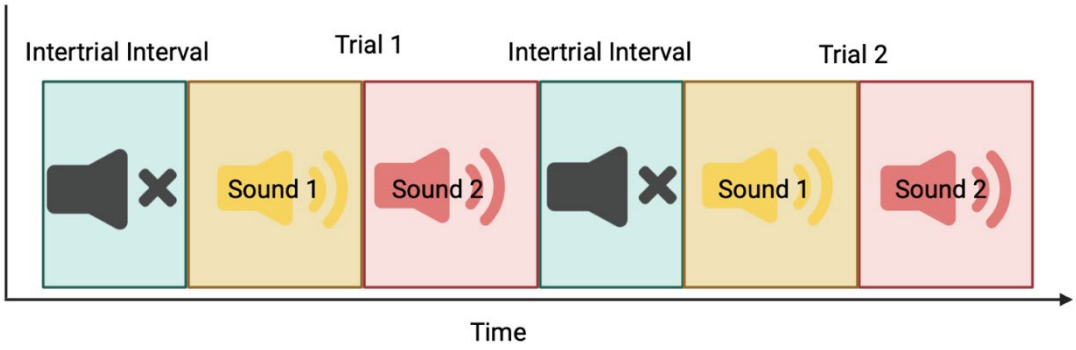

Figure 5: Example Timeline of 2 Trials in Frequency Change Condition

Each trial is signified by the onset of sound 1 and the offset of sound 2. The intertrial interval (ITI) is the interval of silence between each trial.

Table 2: Configurations used for Frequency Change Experiments

Configuration	ITI (s)	ITI +/- (s)	sound intensity (dB-SPL)	First Sound Duration (s)	total duration (s)	fade in (s)	min freq (kHz)	max freq (kHz)	Environmental light
2Sconfig1	15	0	65	1	2	1	6000	13000	level 1
2Sconfig2	15	1	65	1	2	1	6000	13000	level 1
2Sconfig3	15	1	65	5	10	1	6000	13000	level 1
2Sconfig4	15	1	65	5	10	1	6800	7000	level 1
2Sconfig5	15	1	65	15	20	1	6000	13000	level 1
2Sconfig6	15	1	65	15	25	1	6000	7000	level 1

2Sconfig7	15	1	65	5	15	1	2000	5000	level 1
2Sconfig8	15	1	65	5	10	1	5000	6000	level 1
2Sconfig9	15	1	67	5	10	1.5	5000	6000	level 2
2Sconfig10	15	1	67	5	10	1.5	13000	17000	level 2
2Sconfig11	15	1	70	5	10	1.5	6000	17000	level 2
2Sconfig12	15	1	70	5	10	1.5	6000	14000	level 2
2Sconfig13	15	1	70	5	10	1.5	6000	15000	level 2
2Sconfig14	15	1	70	5	10	15.	6000	16000	level 2
2Sconfig15	15	1	65	5	10	1	12750	13000	level 1
2Sconfig15	15	1	65	5	10	1	5750	6000	level 1

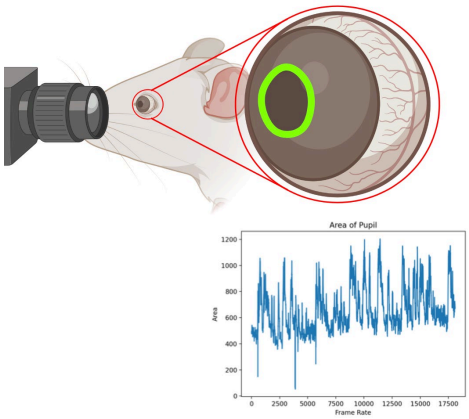

Figure 6: Analysis of pupil data methodology

The video footage is analyzed through a GUI called Facemap, where the pupil is captured, and the program is able to calculate the area of the pupil over the course of the entire session.

We measured the pupil area during each trial using the Facemap graphics bases operating system interface (GUI) video analysis software which automatically estimated the area of the pupil. After the pupil area was calculated, we also determined where

within the session the trial occurred by scoring when the sync LED was turned on using Facemap. Then, Manuel Ospina Meija developed a computer code using the Python programming language to graph the average changes in pupil size at the onset of a trial. Using Manuel's code, we graphed pupil area the seconds before and after the trials occurred to see the changes in the pupil size present when the stimulus occurs.

Results

The animals were first tested using config1 (table 1), positive condition and negative condition. The graphs of the sessions show the average pupil area (pixels^2) across one session. Time 0 s is when the trial occurs, or when the onset of the stimulus happens. The time before the trial is signified by the negative x axis and the time after the trial is signified by the positive x axis. Although only one or two graphs are shown per condition, each condition was tested several times over several days and conclusions were drawn from multiple sessions.

In all sessions of the positive condition, we observed a pupillary constriction (Fig 7, 9, 10). This test confirmed that we could measure changes in pupil area to stimuli presented. The negative condition was performed to confirm that there were no changes in the pupil to the infrared LED that was used to signify when the stimulus occurred. As seen in Fig 8, 9, and 10, there are no changes in the pupil area when the infrared LED is turned on. In config1 of our Chord train condition, there is no change in the pupil area seen to the onset of the sound (Fig 9 & 10).

Figure 7: Pupil Area Behavior in Chad049 on 10/30/2021 during Positive Condition

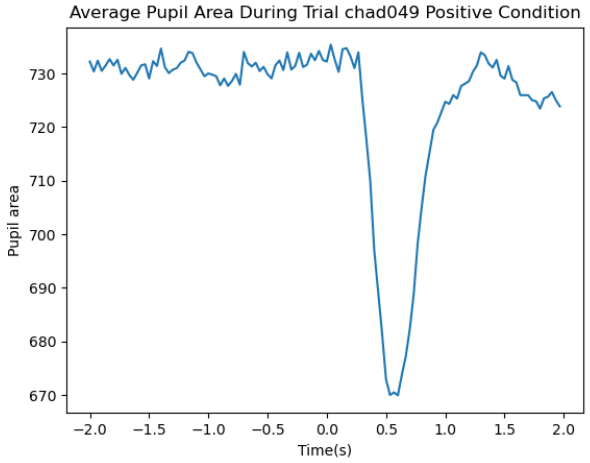

Figure 7: The average pupil area for one mouse was graphed against the timing of the stimulus occurrence for an entire session of 100 trials. The onset of the stimulus occured at t = 0 s.

Figure 8: Pupil Area Behavior in Chad048 on 10/30/2021 during Negative Condition

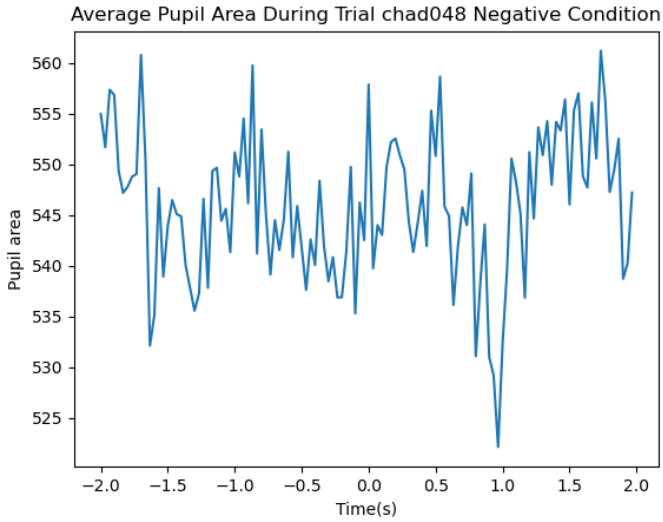

Figure 8: The average pupil area for one mouse was graphed against the timing of the stimulus occurrence for an entire session of 100 trials. The onset of the stimulus occured at t = 0 s.

Figure 9: Pupil Area Behavior in Chad049 during Positive, Negative, and Chord Train Config1 Conditions 10/28/2021

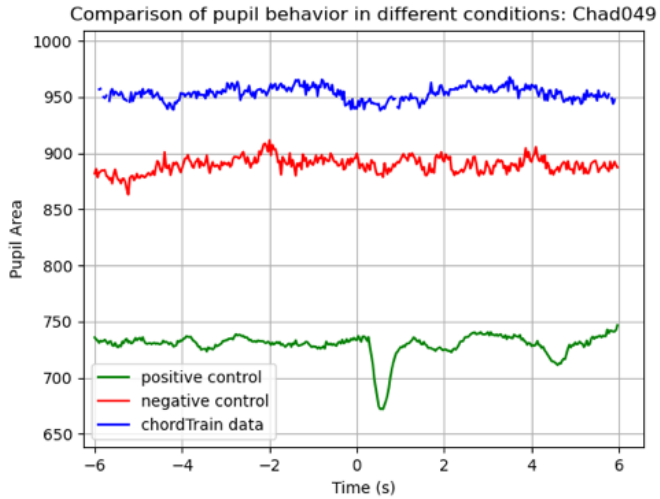

Figure 9: The average pupil area for one mouse was graphed against the timing of the stimulus occurrence for an entire session of 100 trials. The onset of the stimulus occured at t = 0 s. The positive condition (green), negative condition (red), and chord train condition (blue) sessions were graphed.

Figure 10: Pupil Area Behavior in Pure001 during Positive, Negative, and ChordTrain Config1 Conditions 10/28/2021

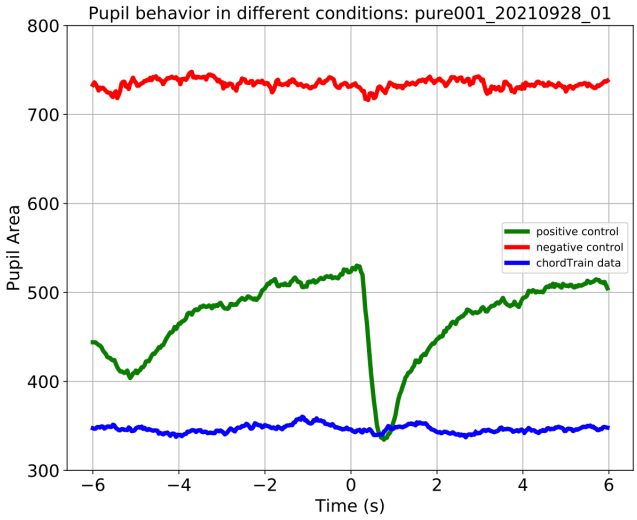

Figure 10: The average pupil area for one mouse was graphed against the timing of the stimulus occurrence for an entire session of 100 trials. The onset of the stimulus occured at t = 0 s. The positive condition (green), negative condition (red), and chord train condition (blue) sessions were graphed.

We were initially confused as to why we could not observe a change in the pupil size to the onset of a sound. Then, Montes-Lourido et al. published a paper on their project using pupillometry in guinea pigs to study auditory discrimination in which they used an ITI of ~1.5 min (Montes-Lourido et al. 2021). This ITI was much longer than the ITI we were using, which was 5 s at the time. So, we tested config6 (ITI = 20s), config7 (ITI = 30s), config12 (ITI = 10s), and config14 (ITI = 20s) to determine if a longer intertrial interval would elicit a change in the pupil size to the onset of a sound. As seen in Figures 11, 12, 13, and 14, the increase in intertrial time led to a response in the pupil of the mice, specifically a pupillary dilation. We tested several frequencies in the range of 1 kHz-32 kHz to determine if all frequencies exhibited a pupillary dilation response. All frequencies tested produced a pupillary dilation response. This suggested that the onset of a chord sound produced a pupillary dilation response.

Figure 11: Chordtrain Condition with Increased Intertrial Interval Time Period for Pure004 using Config6 10/30/2021

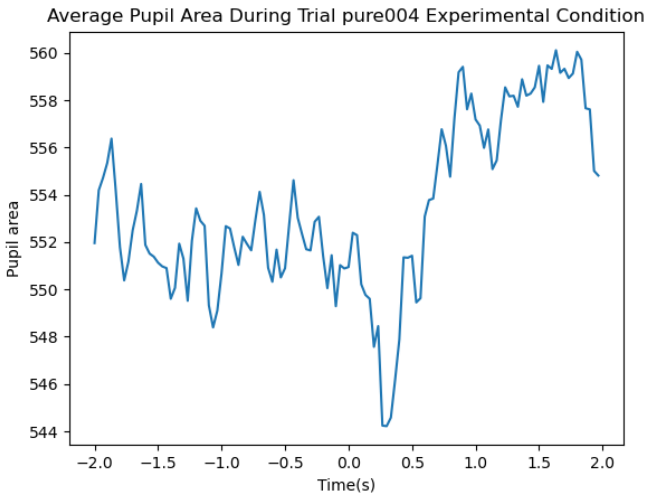

Figure 11: The pupil area was graphed against the timing of the stimulus occurrence. The onset of the sound occurs at t=0.

Figure 12: Chordtrain Condition with Increased Intertrial Interval Time Period for Pure004 using Config7 10/30/2021

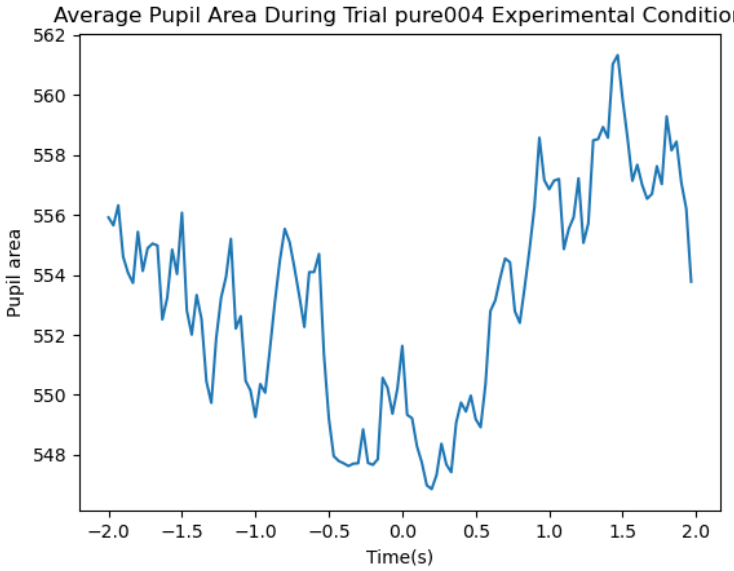

Figure 12: The pupil area was graphed against the timing of the stimulus occurrence. The onset of the sound occurs at t=0.

Figure 13: Chordtrain Condition with Increased Intertrial Interval Time Period for Pure004 11/21/2021

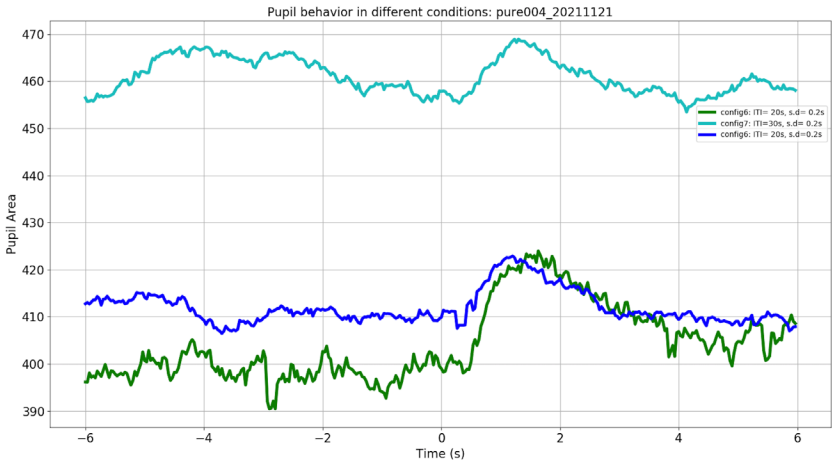

Figure 13: The pupil area was graphed against the timing of the stimulus occurrence. The onset of the stimulus occurs at t=0. Different Intertrial Interval Time periods are displayed: 20s (green & blue), 30s(teal). Data collected and graph created by Manuel Ospina Meija.

Figure 14: Chordtrain Condition with Increased Intertrial Interval Time Period for Pure004 12/07/2021

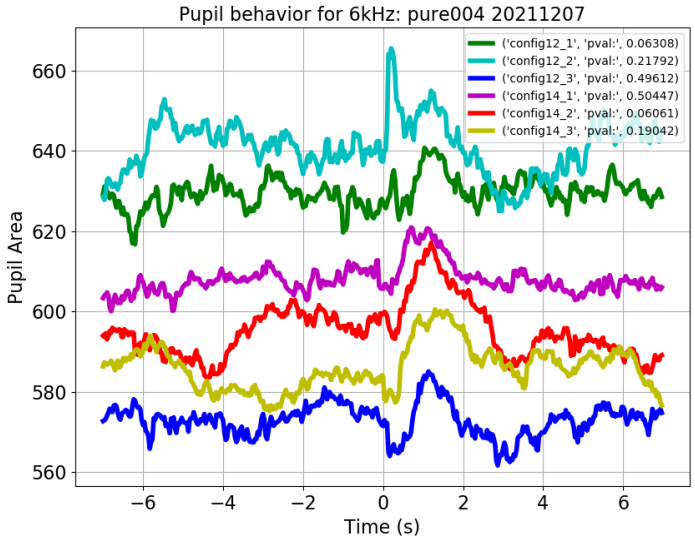

Figure 14: The pupil area was graphed against the timing of the stimulus occurrence. Several sessions across two days are pictured. The onset of the stimulus occurs at t=0. Different intertrial intervals were tested config12 (ITI = 10 s +/- 1s, Frequency = 6000 Hz, intensity = 65 dB) and config14 (ITI = 20 s +/- 1 s, Frequency = 6000 Hz, intensity = 65 dB). The time window from -0.5 s - 0 s before the stimulus and 1.4 s - 2 s after the stimulus were used to determine the p-value. Data collected and graph created by Manuel Ospina Meija.

After we confirmed we were able to observe pupillary dilation responses to the onset of a chord, we then tested if we were able to observe a pupillary dilation response to a change in frequency of a chord. We first tested large changes in frequency like 6000 Hz to 13000 Hz using 2Sconfig5 (table 2). These large changes in frequency produced a change in the pupil size at the onset of the first sound and at t = 0 s where the change in frequency occurs (Fig 15 & 16).

Figure 15: Pupillary Dilation Responses to Changes in Frequency Pure004 2Sconfig5 1/26/2022

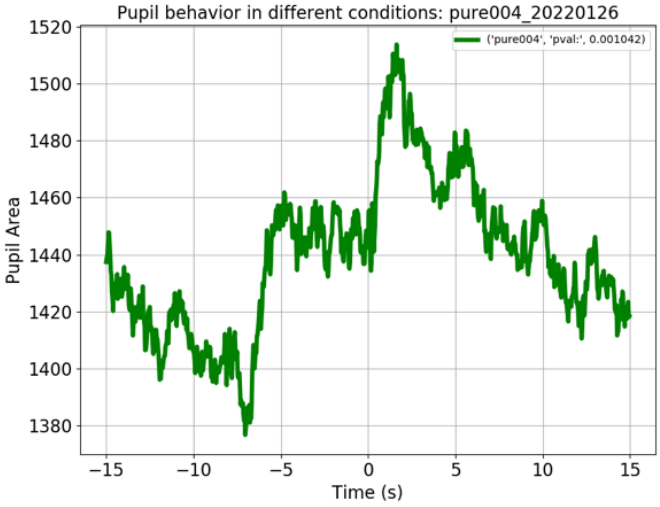

Figure 15: Pupil dilation responses to onset of sound and then subsequent change to a second sound. The pupil area was graphed against the timing of the stimulus occurrence. First sound stimulus 6000 Hz starts at t= -7 s and sound then changes to 13000 Hz at t = 0 s. The time window from -0.5 s - 0 s before the stimulus and 1.4 s - 2 s after the stimulus were used to determine the p-value (0.001).

Figure 16: Pupillary Dilation Responses to Changes in Frequency Pure004 2Sconfig5

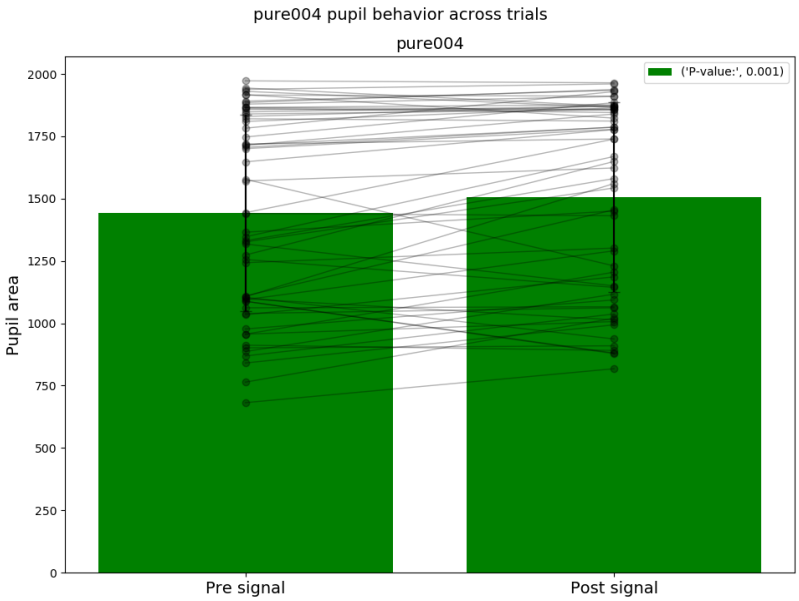

Figure 16: Average pupil area before stimulus change (Pre-Signal) vs. Average pupil area after stimulus change. First sound stimulus 6000 Hz starts at t= -7 s and sound then changes to 13000 Hz at t = 0 s. The time window from -0.5 s - 0 s before the stimulus is the 'pre-signal' and 1.4 s - 2 s after the stimulus is the 'post signal'. Each line represents one trial, where the dot is the pupil area before in pre-signal and after in post-signal.

Finally, after we observed a change in pupil area to large differences in frequency, we tested smaller changes in frequency like 5750 Hz - 6000 Hz, and 12750 Hz -13000 Hz. Both small changes in frequency tested produced a change in the pupil (Fig 17 & 18).

Figure 17: Pupillary Dilation Responses to Changes in Frequency Pure004 2Sconfig17 2/06/2022

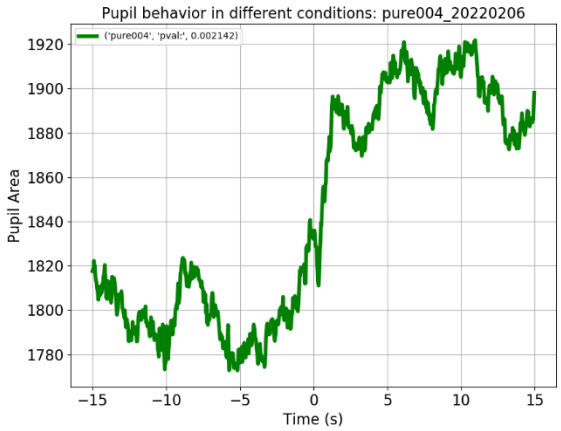

Figure 17: Pupil dilation responses to onset of sound and then subsequent change to a second sound. The pupil area was graphed against the timing of the stimulus occurrence. First sound stimulus 5750 Hz starts at t= - 5 s and sound then changes to 6000 Hz at t = 0 s. The time window from -0.5 s - 0 s before the stimulus and 1.4 s - 2 s after the stimulus were used to determine the p-value (0.002).

Figure 18: Pupillary Dilation Responses to Changes in Frequency Pure005 2Sconfig15 2/13/2022

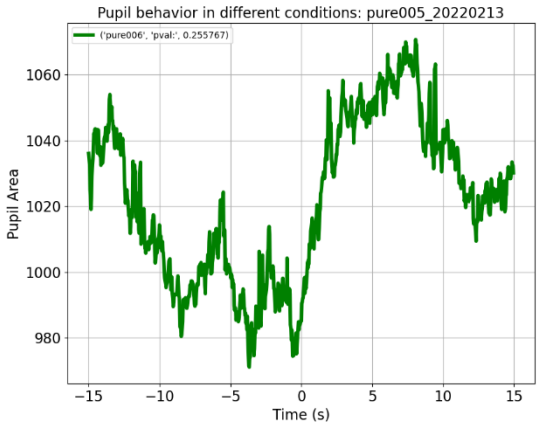

Figure 18: Pupil dilation responses to onset of sound and then subsequent change to a second sound. The pupil area was graphed against the timing of the stimulus occurrence. First sound stimulus 12750 Hz starts at t= - 5 s and sound then changes to 13000 Hz at t = 0 s. The time window from -0.5 s - 0 s before the stimulus and 1.4 s - 2 s after the stimulus were used to determine the p-value (0.25).

Discussion

The identification and discrimination of sounds allows the brain to create meaning from the environment. To understand how the brain interprets sounds, previous research in our lab trained mice to discriminate between different sounds. Training mice to discriminate between different sounds is an arduous endeavor. The goal of this project was to develop a methodology to indirectly measure sound discrimination without training the animals. This project gives us insight as to how our auditory system interprets sounds, thereby allowing us to interpret our surroundings. This project is the first step towards understanding how pupillometry can be used as a method to study sound discrimination in untrained mice.

We hypothesized that pupillometry could be used to study sound discrimination in mice. Further, we hypothesized that animals would exhibit a pupillary dilation response to the onset of a sound and to changes frequency of a sound. According to our data, we can measure changes in pupil size to the onset of different stimuli. The onset of a light stimulus decreased the pupil size during our positive condition. The onset of the sync light exhibited no change in pupil area during our negative condition. This finding was crucial for our experiment because we use the sync light to signify when the stimulus occurs, and we did not want the changes in pupil to be due to the sync light. Initially, we did not observe any changes in the pupil to the onset of a sound. After changing the ITI in our chord stimulus sessions, we observed a pupillary dilation response to the onset of a chord. Finally, we observed a pupillary dilation response to large and small changes in frequency of a chord.

This project provides the foundation for studying complex sounds using pupillometry. Since complex sounds have many frequencies in them, we wanted to confirm changes in the frequency of simpler sounds could produce pupillary responses. All changes in frequency that we tested exhibited a change in the pupil response. Since we found that changes in frequency brought about a change in the pupil area, future studies can begin to explore complex sound discrimination using pupillometry.

Training mice is an arduous endeavor: it often takes months to train mice to discriminate between different sounds. This project provides a way to test the ability of the brain to discriminate between different sounds without training the subjects beforehand. Using pupillometry as a method for experimentation in future research can be useful. Pupillometry is a great way to be able to test a hypothesis relating to the auditory system without having to train the animals for significantly long periods of time. Pupillometry is a simple method to study sound discrimination and a way to acquire different types of data at the same time. For example, pupillometry can be paired with electrophysiology to study the activity of individual neurons during different sound stimuli (Moulin et al. 2021). This can help researchers build an understanding of the circuitry involved in sound discrimination. Further, pupillometry can be paired with other experimental procedures like optogenetic inactivation of specific sections of the brain. This way, researchers can determine which sections of the brain are crucial for sound discrimination. Future research can focus on using pupillary dilation responses and electrophysiology to study complex sounds. Using these methodologies will help researchers begin to build a foundational understanding of how complex sound discrimination occurs in the brain. In this way, researchers can begin to

understand how humans discriminate sounds found in everyday life, like leaves crunching and waves crashing. Pupillometry is a simple experimental method that can be used with other forms of data collection simultaneously to create a more holistic approach to studying the discrimination of sounds.

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