FORMULATION OF A TWO-ALTERNATIVE CHOICE TRAINING REGIMEN FOR MICE

by

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Linguistic research has revealed that humans and animals can learn to discriminate sounds through both active training and passive exposure, but the changes in neural coding that occur as a result of these two types of training have still not been characterized. To investigate these neural changes in an animal model such as mice, it is first necessary to create protocols that can teach mice to discriminate between features of speech sounds. We were able to create successful training protocols for discriminating frequencies in a head-fixed setup and amplitude modulated (AM) sounds in a freely-moving setup. In our preliminary attempts, however, we found little success in training mice to discriminate between AM sounds in head-fixed setup. However, we have identified potential strategies that may improve the process. Through our findings, we have helped to solidify the active behavioral training methods for a two-alternative choice discrimination task, which can then be used for our explorations of active vs. passive training regimens and subsequent physiological investigations of the neural encoding of these sounds.

Acknowledgements

I would like to extend my deepest gratitude to the principal investigator of this lab and my primary reader Dr. Santiago Jaramillo, whose mentorship for the past four years has allowed me to become a better scientist and critical thinker. I cannot emphasize the skills and personal fulfillment I have derived from being a part of this research group. I would also like to thank my second reader, Dr. Jenny Mohn, who truly leads by example in her thoroughness and clear communication. Additionally, I thank my CHC representative and the dean of the honors college, Dr. Carol Stabile, for her constant guidance and encouragement.

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Introduction

Learning a foreign language is a notoriously difficult task, yet almost half of all enrolled students in educational institutions people pursue this challenge within the United States and Europe (Goldberg, et. al). One aspect of language learning that serves as a barrier is the ability to categorize unfamiliar speech sounds. For example, people who are raised with an East Asiatic language as their primary language often face difficulties differentiating between the English **phonemes** /r/ and /l/ (Lively, 1994). Meanwhile, native English speakers often have difficulty distinguishing amongst the different tonalities (changes in pitch) in Mandarin Chinese that give different meanings to syllables (Wang, 1999).

One intriguing linguistic study (Wright, 2015) found that in human participants attempting to learn new phonemes from a target language, the most robust category learning occurred in the group that combined active practice with passive exposure to unfamiliar speech sounds. This group advanced in their target language faster than the other group of participants who utilized either active practice or passive exposure alone. While this finding was fascinating in the sphere of research in linguistics, the theories of language learning have not yet been explored from the lens of cognitive neuroscience.

On the other hand, the field of cognitive neuroscience is replete with research related to brain plasticity after simple sound learning. For example, one study shows that after animals learn to discriminate sounds of different intensities, the responses of individual **auditory cortex** neurons were reshaped to became selective to certain intensities, demonstrating the brain's plasticity in response to noise level (Polley et al. 2004). Another experiment found that after ferrets engaged in a task to detect a target

frequency, the ferrets' auditory cortical neurons exhibited modulatory changes to target tones that they had been trained to detect, even hours after the task was finished (Fritz, et. al, 2013), This neural plasticity also extends to larger systems within the brain as well. One study by Recanzone and colleagues showed that attended stimuli can modify auditory **tonotopic organization** – the mapping of sound frequencies in auditory regions – within primates after undergoing a frequency discrimination task (Recanzone et al. 1993).

In addition to sound learning, another field of studies in neuroscience studies how the brain responds differently to auditory stimuli between active training and passive exposure. With rats, Carcea and colleagues demonstrate that when rats initiate their auditory task (an example of active training), this resulted in decreased responses to sounds in AC neurons, but improvements in auditory detection and recognition (Carcea et. al, 2017). Another study found that in auditory cortex neurons that have the same tuning (the degree to which two neurons respond similarly to a stimulus set), correlated variability tended to decrease with task engagement, compared to variability with passive engagement (von Trapp, 2016). To conclude, it seems that with active engagement to auditory stimuli, auditory cortex neurons fire less frequently but more specifically for certain stimuli.

While there have been studies on brain responses to both sound learning and to varying levels of auditory engagement, there has not been a comprehensive systematic investigation into how the latter affects the former. This study hopes to investigate how the combination of active training and passive exposure affects the learning of sounds from both a behavioral and cellular lens.

Project Development

This project was started as a collaboration between the linguistics research group of Dr. Baese-Berk and the neuroscience research group of Dr. Jaramillo. The linguistics side of the project utilizes human subjects to explore different language learning theories, while the neuroscience side of the project works with mice as an animal model for exploring the neural processes that occur during language learning.

The overall goal of the neuroscience side of the project is to characterize the changes in neural coding of speech sounds by auditory cortical neurons as a result of active training and passive exposure. The characterization will be accomplished through **electrophysiological recordings** of cells in the auditory cortex, the region of the brain responsible for processing auditory signals.

However, before these recordings take place, it was first necessary to have a training protocol that is successful in teaching mice to discriminate between sounds. My role in this project was to solidify the behavioral training protocol that could serve as the "active training" portion of these experiments.

Materials and Methods

Mouse Model

Mice were selected to be used as this project's animal model for multiple reasons. First, mice have been shown to have the cognitive complexity to be able to learn human phonetic categories when these sounds are frequency-shifted to the appropriate hearing range (Saunders and Wehr, 2019). Furthermore, the mouse model allows for the ease of manipulation and monitoring of neuronal activity that is not feasible in humans.

This project utilized C57BL/6J mice, the strain of mice used most commonly across research laboratories (Capri, 2019). Mice underwent water restriction to foster motivation for completion of the task, since water was used as their primary incentive. Water was provided during the animals' daily training, but they had continual access to food. All animal procedures were overseen by the University of Oregon Institutional Animal Care and Use Committee.

Surgical procedures

Isabella Salinas, a graduate student in the Jaramillo lab, and Jenny Mohn, a postdoctoral researcher in the Jaramillo lab, completed all the surgeries for headbar installations, which were needed for the head-fixed behavioral task.

First, the mouse was anesthetized by 3% isoflurane. An incision was made to expose the top of the skull. A headbar was lowered onto the skull and secured in place with layers of cured superglue. After the glue dried sufficiently, mice underwent post-op observations for three days.

Auditory Stimuli

The goal of this project is to characterize the brains' ability to categorize speech sounds. Preliminary data from the Jaramillo lab have shown that it is possible to teach mice to discriminate between human phonemes such as /ba/ from /da/ or /pa/ when the frequency is shifted to the mice's hearing range. Yet, while these results were promising, the training process for discriminating between human phonemes took the mice over 30 days, raising an issue for electrophysiological recordings. It is difficult to record from the brains of mice for many weeks at a time, given the limited viability of craniotomies. It was necessary to find auditory stimuli that were simple enough for mice to learn within a few weeks, but still carried features relevant to speech sounds.

Two aspects of sounds were determined to be important: modulation in spectral components and modulation in temporal components. Spectral component modulation refers to how the types of frequencies of soundwaves that comprise a sound change over time. For example, it is the different wavelength frequencies of the phonemes /ba/ and /da/ that allow us to tell the difference between the words "dog" and "bog" (Figure 1, left two panels)

Temporal component modulation refers to how amplitude of a sound changes over time. An example of speech sounds that differ in this regard are the phonemes /ba/ and /pa/. Spectral features are largely the same between the two sounds, except the voice onset time is later for the /pa/ phoneme (Figure 2, right two panels).

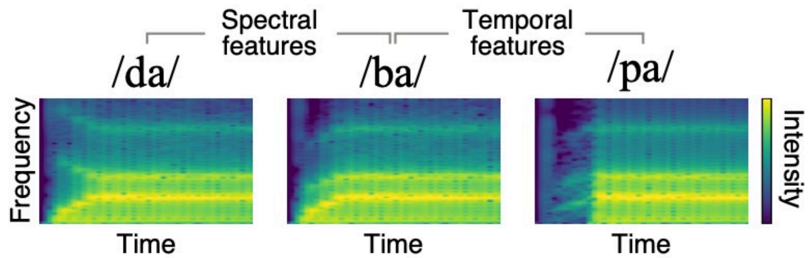

Figure 1. Spectrograms of human phonemes

The spectrogram created from human phonemes demonstrate the features that make them sound different. /da/ and /ba/ differ in the directional sweep of their frequency components, a spectral feature. /ba/ and /pa/ differ in voice onset time, a temporal feature. Figure was adapted from the grant written by Dr. Jaramillo and Dr. Baese-Berk for this project.

Pure tones also differ in spectral modulation, through their differences in wavelength frequencies. Pure tones served as the simplified version of complex spectrally modulated sounds. Examples of pure tones can be seen below (Figure 2).

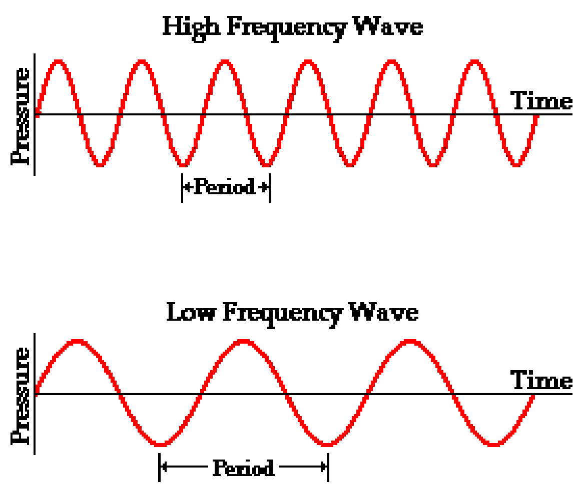

Figure 2. Sound signal plot constructed from two pure tones

Figure was adapted from The Physics Classroom (n.d.).

Amplitude modulated (AM) sounds also differ in temporal components because their amplitude changes as a function over time (Figure 3). AM sounds served as the simplified models of temporal modulation. The mice were trained to discriminate either between modulated and unmodulated sounds or between sounds of different rates of modulation.

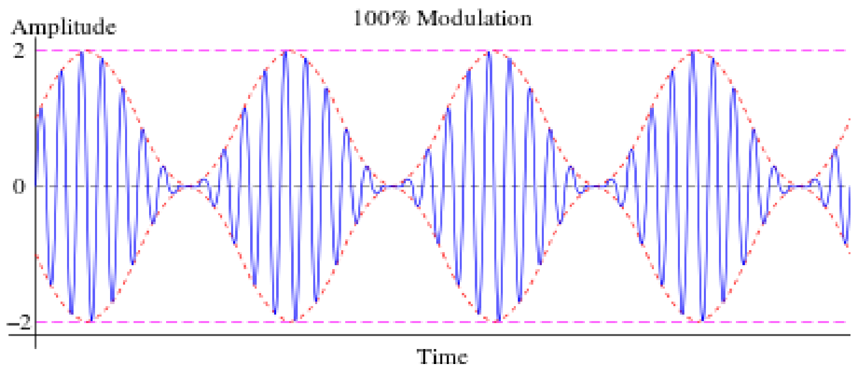

Figure 3. Sound signal plot of an amplitude modulated (AM) sound

AM sounds differ in loudness over time. The AM sounds may vary between AM rates, or level of modulation (differences in amplitude between peaks and troughs). Figure was reprinted from Wikimedia Commons (2015). https://en.wikipedia.org/wiki/Amplitude_modulation

Behavioral Training Paradigm

The mice underwent training paradigms for at least one hour daily. Mice typically performed hundreds of trials per training session. Trials are comprised of the presentation of the stimulus, followed by a period allotted for the mouse to make a choice, and then time allotted to collect their reward, if they chose correctly.

There were two types of setups used in lab: freely-moving, which is often used to observe behavior, and head-fixed, which is often used when recording neurons.

In the freely-moving setup (Figure 4), mice are free to run around a box that contain three adjacent water ports with lick sensors. There are correct sounds associated with the left and right lick port. For example, in the AM discrimination protocol, the slower rated sound is associated with the left, but the faster sound is associated with the right. By the end of the training, the mouse should be able to complete the full task: begin a trial by poking the center port, listen to the presentation of the sound, and then poke the corresponding port on either the left or the right. If the mouse's choice was correct, they will receive a water reward.

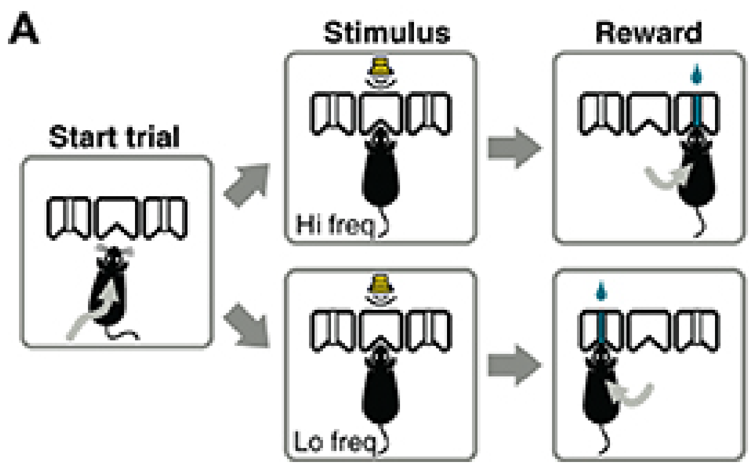

Figure 4. Freely-moving training setup

Figure was adapted from Jaramillo & Zador (2014).

In the head-fixed protocol (Figure 5), the mice's head are secured to a rig while they are free to run on a wheel. Waters spouts with lick sensors are adjusted to be directly in front of the mouse's mouth. Trials begin automatically as soon as the paradigm is launched. By the end of training, the mouse should be able to complete the full task: listen to the entire sound and then lick the corresponding spout on the left or right. If the mouse licks the correct spout after the sound is played, they will receive a water reward.

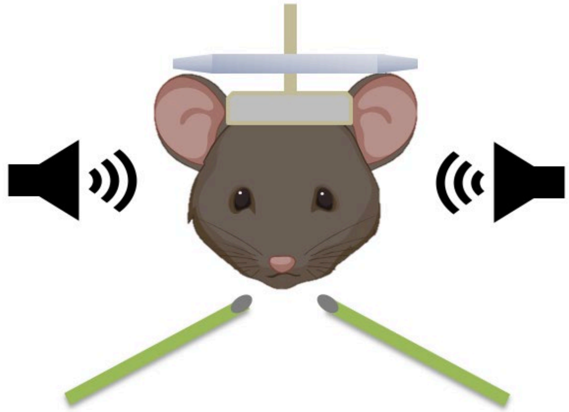

Figure 5. Head-fixed training setup

Image created by Isabella Salinas via BioRender.

Head-fixed training is the preferred method of training because it allows for recording of the cells through electrophysiology.

In both head-fixed and freely-moving setups, the mice's behavior was shaped by starting very simply and introducing new expectations in stages until they were performing the final task. A list of all the training protocols that were tested in the freely-moving and head-fixed setups can be found below.

Training Parameters for Freely-Moving Setup

In a freely-moving setup, five main training parameters changed from stage to stage.

1. The "outcome mode" parameter determines what actions the animal must take before they receive their water reward. The list of choices for the "outcome mode" parameter is shown below and increases with difficulty down the list.
    - Sides direct: whenever an animal pokes in a side port, the sound for that corresponding side plays and water is delivered to that spout immediately. If the mouse pokes the center port, the sound and associated reward are presented at random.

- Direct: whenever an animal pokes in the center port, water is delivered immediately in the corresponding side port for that trial.
- On next correct: after poking the center port, the animal must lick the corresponding port at any point during the allotted time following the sound presentation to receive their water reward. Even if they lick the incorrect side, they can still obtain the reward as long as they “correct” their choice by licking the correct port during the allotted time.
- Only if correct: after poking the center port, the animal must lick the corresponding port on their first try to receive their reward

2. The “mean delay to target” parameter determines the length of time between animal poking the center port and the sound playing. Default for this parameter is 0.0 sec.
3. The “automation mode” is a special parameter that determines if the “mean delay to target” parameter increases or remains fixed. The default mode for this parameter is off.
   - Increase delay: this increases the delay period by 0.01 seconds every 10 trials
   - Off: delay period remains fixed
4. “Anti-bias mode” is a special parameter that is used in case the animal is biased towards one side. The default mode for this parameter is off.
   - Repeat mistake: if the mouse chooses the incorrect port after the presentation of one of the two stimuli, that stimulus will repeatedly be used for the next trial until the animal chooses correctly.
   - Off: sounds are presented at random.

5. “Psycurve mode” is a special parameter that is used to test animals’ ability to discriminate amongst a range of sounds, rather than between two sounds. Users must also specify the number of “steps” between the two extremes.
   - On – The auditory stimuli are presented that exist in a range between two extremes.
   - Off – only the two stimuli are presented

Additionally, there were four important parameters that remained constant throughout all the stages:

1. The “High AM rate” parameter determines the AM rate of the faster stimulus of the two. This parameter was set to 32 Hz for all stages
2. The “Low AM rate” parameter determines the AM rate of the slower stimulus of the two. This parameter was set to 8 Hz for all stages.
3. The “steps” parameter determines how many other AM rates are presented between the extreme AM rates in the Psycurve mode. This parameter was set to 6 for the stages where Psycurve mode was on.
4. “Target duration” parameter determines how long the stimulus was presented. This was set to 0.2 seconds for all stages.

Training Protocol for AM Discrimination in Freely-Moving Setup

The training protocol used to teach mice to discriminate between rates of AM sounds can be found in Table 1. The stages that utilize a parameter that is off by default are described in the “stage-specific parameters” column.

Stage	Goal for Mice	Outcome Mode	Mean Delay to Target	Stage-specific parameters	Criteria to Pass
0	To become familiar with the reward delivery ports	Sides Direct	0.0 +/- 0.0	n/a	100 rewarded trials
1	To start trials by poking in the center	Direct	0.0 +/- 0.0	n/a	200 rewarded trials
2	To wait for the beginning of the sound and to reach the correct port for reward to be delivered	On next correct	0.0 +/- 0.0	Automation mode: Increase delay	300 rewarded trials
3	To associate each sound with the corresponding reward port	Only if correct	0.2 +/- 0.05	n/a	To move to stage 4: 70% correct on each side and at least 300 trials total. To move to stage bias correction: Accuracy for one side is <20%
Bias Correction	To reverse the animal's bias towards one port	Only if correct	0.2 +/- 0.05	Anti-bias mode: repeat mistake	To return to stage 3: performance must be > 30% accurate in the side against which the animal was originally biased
4	To test how animals respond to range of sounds between the extreme sounds	Only if correct	0.2 +/- 0.05	Psycurve mode: On	n/a

Table 1. Stages of the freely-moving training AM discrimination training protocol

Stages with their associated changing parameters used for teaching mice to discriminate between AM sounds in a freely-moving setup are shown.

Training Parameters for Head-Fixed Setup

In a head-fixed setup, there were four main training parameters that changed from stage to stage:

1. The “Lick before stim offset” variable determines what occurs if a mouse licks before the sound is done being played. There are four different outcomes:
    - reward: if the mouse licks before the sound ends, they are rewarded anyway
    - abort: if the mouse licks before the sound ends, the sound aborts and the reward is not given
    - ignore: only the licks after the sound ends are counted as licks for a certain "choice"
    - Warning beep: if the mouse licks before the sound ends, a warning beep is played, and the reward is not given
2. The “reward side mode” determines which stimuli are presented during each trial.
    - random: the stimuli are played at random
    - repeat_mistake: if the mouse chooses the incorrect spout after the presentation of one of the two stimuli, that stimulus will repeatedly be used for the next trial until the animal chooses correctly
    - onlyL: only the sound corresponding to the left port will play
    - onlyR: only the sound corresponding to the right port will play

3. “Task Mode” determines what actions the animal must take before they receive their water reward. The list of choices for this parameter is shown below and increases with difficulty down the list.
    - water_on_sound: water reward is available after the initial presentation of the sound.
    - water_after_sound: water reward is available after the full presentation of the sound.
    - water_on_lick: water is given when the animal licks the spout. Upon licking the corresponding sound is presented.
    - lick_after_stim: to obtain a water reward, the animal must lick the corresponding spout at any point during the allotted time following the sound presentation. Even if they lick the incorrect side, they can still obtain the reward as long as they “correct” their choice by licking the correct spout during the allotted time.
    - discriminate_stim: to obtain a water reward, the animal must lick the corresponding spout on their first attempt.
4. “Sound type” determines what auditory stimuli are played.
    - AM depth: tones that differ in rates of modulation
    - chords: pure tones that differ in frequency

Additionally, there were five important parameters that remained constant throughout all the stages:

1. If the “sound type” parameter was set to AM depth, the “High AM depth” parameter determines the level of modulation for greater modulated stimulus of the two. This parameter was set to 100% for all stages.
2. If the “sound type” parameter was set to AM depth, the “Low AM depth” parameter determines the level of modulation for the decreased modulated stimulus of the two. This parameter was set to 0% for all stages.
3. If the “sound type” parameter was set to chords, the “High frequency” parameter determines the higher frequency of one of the two stimuli presented. High frequency was set to 13,000 Hz for all stages.
4. If the “sound type” parameter was set to chords, the “Low AM depth” parameter determines the lower frequency of one of the two stimuli presented. Low frequency was set to 6,000 Hz for all stages.
5. “Target duration” parameter determines how long the stimulus was presented. This was set to 0.2 seconds for all stages.

Training Protocol for Frequency Discrimination in Head-Fixed Setup

We utilized one training protocol (Table 2) for teaching mice to discriminate between AM sounds in a head-fixed setup. In all stages, the “sound type” was set to chords.

Stage	Goal	Reward Side Mode	Task Mode	Criteria to Pass
1	To teach the mice to lick the spouts for water	Random	Water on sound	100+ licks on each side
2	To teach the mice to lick at the correct time	Repeat mistake	Lick after stimulus	100+ hits on each side
3	To teach the mice to lick the correct side to obtain water reward	Repeat mistake	Discriminate stimulus	100+ licks and >70% accuracy on each side

4	To test if the mice can discriminate between the presented sounds	Random	Discriminate stimulus	100+ licks and >70% accuracy on each side

Table 2. Stages of the head-fixed frequency discrimination training task

Training stages with their associated changing parameters used for teaching mice to discriminate between frequencies in a head-fixed setup are shown.

Training Protocols for AM Discrimination in Head-Fixed Setup

We utilized three different training protocols for teaching mice to discriminate between AM sounds in a head-fixed setup. In all these three protocols, the "sound type" parameter was set to AM depth.

First AM Head-Fixed Protocol: Original protocol

This protocol was identical to the one used in Table 2, except the "sound depth" parameter was set to AM depth instead of chords.

Second AM Head-Fixed Protocol: Implementation of the Warning Beep

This protocol represented the first time implementing the Warning Beep mode in the "lick before stim offset" parameter.

Stage	Goal	Reward Side Mode	Lick Before Stim Offset	Task Mode	Criteria to Pass
1	To teach the mice to lick the spouts for water	Random	Reward	Water on sound	100+ licks on each side
2	To teach the mice to lick at the correct time	Repeat mistake	Ignore	Lick on stimulus	100+ hits on each side
3	To teach the mice to lick the correct	Repeat mistake	Warning Beep	Discriminate stimulus	100+ licks and >70%

	side to obtain water reward and that they cannot lick before full sound presentation				accuracy on each side
3.5	To slowly wean mice off the warning beep mode	Repeat mistake	Abort	Discriminate stimulus	100+ licks and >70% accuracy on each side
4	To test if the mice can discriminate between the presented sounds	Random	Ignore	Discriminate stimulus	n/a

Table 3. Second head-fixed AM discrimination training protocol

This protocol is notable for being the first protocol in which the “lick before stim offset” parameter was changed between stages.

Third AM Head-Fixed Protocol: Earlier Implementation of the Warning Beep

This protocol attempted to incorporate the warning beep mode earlier within the protocol to reinforce the importance of lick timing in mouse trials.

Stage	Goal	Reward Side Mode	Lick Before Stim Offset	Task Mode	Criteria to Pass
1	To teach the mice to lick the spouts for water and that the water will not come before the sound ends	Random	Reward	Water after sound	100+ licks on each side
2	To teach the mice that they cannot lick before the sound is done being presented	Random	Warning Beep	Water after sound	70% of trials are rewarded
3	To teach the mice to lick a specific port when a	Repeat mistake	Warning Beep	Lick on stimulus	100+ hits and >70% accuracy on each side

	certain sound plays				two days in a row
4	To reinforce mice's idea that certain ports are specific to certain sounds	Repeat mistake	Warning Beep	Discriminate stimulus	100+ licks and >70% accuracy on each side two days in a row
4.5	To slowly wean mice off the warning beep mode	Repeat mistake	Abort	Discriminate stimulus	100+ licks and >70% accuracy on each side for two days in a row
5	To test if the mice can discriminate between the presented sounds	Random	Ignore	Discriminate stimulus	n/a

Table 4. Third training head-fixed AM discrimination protocol

This protocol is notable for its use of the warning beep mode early in the stages.

AM Head-Fixed Protocol Modification: Testing a "Remedial" Stage

A "remedial stage," was created to combat low accuracy rates, to which mice could be temporarily moved from Stage 4 in third AM head-fixed protocol. During this remedial stage, mice would only hear one sound for half an hour and then the other sound for the other half of the hour with an automatic reward, as long as they did not lick early.

- Reward side: onlyL for 30 minutes, and then onlyR for 30 minutes
- Lick before stim offset: warning beep
- Task mode: water after sound

Results

Freely-moving training protocol for AM discrimination resulted in learning

In the span of 20 days on average, 5 out of the 6 animals put on this protocol passed the three learning stages of the freely-moving AM discrimination task. The number of days taken to complete these stages ranged between 15 and 27 days. To completely learn the task means that, within an hour's training session, the animal had to have completed at least 300 trials, with at least their 70% of their total choices on either side being correct.

Furthermore, after passing the three learning stages, the animals demonstrated their ability to categorize a range of AM modulation rates as low or high by choosing a side (Figure 6).

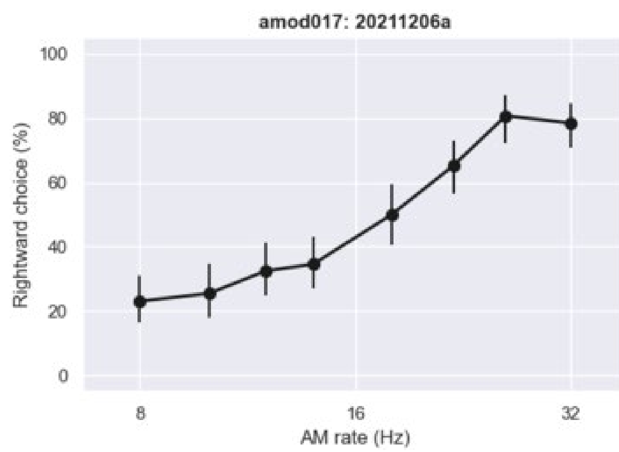

Figure 6. Mice can discriminate between a range of AM rates in head-fixed setup

Psycurve graph generated from one mouse's training session on 2021-12-06 shows an example of what we want the animal's behavior to be at the end of training. Although this mouse appeared to choose sides randomly at intermediate frequencies, his accuracy rates for the sounds near the extreme sides of AM rate spectrum had high accuracy.

It is worth noting, however, that one animal was unable to pass the three learning stages, even after multiple rounds of bias correction. Additionally, while the psychometric function in Figure 6 demonstrates an example of the ideal behavior of animals in the psycurve mode, animal performance was often variable amongst different sessions, with performance sometimes being skewed to one side.

The training protocol was successful in teaching the majority of animals to complete the task, yet their performance while being tested left gaps to be desired, such as demonstrated bias to one side.

Head-fixed training protocol for frequency discrimination resulted in learning

In the span of 12 days on average, 4 out of the 4 animals put on this protocol passed the last stage of frequency discrimination, meaning that within one training session, they had licked at least 100 times and had an accuracy rate of 70% or higher. As in the case of the AM freely-moving training protocol, the animals demonstrated an ability categorize a range of frequencies modulation rates as low or high by choosing a side (Figure 7).

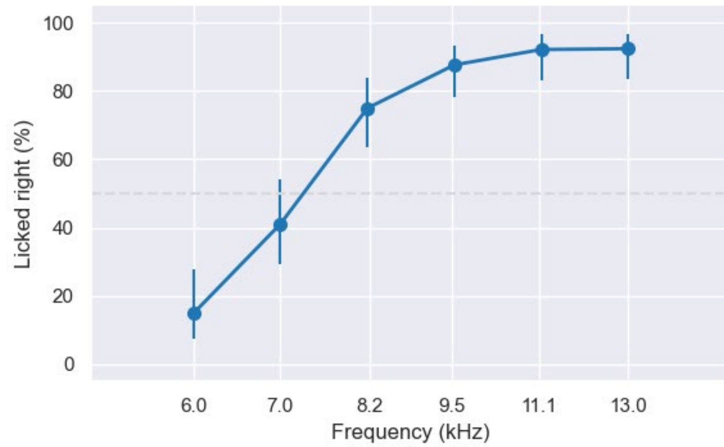

Figure 7. Mice can discriminate between a range of frequencies in a freely-moving setup

This psychometric function, generated from one of the mice's training sessions, demonstrates a slight rightward bias, but an accuracy rate of over 80% at the extreme sides of the frequency spectrum. These results were consistent among this mouse's other sessions on the psycurve sessions as well as among all animals put on this training protocol.

First head-fixed training protocol for AM discrimination failed to result in sufficient learning

Despite undergoing this training protocol for over four weeks, 10 out of the 10 animals put on the first AM head-fixed training protocol stalled on Stage 3.0, the stage in which mice had to lick the correct side on their first try for their attempt to count as a correct choice. The criteria for mice to pass Stage 3.0 was to lick at least 100 times per side and get an accuracy rate of 70% or higher per side, and no mouse was able to achieve this (Figure 8).

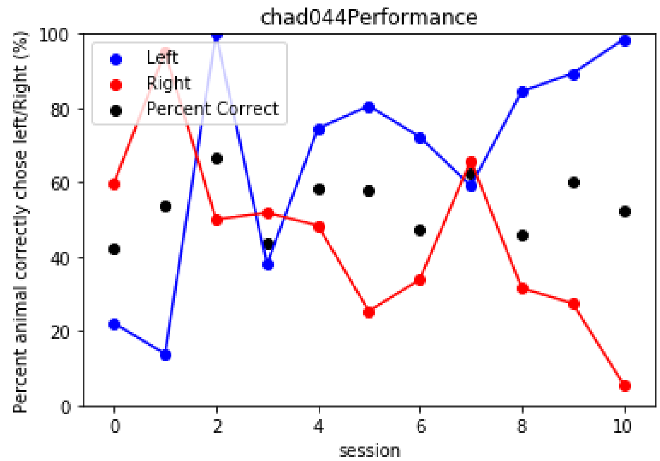

Figure 8. Mice's accuracy rates flatlined in the first head-fixed AM training protocol

This performance graph was generated from one mouse's sessions on Stage 3.0. The graph is noticeable for its minimal improvement beyond baseline. Chad044 starts at an accuracy rate of around 40%, which only climbs to 50% over the course of 10 sessions. The results amongst the other 10 animals on this protocol also reflect minimal improvement.

We hypothesized that perhaps the low accuracy rates in this training protocol were due to the mouse's lick timing. A common trend amongst all the mice were that they tended to make choices before the sound was done being played at t = 0.2 seconds (Figure 9).

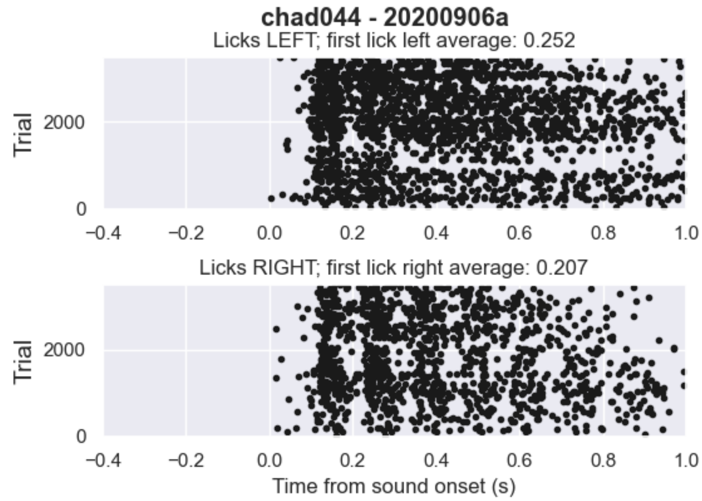

Figure 9. Mice lick prematurely on the first AM head-fixed training protocol

This lick raster plot, generated from a mouse's training session on 2020-09-26, shows how long after the sound starts being played (t = 0.0 seconds) the mouse begins to lick. Each black dot represents one lick. The clustering of licks from t = 0.0 sec to t = 0.2 show that the mouse tends to make their first licks sometime before the sound is done being played. This tendency was shared amongst all the animals in most of their sessions.

Unlike the two frequency-based stimuli, the two AM stimuli sound very similar at the onset. If the mice made choices before they heard the full presentation of the sound, they might be making "uninformed decisions," which would explain their low accuracy rates. To attempt to foster a later licking time and consequently higher accuracy rates, we decided to implement a warning beep mode. If the mice lick prematurely, the sound aborts, a "warning beep" plays, and the mice receive no reward.

Implementing a warning beep did not result in a sufficiently delayed lick-time

Over the course of over five weeks, 5 out of the 5 animals put on the second head-fixed AM training protocol again stalled on Stage 3.0. Like the first protocol, Stage 3.0 is the stage in which mice had to lick the correct side on their first try for their attempt to count as a correct choice. The criteria for moving to the next stage was also the same—to lick at least 100 times per side and get an accuracy rate of 70% or higher per side. No mouse was able to achieve this (data not shown).

Furthermore, the mice's lick-times had still not increased to a sufficiently long enough time. Ideally, since the sound ends at around 0.2 seconds and we want the mice to process the sounds they just heard for at least 0.05 seconds, the ideal first lick-time would be later than t = 0.25 seconds. However, despite the implementation of the warning beep mode, the majority of mice tended to still lick before t = 0.25 seconds (Figure 10).

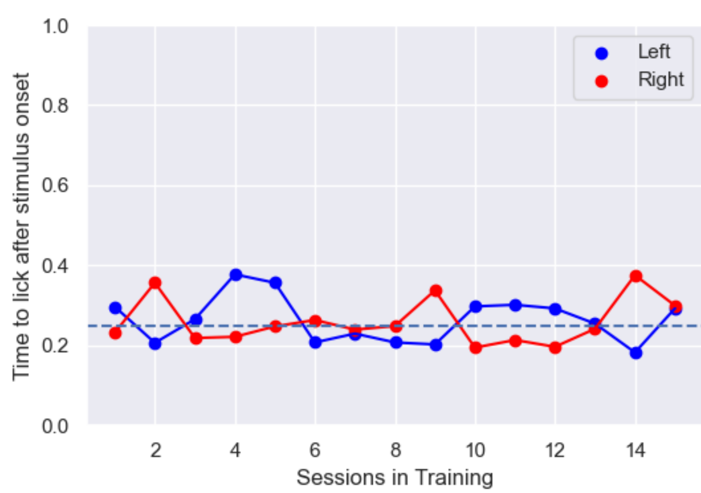

Figure 10. Mice's first lick-times still occurred before desired time in warning beep protocol

This lick-time graph was generated from one mouse's sessions in Stage 3. For almost every day in training, there is always an average lick-time for at least one side that is before t = 0.25 seconds. This trend was common amongst all mice on this protocol.

However, in this protocol, the use of the warning beep mode did not come into effect until Stage 3.0. Mice could lick early in Stage 1.0 or Stage 2.0 of the protocol and still obtain their reward.

For this protocol, we hypothesized that the mice may have become too comfortable licking early in the first two stages, which may have been what led to their consistent premature lickings. We decided to test another cohort of mice on another protocol that implements the warning beep earlier in the stages.

Implementing a warning beep earlier in the protocol resulted in a later lick-time

Implementing a warning beep in the second stage, rather than the third stage, was successful in increasing the mouse's average lick-time (Figure 11).

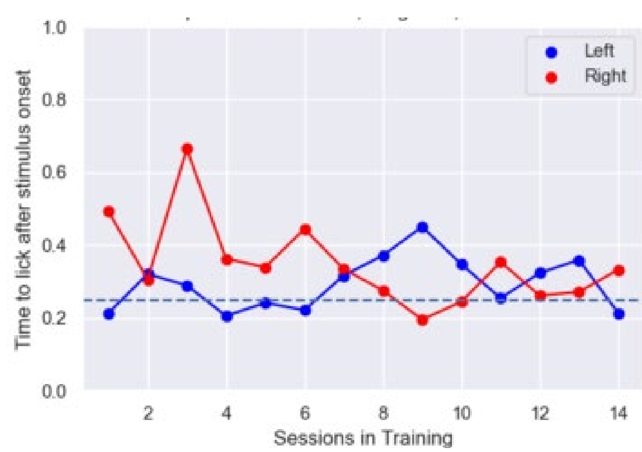

Figure 11. Mice's first lick-times increased after earlier implementation of the warning beep

This lick-time graph was generated from one mouse's sessions in Stage 4.0. On most days, the animal's lick-times on both sides occurred after t = 0.25 seconds.

However, the accuracy rates of the mice's performance still remained low. The mice stalled on Stage 4.0, which for this protocol is identical in expectations as the stage on which the mice stalled for the first two protocols. Again, none of the mice were able to lick over 100 times per side and achieve an accuracy rate of 70% or higher per side.

We hypothesized that, by introducing the warning beep mode so early in the protocol, more emphasis had been placed on the timing of the licks rather than the association of sides with sounds. Next, we attempted to create a "remedial" stage that could help solidify the association of certain sounds with certain sides if the mice could not pass Stage 4.0.

The remedial stage was not sufficient in improving accuracy rates

To assess the success of the remedial stage, we monitored the performance of 3 mice in Stage 4.0 both before and after being on the remedial stage. Both performance graphs of one of the mice can be seen below (Figure 12 and 13).

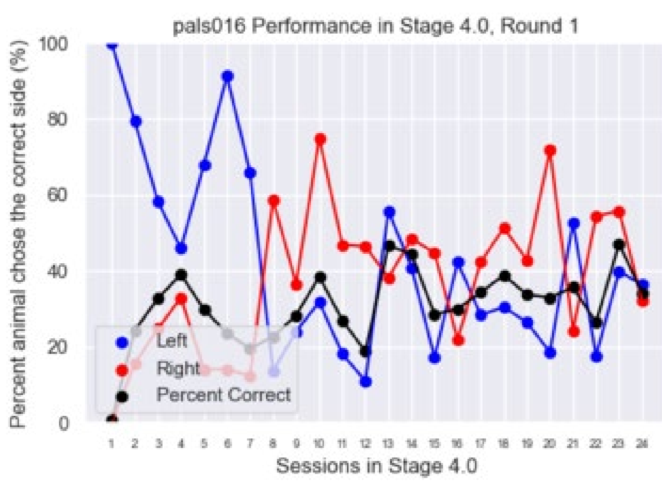

Figure 12. Mouse's performance in Stage 4.0 before the remedial stage

While the mouse demonstrated some increased accuracy from 0% to around 40%, the mouse still did not reach criteria to move to the next stage.

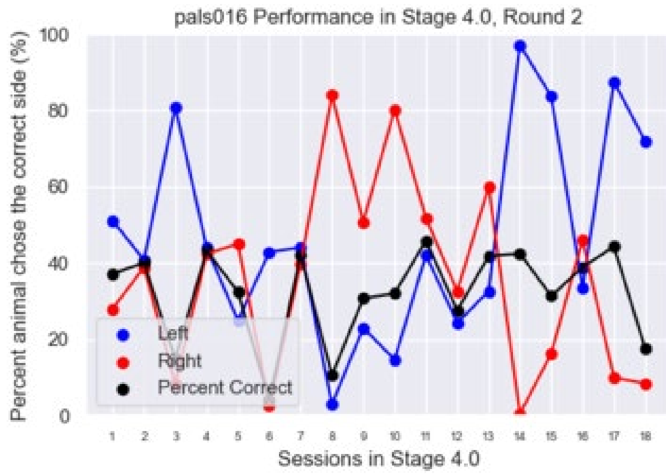

Figure 13. Mouse's performance in Stage 4.0 after the remedial stage demonstrates no improvements

The mouse's performance did not increase beyond roughly 40%, which was its accuracy rate before the implementation of the remedial stage. This lack of improvement was reproduced among the three animals put under this protocol.

All three mice that underwent this remedial stage demonstrated no appreciable difference in accuracy rates before and after the remedial stage, indicating that the remedial stage was unsuccessful.

Discussion

Future Directions in Solidifying the AM Head-Fixed Protocol

While we have not yet found a protocol that could successfully teach mice to discriminate between AM sounds, we have found protocols that were successful in teaching mice to either discriminate between a different set of sounds in the head-fixed setup or to discriminate between the same set of AM sounds in a different setup. We can use the following lessons from those successful protocols to help further solidify the existing head-fixed protocol for AM discrimination.

First, it was surprising to see that the AM discrimination protocol was not successful in the head-fixed set up, but the frequency discrimination protocol was. Both protocols used the same setup, and, for the first AM discrimination protocol, the training stages were identical except for the sound types. This demonstrates that there was not something functionally wrong with the setup itself, but that the stimuli themselves differed.

Originally, we attributed the difficulty of teaching sounds mice in the head-fixed rig to timing. In temporally modulated stimuli, timing is the only feature that sets the two stimuli apart. The AM discrimination protocol presented two stimuli – the modulated sound and the unmodulated sound. The unmodulated sound had a consistent amplitude throughout the entire presentation of the sound. On the other hand, the modulated sound had a frequency of 10 Hz, meaning that the amplitude waxed and waned ten times per second. However, many of the animals licked before a tenth of a second could pass. They did not hear the stimulus start and stop, which was its characteristic feature that set it apart from the unmodulated frequency. In the first 100 milliseconds, it is impossible for the animals to tell the difference between the two sounds. However, while our attempts to foster later timing through an early implementation of the lick timing were successful, the mice's accuracy rates did not increase.

Another interesting revelation was the fact that the AM discrimination protocol was successful in a freely-moving setup but not in a head-fixed setup. This demonstrated that it was possible to teach mice to learn to discriminate between AM sounds, it was just more difficult to teach them in a head-fixed setup instead.

We suspect that the key difference in the freely-moving setup is that its layout helps alleviate some of the problems with timing encountered before. In the freely-moving setup, is more difficult for mice to make a premature decision when they must travel all the way to the other side of the box to make their choice. In this setup, premature decisions are less likely to be made.

Another key difference lies in the type of stimuli presented for the two protocols. In the freely-moving protocol, we used different rates of modulation. On the other hand, in the head-fixed protocol, we used modulated vs unmodulated sounds. The reason we picked modulated vs unmodulated sounds at first was because we suspected that this would be easier for the mice to discriminate. However, this was just a hypothesis. While the mice's success in the freely-moving protocol may be due to a variety of factors, perhaps we should try utilizing different rates of modulation in the head-fixed protocol to see if it makes a difference.

One final factor that I believe makes the most difference between head-fixed and freely-moving setups is the initiation of trials. Unlike in the freely-moving protocol, mice do not initiate trials in the head-fixed protocol. Sounds automatically play without their discretion. Mice could have long lapses in attention during the protocol without regarding to the sounds being presented. One avenue for the future would be introducing a third lick spout in the center for the head-fixed protocol so that animals can initiate trials in the same way that they do for the freely-moving protocol.

Additionally, regardless of the sounds presented, the head-fixed setup is very sensitive to small changes. Because the head-fixed setup involves putting the lick spouts directly next to each mouse's mouth, the setup needed to be adjusted between uses for every mouse that used it. Having variable manual setups created room for variation in positioning. Additionally, we have found that if one of the lick spouts was closer to the mice's mouths than the other, this often created biases in choices for the entire duration of the training session. One avenue for the future would be to standardize distances between the mouse's mouth and the spouts, then make physical measurements to make sure these standards are met every single training session.

Future Directions in Electrophysiological Recordings

The objective of creating this protocol is to be able to characterize neurons' response to these sounds as a function of active and passive training, which will eventually be accomplished through electrophysiological recordings.

Currently, the Jaramillo lab is investigating how auditory cortex neuron respond to features of speech sounds (such as voice onset time and frequency sweeps). For this study, mice are set up in the head-fixed rig their neural activity is measured with a Neuropixel probe while they are exposed to a variety of speech sounds. Afterwards, the brain is sliced, imaged, and undergoes histological analysis to make sure that the recordings were occurring in the target area. This study is adjunct to my thesis, since it does not involve learning of sounds, just responses. However, this project shows a preview of the setup that will be used after the behavioral training paradigm is solidified. The only modification is that the mice will be undergoing combinations of active learning of and passive exposure to these sounds while having their neural activity recorded.

The data collected from this project will continue to advance our knowledge of how humans learn and process speech sounds. We hope that advancements in this field can further our knowledge of neural speech coding and reveal novel methods through which people can learn second languages.

Glossary

Auditory cortex: The region in upper side of the temporal lobe of the cerebral cortex that receives, filters, and processes auditory information in humans and many other vertebrates.

Electrophysiology: The method of measuring the electrical activity in cells in tissues. In neuroscience, electrophysiology is used to measure the electrical activity of neurons, which is how neurons relay information throughout the brain and body.

Frequency: The number of occurrences of a repeating event per unit of time. The frequency of a tone determines how high or low-pitched it sounds.

Phoneme: A unit of sound in a human language that distinguishes one word from another.

Tonotopic organization: The phenomenon in auditory areas in which certain cells are arranged by their responsiveness to different frequencies.

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