**Distribution Agreement**

In presenting this thesis as a partial fulfillment of the requirements for a degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis in whole or in part in all forms of media, now or hereafter now, including display on the World Wide Web. I understand that I may select some access restrictions as part of the online submission of this thesis. I retain all ownership rights to the copyright of the thesis. I also retain the right to use in future works (such as articles or books) all or part of this thesis.

Chengcheng(Jeffrey) Yang November. 22. 2023

# Using optogenetics to test the role of auditory cortex in memory retrieving

by

Chengcheng(Jeffrey) Yang

Dr. Robert C. Liu
Adviser

Biology

Dr. Robert C. Liu

Adviser

Dr. Dieter Jaeger

Committee Member

Dr. Kate Joy McKnelly

Committee Member

2023

# Using optogenetics to test the role of auditory cortex in memory retrieving

By

Chengcheng(Jeffrey) Yang

Dr. Robert C. Liu

Adviser

An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Science with Honors

Biology

2023

Abstract

Using optogenetics to test the role of auditory cortex in memory retrieving
By Chengcheng(Jeffrey) Yang

Understanding the role of the auditory cortex (ACx) in auditory information retrieval is critical for insights into cognitive processes and sensory integration. This study utilized optogenetic tools to transiently inhibit the ACx in mice trained to use auditory cues for pup retrieval, aiming to discern the functional significance of the ACx in memory retrieval. Inexperienced female CBA/CaJ mice underwent a series of trainings in a T-maze, followed by assessments under conditions of optogenetic ACx inactivation.

The experiment was conducted in two conditions: Light (with optogenetic stimulation) and No-light (without stimulation), serving as a control. Subjects' performances were analyzed using binomial tests to compare success rates against chance, and chi-square tests to compare performances across conditions. Results indicated that under non-light conditions, both subjects displayed a proficiency in auditory cue-based localization, with performances significantly above chance ($p < 0.001$, while during Light trials, subject's success rate did not differ significantly from chance.

The study's findings underscore the complexity of the ACx's involvement in auditory processing, highlighted by the variability in response to optogenetic inhibition. This variability could suggest individual differences in neural circuitry or sensitivity to optical stimulation. The results emphasize the need for further research to refine optogenetic applications, understand individual variability, and explore the intricate relationship between neural circuitry, sensory processing, and behavior.

# Using optogenetics to test the role of auditory cortex in memory retrieving

By

Chengcheng(Jeffrey) Yang

Dr. Robert C. Liu

Adviser

A thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Science with Honors

Biology

2023

**Acknowledgement**

I express my sincere appreciation to Kai Lu, Lily Ling, and Kelwin Wong from our lab for their significant contributions to this research. Kai's training and assistance with experimental procedures were invaluable. Lily's dedication to the care and breeding of CBA/CaJ animals was crucial for our studies. Kelwin's role in maintaining lab organization and supplies was fundamental to our daily operations. I am particularly thankful to Dr. Robert Liu for his mentorship and guidance during my time as an honors student. Finally, my gratitude goes to all members of the Liu Lab for their ongoing support and insightful feedback throughout this research endeavor.

## Table of Contents

# Introduction

In the dynamic realm of natural biology, the capacity of organisms to adapt their behavior based on novel sensory information is a cornerstone of cognitive development and evolutionary adaptability. This concept, originally proposed by Mayr et al. in 1974, emphasizes the intricate interplay between genetic predispositions and experiential inputs, which modulate inherent behavioral patterns.[1] Within this context, mice have proven to be invaluable model organisms for investigating these complex neural processes due to their well-documented behavioral responses and genetic tractability. Their use in neuroscience research has shed light on various aspects of neural functioning, especially in understanding behaviors like maternal care and the processing of sensory cues, such as ultrasonic pup vocalizations.[2,3]

Optogenetics, namely combining genetic and optical methods together, allows tight spatial and temporal control of the activity of specific neurons in the living brain. This revolutionary advance ushered in a new era in which scientists gained an unprecedented understanding of how neural circuits influence animal behaviors.[4] In optogenetics, opsins, or naturally occurring light-sensitive proteins, are engineered and illuminated by specific light frequency, leading to modulation of later cascade of reactions, depending on the types of opsins. Opsins are generally divided into two categories: microbial opsins and vertebrate opsins.: excitatory opsins (channelrhodopsins) and inhibitory opsins (halorhodopsin).[5,6]

Building upon this foundation, our research pivots to a novel and ambitious objective: evaluating the feasibility of using optogenetics as a tool to study the role of the auditory cortex (ACx) in auditory associative learning in an innate maternal behavior contex. In this study,

female mice were trained to learn to use a novel sound to retrieve pups in a t-maze. The previous works have shown that the inactivation of ACx impairs learning, suggesting the role of ACx in memory formation during this type of learning.[7] The aim of the current work is to test the role of ACx in memory retrieval. I hypothesize that optogenetic inactivation of the ACx impairs the performance of the learned sound-cued search behavior. The second aim of the experiment is to determine the optimal light intensity to inactivate ACx. This study, therefore, not only explores the role of ACx in memory retrieval, but also seeks to establish optogenetics as a viable method for studying complex neural circuits involved in sensory processing and learning.

# Method

## Animals

All experimental procedures involving mice received approval from the Emory University Institutional Animal Care and Use Committee. The study utilized four naive female CBA/CaJ, specifically those aged between 8 weeks. These mice were housed together in same-sex groups, under a reversed light cycle of 14 hours of light and 10 hours of darkness, with unrestricted access to both food and water. However, two of them failed to learn retrieving pups based on sound cues and then were excluded from the study.

## T-maze setup

The behavioral training of mice took place in an acoustically isolated, double-walled chamber measuring 8 feet 2 inches by 10 feet 6 inches, provided by IAC Acoustics, under subdued red lighting. The training involved an elevated T-maze, lined with Alpha-Dri bedding from Shepherd Specialty Papers. At the lower end of the T-maze's stem, a rectangular area measuring 11 square centimeters, set 1 centimeter below the stem level, was designated as a nesting area and supplemented with additional bedding. Each arm of the maze was equipped with a high-frequency ribbon tweeter speaker (model PT-R4 by Pioneer), situated 27 centimeters from the arm's end and directed towards the junction of the maze's arms and stem. An additional speaker (model EMIT by Infinity) was installed 30 centimeters above the nesting area, angled slightly at 5 degrees towards it to reduce standing wave effects. Ultrasonic vocalizations (USVs) were captured using a Mini-3 heterodyne bat detector from Ultrasound Advice, placed 15 centimeters to the side of the nest, connected to a RP2 processor from Tucker Davis Technologies, which sampled at 20 kHz. For behavioral monitoring, a Flea 3 camera from Teledyne FLIR was mounted above the maze, recording at a rate of 20 frames per second. This video feed was synchronized with the electrophysiological data via a TTL trigger emitted by the camera.

## Viral Injection

Craniotomies (1 mm diameter) were made above ACx of both hemispheres at the stereotaxic coordinate (ML +/-4.25, AP 2.80). A syringe (NanoFil, WPI) with a 36 GA needle was positioned at the center of the craniotomies. The needle was advanced into the cerebral tissue for 1.5 mm

and was then backed up for 0.2 mm (figure 2). Five minutes after needle insertion, AAV-h8yn-eNpHR 3.0-EYFP (800nL on each side) was infused into the ACx bilaterally at a rate of 100um/s (controlled by MICR 021, WPI). I then waited five minutes before withdrawing the syringe.

## Ferrule Implantation

Immediately after viral injection. Fiber optic ferrules (phy 1.25, 300um core, 0.39NA, 3mm length) were implanted in ACx bilaterally. Ferrules were forwarded 0.5 mm under the surface, then silicon elastomer (Kwik-Cast, WPI) was used to cover the brain tissue. Dental cement (C&B Metabond, Parkell) was then applied to implant ferrules on the skull. I waited 15 min to cure dental cement and finally covered the implant with acrylic resin dental cement (ortho-jet BCA) mixed with carbon glassy powder.

## Habituation

The mice were allowed to recover 2 weeks after surgery. Initially, for a period of two days (45 minutes each day) after recovery from the implantation surgery, the mice were habituated in the cage with trainer's scent. After that, the mice were acclimatized to the T-maze environment for two days and 45 minutes each day. Following this, for the next two days, the mice were accompanied in the maze by two pups during their 45-minute daily habituation sessions. Mice that displayed nurturing behavior by huddling over the pups were selected for the subsequent procedures, while those that exhibited aggression towards the pups or did not interact were excluded from further participation in the study. A period of six days post-implant surgery was

allocated for recovery, after which the animals were reintroduced to the T-maze for an additional 45-minute session with two pups to reestablish familiarity with the environment.

## Stimuli

Two types of stimuli were used to train mice. I started to use noise burst stimuli (3 bursts/1s, dur:200ms, center frequency 20khz, bandwidth 0.5 oct) for training. However, after 9 days of training, the mice failed to learn the behavior. Thus, I switched to use AM-modulated noise stimuli to train animals (based on prior study am-noise is effect for training). AM-modulated noise stimuli possess the properties of center frequency 40 kHz, bandwidth 0.5 oct, modulated by 5Hz sin-wave. Both stimuli were generated by Matlab (MathWorks) at a sampling rate of 223.214 kHz. During experiment. Stimuli were presented by OpenEx operating on an RX6 processor (Tucker DavisTechnologies).

## Behavioral Training

Each training day commenced with the placement of two neonatal pups (aged between postnatal days 2 to 5, sourced from a different cage) in the nest area to encourage the subject mouse to remain at the nest. In each session, training started with dispersing two pups across the maze to motivate the subject mouse to commence its search. The first trial of sound pairing initiated as soon as the mouse brought the last dispersed pup back to the nest. This sound cue was emitted from one of the two arm speakers. If the mouse entered the arm corresponding to the sound source, it was rewarded with a pup, coinciding with the termination of the sound. Conversely, if the mouse entered the incorrect arm, the sound continued until the correct arm was explored. Following pup retrieval, the next trial was initiated (Figure 1). The delivery side

for the sound and pup was pre-set in a pseudorandom pattern, ensuring an equal probability (50%) of either side being chosen throughout the 100 trials conducted each day, and limiting consecutive assignments of the same side to no more than 40% of the trials. The volume of the target sound was alternated between 70 dB and 66 dB (calibrated at the T-maze intersection) in a pseudorandom sequence with equal likelihood of selection for either level.

## Testing Memory Retrieval with Optogenetic Inactivation.

After animals learned to use sound-cue to search for pups. I tested the effect of optogenetic inactivation of ACx on task performance. Before testing, optical fibers were connected to the ferrules implanted on the skull of animals. I waited 30 min before starting the test to reduce the effect of stress on the performance. The task procedure and stimuli are same as the second training phase (with AM-modulated noise). In randomly selected 30% percent of trials, LED light (25 Hz pulse train, 4 mW) was emitted to the bilaterial ACx during sound playback. Both animals were tested for two sessions. The performance (percent of correct trials) of the two sessions were combined within each animal for data analysis.

## Statistics

### Binomial Test

To assess the statistical significance of the mice's performance in correctly identifying pup locations based on vocalization cues, we employed the binomial test. This test was specifically chosen to determine whether the observed proportion of successful trials in both Light (with optogenetic stimulation) and No-light (without stimulation) conditions significantly differed

from chance level, which was set at 50%. The binomial test is particularly suited for this analysis as it evaluates the success rate against a hypothesized probability in a binary outcome experiment, which in our case were the correct or incorrect identifications in each trial. This test was applied separately to the results of the Light and No-light trials for each animal.

### Chi-Square Test

In addition to the binomial test, we used the chi-square test to compare the performance of each animal between the Light and No-light conditions. The chi-square test was selected for its effectiveness in determining if there is a significant difference in the distribution of categorical variables between two or more groups. In our study, this test enabled us to statistically evaluate whether the optogenetic manipulation led to any significant change in the ability of the mice to locate pups based on auditory cues. This comparative analysis was crucial in understanding the impact of optogenetic stimulation on auditory information retrieval.

# Results

The primary objective of this study was to investigate the role of the auditory cortex in information retrieval under optogenetic manipulation, as evidenced by the ability of mice to locate pups based on vocalization cues. The experiment was carefully designed to include trials under two distinct conditions: Light (with optogenetic stimulation) and No-light (without stimulation). This approach provided a comparative framework to examine the effects of optogenetic intervention on auditory cue-based localization.

All mice reached the train criteria after 8 days training. Animal 1 displayed an 72% success rate, correctly identifying the location in 144 out of 200 trials. Animal 2 achieved an 70.5% success rate with 141 correct identifications out of 200 trials (Figure3). This high rate of accuracy suggests animals learned to use the sound cue to search for pups. This proficiency serves as a crucial baseline against which the effects of optogenetic manipulation can be measured.

## Optogenetic inactivation of ACx impaired memory retrieval

Subject 1 demonstrated a 50% success rate in locating pups in trials with lights (27 successes in 54 trials) (figure3), which is then tested on whether the performance purely rely on chance or not (binomial test: $p = 0.554$). The p value does not reach the alpha significance level of 0.05, implying that the success rate of Subject 1 in trials with light is not statistically significant different from the chance level (50%), thereby questioning the reliability of visual cues in this context for the subject. When light was absent, the subject's performance to locate pups was significantly higher than the chance level (binomial test: $p < 0.001$). This significant result suggests that Subject 1's ability to retrieve information was inhibited due to the inactivation of auditory cortex. A chi-square test further tested the difference in performance between the two conditions. The test revealed a significant difference between Light and No-light conditions ($p = 0.003$), indicating that Subject 1's performance to search for pups with the sound cue was significantly impaired by the light inhibition. This finding supports the hypothesis that the optogenetic manipulation can interrupt the process of information retrieve by inhibiting the auditory cortex.

Parallel to the first, Subject 2's performance was quantified, revealing a 56.7% success rate in trials with light (34 out of 60 trials) (figure 3). The performance was not significantly difference from the chance level (binomial test: p= 0.183), indicating an impairment in performance. This consistency across subjects reinforces the effectiveness of optogenetic manipulation. In the absence of light, Subject 2's performance was significantly higher than the chance level (binomial test: p-value < 0.001), mirroring the findings of Subject 1 and suggesting a shared effect from the manipulation. The two-tail chi-square test result for Subject 2 is a p-value of 0.058, which then could be divided half to 0.029 as a single-tail test result, also misses the threshold of 0.05. Thus, it proves a statistically significant difference in performance between the light and non-light conditions, combined with two binomial test results, supporting that hypothesis inactivation of ACx by optogenetic impairs the performance of the learned task. This outcome invites further research to test more subjects and possibly to explore a refined sensory threshold for statistical significance in this context.

The significant difference between performances under two conditions highlighted the role of auditory cortex in information retrieving. Optogenetics approach manipulates neural circuits both temporally and spatially by precisely inhibiting the auditory cortex only when light presence, providing a new cutting point to study animals' innate behaviors.

The variability in responses between animals, particularly in the light trials, highlights the complex interplay of neural mechanisms and individual differences in sensitivity to optogenetic intervention. It raises important questions about the specificity and efficacy of optogenetic techniques in modulating neural activity for behavioral outcomes. Furthermore, the high

proficiency in non-light trials underscore the effectiveness of the mice's natural auditory processing mechanisms. This proficiency forms a crucial backdrop for understanding the role of the auditory cortex and sets a benchmark for evaluating the impact of experimental interventions like optogenetics.

The detailed analysis of each trial, the consistency of results across different conditions, and the statistical rigor applied in this study provide a comprehensive understanding of the auditory cortex's role in sensory processing. These findings pave the way for future research, suggesting avenues for refining optogenetic techniques and exploring the neural underpinnings of auditory information processing in more depth.

# Discussion

The results of this study offer significant insights into the role of the auditory cortex in auditory information retrieval under optogenetic manipulation. This discussion aims to interpret these findings within the broader context of neuroscience, particularly focusing on the implications for our understanding of sensory processing and the efficacy of optogenetic techniques in behavioral studies.

## Interpretation of Optogenetic Influence

The significant deviation from chance performance in the Light trials, particularly for animal 1, suggests a notable influence of optogenetic stimulation on the auditory cortex's role in sensory processing. This finding indicates that optogenetic manipulation, under the parameters used in our study, effectively altered the mice's ability to accurately locate pups based on vocalization

cues. The significant difference in performance between Light and No-light trials for Animal 1 ($p = 0.0003$) demonstrates that optogenetic stimulation can have a measurable impact on auditory information retrieval behaviors. For Animal 2, although the difference in performance between Light and No-light conditions did not reach conventional levels of statistical significance ($p = 0.058$), there was a trend suggesting some level of influence by optogenetic manipulation. This variation in response between the two animals underscores the potential for individual differences in the neural circuitry's responsiveness to optogenetic intervention. These results collectively suggest that the auditory cortex, when subjected to optogenetic manipulation, plays a more dynamic role in auditory information processing than previously understood. The ability to influence auditory cue-based behavior through optogenetic stimulation of the auditory cortex opens new avenues for exploring the neural mechanisms underlying sensory processing and cognitive functions.

## Innate Auditory Processing Proficiency

The significantly higher than chance performance observed in the non-light trials for both animals highlight their innate proficiency in auditory information retrieval. This finding aligns with existing literature on the natural capabilities of mice in sensory processing and suggests that their ability to interpret auditory cues is robust and reliable in the absence of external neural manipulation.

## Individual Variability in Response to Optogenetic Stimulation

The variability in response to optogenetic stimulation, as evidenced by the significant difference in performance between Light and non-light trials for Animal 1 and the marginal non-significance for Animal 2, raises important questions about individual differences in neural circuitry or sensitivity to optical stimulation. This suggests that optogenetic effects may not be uniformly expressed across subjects, possibly due to variations in opsin expression levels, neural connectivity, or other individual-specific factors.

## Implications for Optogenetic Applications

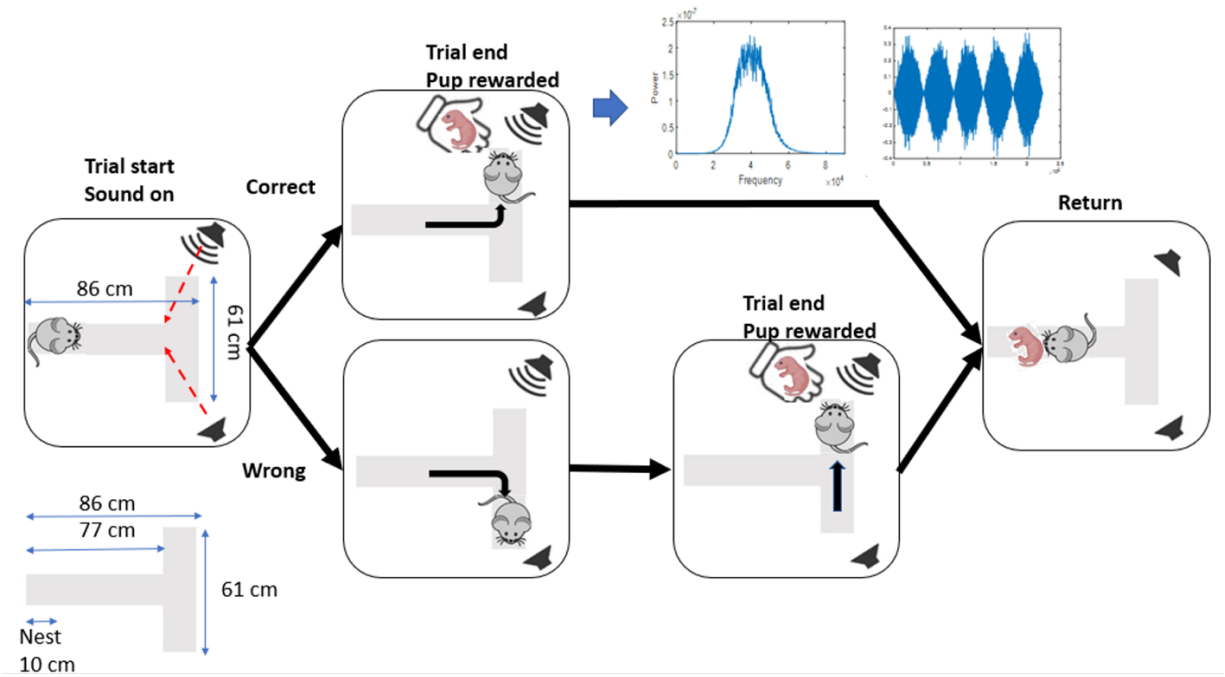
The findings from this study underscore the need for careful consideration of the parameters and methods used in optogenetic manipulation. While optogenetics is a powerful tool for investigating neural circuits, our results suggest that its efficacy may vary depending on the specific neural functions or behaviors being studied. This highlights the importance of optimizing optogenetic approaches for each experimental context and considering individual variability in neural responses.

# Future Directions

Further research is warranted to explore the nuances of optogenetic effects on different aspects of neural function and behavior. Studies with varied optogenetic parameters, perhaps coupled with other neuroscientific methods such as functional imaging or electrophysiology, could provide deeper insights. Additionally, investigating the role of the auditory cortex in more

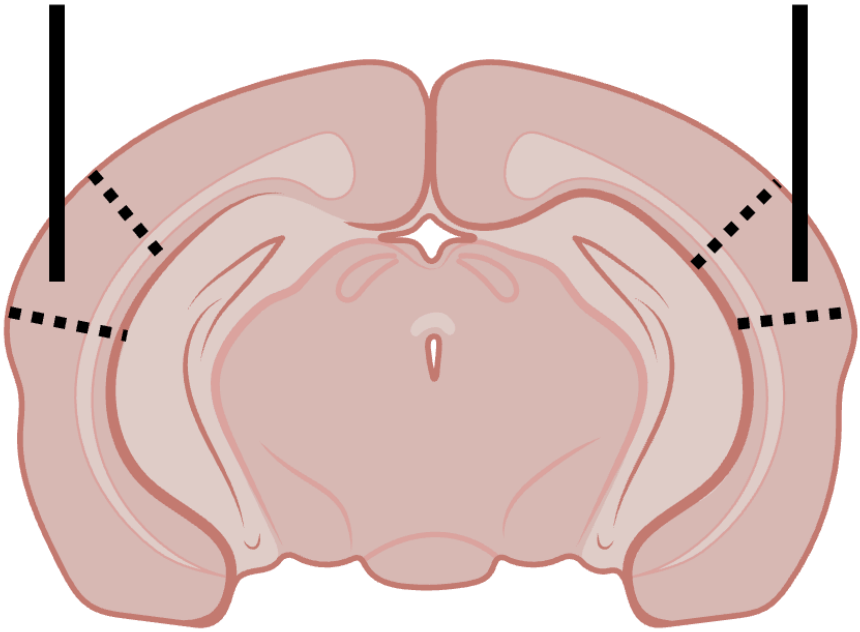
complex or diverse auditory processing tasks may reveal different aspects of its functional significance.

In conclusion, this study contributes to our understanding of the auditory cortex's role in sensory processing and highlights the complexities involved in applying optogenetic techniques to behavioral neuroscience. The findings emphasize the need for a nuanced approach to interpreting the effects of neural manipulations and suggest directions for future research that can further elucidate the intricate relationship between neural circuitry, sensory processing, and behavior.



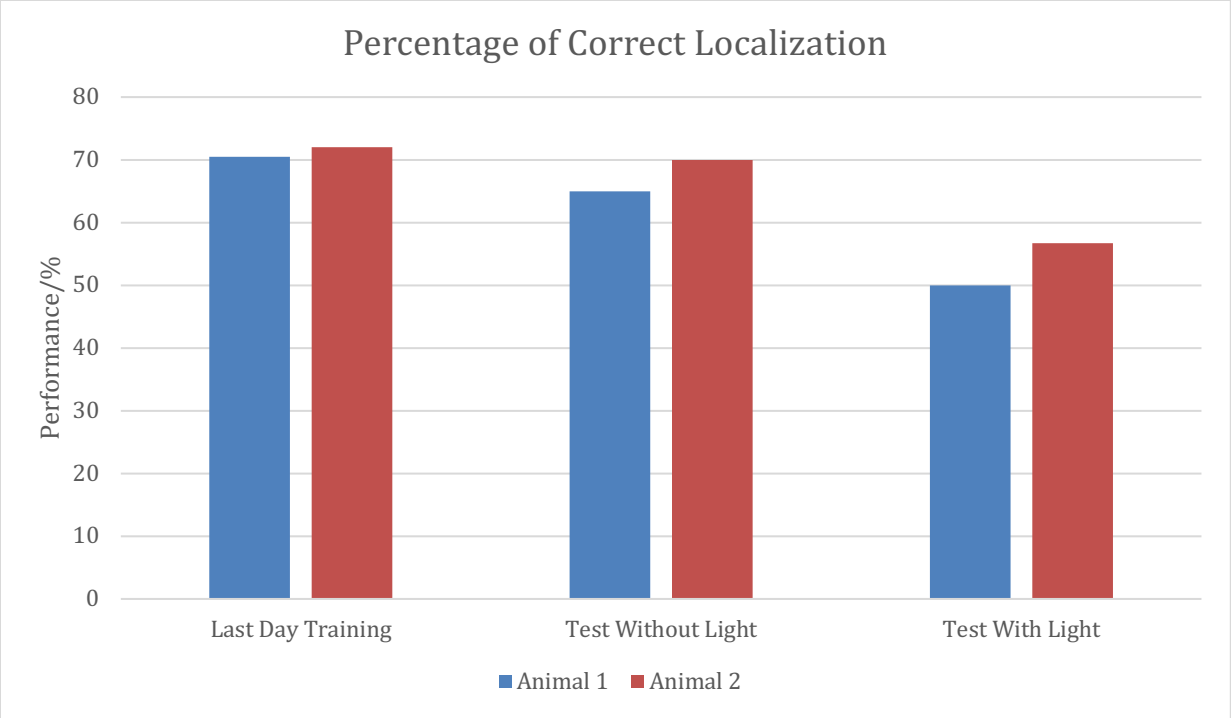

# Figure 1: Training Protocol for Auditory Cue-Based Localization in Subject Mice

Schematic of the T-maze training protocol showing the initiation of trials with pup dispersion, followed by auditory-cued retrieval. Correct arm entries were rewarded with a pup and cessation of sound, while incorrect choices prompted continued sound until the correct arm was chosen. Trial conditions, including sound delivery and volume (alternating between 70 dB and 66 dB), were pseudorandomized across 100 daily trials to prevent pattern recognition and ensure equal probability of arm selection.



# Figure 2: Depiction of ACx Craniotomy and Optogenetic Vector Infusion

Illustration of bilateral craniotomy procedures over the auditory cortex (ACx) at coordinates ML ±4.25, AP 2.80, with subsequent introduction of a 36 GA needle into the ACx (1.5 mm deep, retracted 0.2 mm post-insertion). The graph visualizes the precise infusion of AAV-hSyn-eNpHR3.0-EYFP (800 nL per hemisphere) at a controlled rate of 100 nL/s, followed by a stabilization period before syringe withdrawal.




# Figure 3: Percentage of correct localization

Comparism between performance of mice in trials under different conditions.

# References


1. Mayr, E. Behavior Programs and Evolutionary Strategies: Natural selection sometimes favors a genetically ‘closed’ behavior program, sometimes an ‘open’ one. *Am. Sci.* **62**, 650–659 (1974).
2. Dunlap, A. G. *et al.* Becoming a better parent: mice learn to use predictive auditory cues to improve a stereotyped maternal behavior.
3. Dunlap, A. G. *et al.* Becoming a better parent: Mice learn sounds that improve a stereotyped maternal behavior. *Horm. Behav.* **124**, 104779 (2020).
4. Guru, A., Post, R. J., Ho, Y.-Y. & Warden, M. R. Making Sense of Optogenetics. *Int. J. Neuropsychopharmacol.* **18**, pyv079 (2015).
5. Boyden, E. S., Zhang, F., Bamberg, E., Nagel, G. & Deisseroth, K. Millisecond-timescale, genetically targeted optical control of neural activity. *Nat. Neurosci.* **8**, 1263–1268 (2005).
6. Zhang, F. *et al.* Multimodal fast optical interrogation of neural circuitry. *Nature* **446**, 633–639 (2007).
7. Lu, K., Wong, K., Zhou, L. N., Shi, Y. T. & Yang, C. J. to guide an innate social behavior.