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NEURON ACTIVATION IN RESPONSE TO AUDITORY STIMULATION

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in

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by

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CHAPTER 1

Abstract

Neuron Activation in Response to Auditory Stimulation

Gabriela Llarena

Gaining genetic access to neurons allows us to study the structure and function of the mammalian auditory system. Audition captures sound stimuli detected from the environment and sends this information to the brain through the auditory pathway. The information gathered by the auditory system is integrated with information gathered from the other sensory modalities: vision, olfaction, touch, and gustation; this allows us to modify our behavior accordingly and is crucial for survival. Methods to identify neuron populations based on their response properties are limited, as most genetic manipulation experiments identify neurons based on their anatomy or genetic composition. Neuroscientists have developed a novel tool that genetically targets different neuron populations based on their functional criteria. The *fos*/TRAP2 method, or "Targeted Recombination of Active Populations" (TRAP), genetically targets populations of auditory-responsive neurons when exposed to an auditory stimulus (Guenthner, Casey J, et al., 2013). Using a transgenic mouse line of $Fos^{2A\text{-}iCreERT2}$, or TRAP2, knock-in mice, I designed an auditory stimulation experiment that utilizes a *cre*-inducible system in cells that express *fos* in the presence of 4-Hydroxytamoxifen to identify and locate the auditory-responsive neuron populations. I found that mice exposed to an auditory stimulus expressed greater numbers of active cells in areas receiving auditory inputs, such as the primary auditory cortex (A1), superior colliculus (SC), and inferior colliculus (IC), than in mice that had their ears

plugged; in the primary somatosensory cortex (S1), both experimental conditions showed similar numbers of active neurons. These results demonstrate how the *fos*/TRAP2 method could serve as a tool to label neurons that respond to auditory stimuli; this method uses the effector gene *R26tdTomato* to fluorescently label cells, which can be analyzed using immunohistochemistry. The *fos*/TRAP2 method and analyses can provide insight into different auditory-responsive neuron populations; it can be used to determine which neuronal markers the cells express to identify their type, such as whether they are excitatory or inhibitory neurons, and the cells' defining characteristics. Additionally, this method can be used to sort cells for RNA sequencing (RNA Seq) analysis or to induce expression of optogenetic tools used to assay auditory circuitry. Identifying the function of different auditory-responsive neuron populations can be used to further investigate the auditory pathway and sensory integration to better understand how mammals detect and process sound.

Dedication

To my grandparents, Angel and Elena.

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Chapter 1: The Auditory System and Neuronal Activation in Response to Auditory Stimuli

1.1 Introduction

1.1a Audition detects sound information from the environment

The five senses, olfaction, audition, vision, gustation, and touch, are the means by which we collect information from the environment. Our sense of audition captures sound information from the environment and processes it in a way which allows us to interpret these sounds in order to make sense of the world around us. The auditory system begins by filtering vibrations from sound waves; mechanical signals produced by sound waves are transduced into electrical signals, which are then processed and interpreted by our brain in order to appropriately respond to the environmental stimuli detected. The auditory information received is then combined with information gathered by our other senses: olfaction, vision, gustation, and touch. The combination of sensory inputs collected is transformed into useful information that can be interpreted by our brains, allowing us to interact with and interpret the different types of stimuli in our environment.

1.1b Sound and mechanical signaling in the auditory pathway

The auditory system collects information from the environment in the form of sound. Frequencies of the different sounds detected are determined by the air pressure fluctuations caused by the movement of sound waves, resulting in particle motion. Sound frequencies are measured in hertz, which represents the number of compression-refraction cycle repetitions for a specific time interval. Variations in air

pressure produced by objects in our surroundings result in the production of sound waves, which then enters the ear. The standard range of sound frequencies that can be heard by humans fall between 20-20,000 hertz. When these sound waves enter the ear through the auditory canal, it results in the vibration of the tympanic membrane, also called the eardrum; mechanical signals in the form of vibrations marks the beginning of the auditory signal transduction process (Peterson DC, Reddy V, Launico MV, Hamel RN, 2024). Collectively, the auditory system's complex pathways allow us to transform sound waves into meaningful information we can use to interact with our environment.

1.1c The mammalian auditory system and signal transduction

The mammalian auditory system is made up of two pathways, the central auditory pathway and the peripheral auditory pathway; together, these pathways transform mechanical signals in the form of sound waves into electrical signals that are sent to the brain. These auditory system pathways are conserved across mammalian species, including humans and mice. The peripheral auditory pathway collects mechanical signals in the form of sound waves detected from the environment; these sound waves enter the external ear and are converted into electrical signals, which are then transduced in the cochlear nerve (Standring, Susan., et al, editor, 2008). Axons from the cochlear nerve terminate in the dorsal-ventral cochlear nuclei, and mark the start of the ascending central auditory pathway (Standring, Susan., et al, editor, 2008). This process begins with the external ear structures, the pinna and the external auditory canal, which are responsible for taking

the sound stimuli detected from the environment and sending it to the middle ear structures, such the tympanic membrane and auditory ossicles, in order to reach the inner ear (**Fig. 1** and **Fig.2**). The auditory ossicles, consisting of the malleus, incus, and stapes, relay the vibrations from the tympanic membrane through the oval windows to reach the vestibular apparatus, which is located externally on the cochlea (Peterson DC, Reddy V, Launico MV, Hamel RN, 2024).

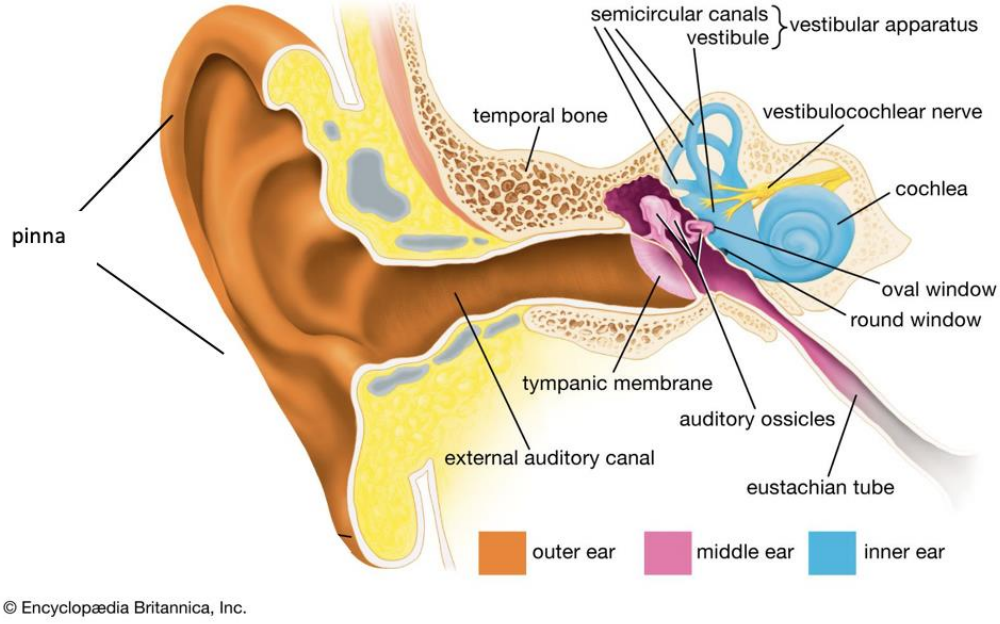

Figure 1 Schematic of the human ear structures

The pinna is the visible part of outer ear that collects the sound waves. The sound travels into the external auditory canal which amplifies the sound waves. The sound then reaches the tympanic membrane whose vibrations are relayed by the auditory ossicles. The auditory ossicles consist of the malleus, incus, and stapes, which are three small bones in the middle ear. The auditory ossicles transmit air vibrations from the outer ear into the inner ear. The cochlea contains organ of corti, where sound waves are transduced into electrical signals. The vestibulocochlear nerve transmits impulses from the inner ear to the brain (adapted from Hawkins, Joseph E. "human ear". Encyclopedia Britannica).

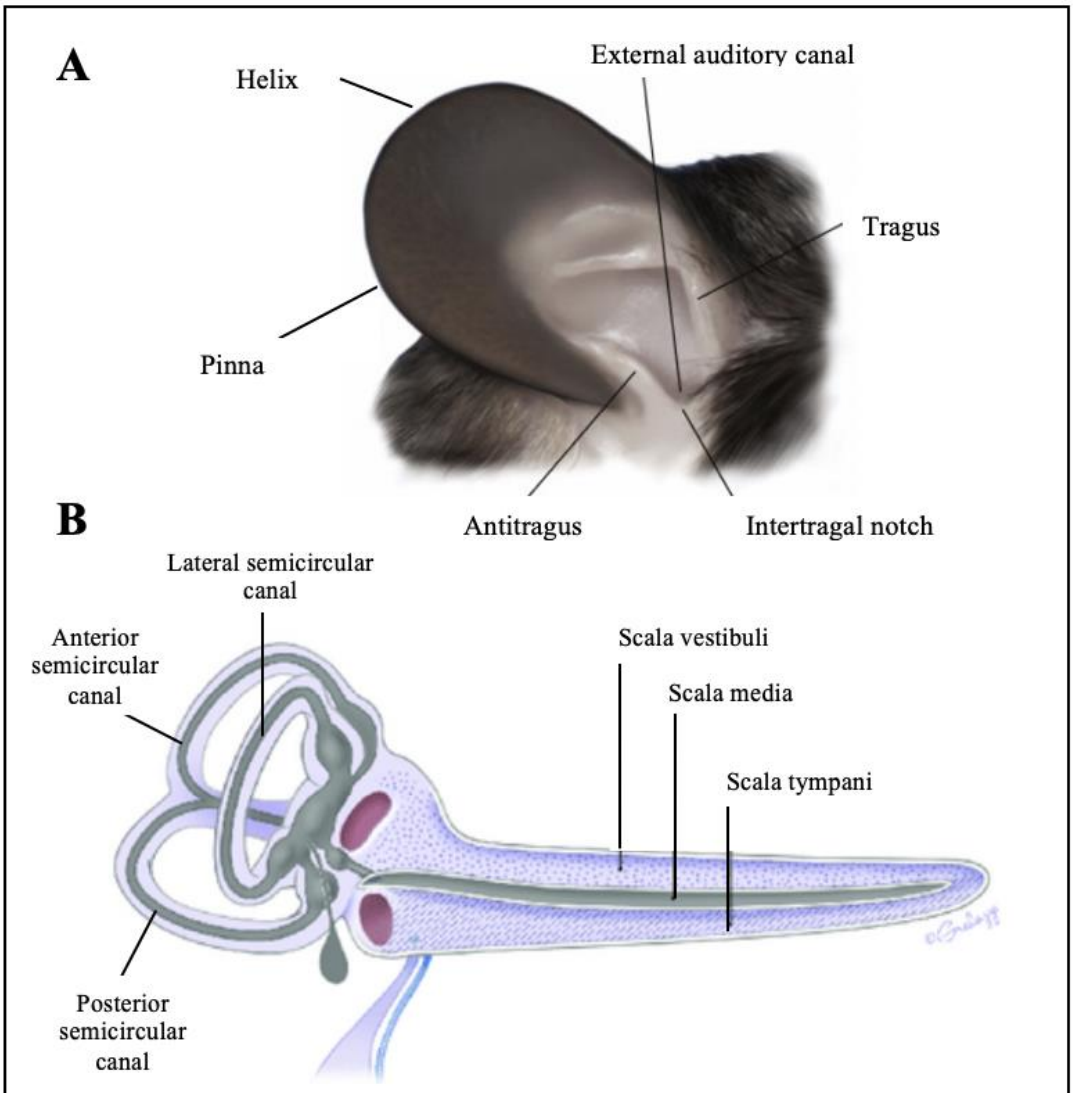

Figure 2 Schematic of mouse ear structures

(A) Mouse external ear structures resemble human external ear structures. The helix and pinna have less folds than the human pinna. The other structures, such as the antitragus and external auditory canal, remained conserved between both species (figure modified from Treuting, Piper M, and Suzanne M Dintzis, 2012). **(B)** Uncoiled cochlea in the inner ear of a mouse. The mouse cochlea shows a strong resemblance to the human cochlea (modified from Talaei, S. & Schnee, et al., 2019).

The cochlea in the inner ear vibrates in response to the different sound wave patterns through a complex mechanism involving mechanical receptors in the form of hair cells that are embedded in the basilar membrane. The cochlea is made up of three chambers: the outermost region is called the scala tympani, the scala vestibuli makes up the innermost region, and the scala media is located between these two chambers (**Fig. 2**) (Peterson DC, Reddy V, Launico MV, Hamel RN, 2024). In the scala media lies the organ of corti on the basilar membrane, which contains these middle and inner ear hair cells that serve as mechanical receptors. These hair cells have small projections, called stereocilia, located at their apex that contain superficial potassium and calcium channels. Their displacement activates mechanoelectrical transduction ion channels that respond to potassium and calcium ions present in the endolymph, allowing or refusing their entry into the hair cell (Standring, Susan., et al, editor, 2008).

In the inner ear, a fluid-like substance called endolymph is displaced by vibrations produced by sound waves and serves an important role in sound transmission in the mammalian auditory system (Casale J, Agarwal A., 2024). Endolymph has the greatest resting potential in our bodies and serves as the driving force for mechanotransduction of sound waves into electrical impulses that travel through the brain to be integrated in order to interpret this auditory information collected from our environment (Standring, Susan., et al, editor, 2008); endolymph also plays an important role in balance (Casale J, Agarwal A., 2024). As the basilar membrane shifts, the cochlear oscillations produced, along with the movement of

endolymph, stimulates and displaces the stereocilia, causing them to bend as a result of their close proximity to the tectorial membrane (Standring, Susan., et al, editor, 2008). The direction in which the stereocilia bend determines whether they will be hyperpolarized or depolarized, which is determined by the opening or closing of potassium channels in the stereocilia's cell membranes. If the potassium channels remain closed, the cell depolarizes, resulting in an electrical signal being communicated through connections to the vestibulocochlear nerve, also named cranial nerve VIII, which innervates the hair cells (Peterson DC, Reddy V, Launico MV, Hamel RN, 2024). The electrical impulses received by the stereocilia will then travel through the brainstem and are ultimately sent to the auditory cortex through the central auditory pathway.

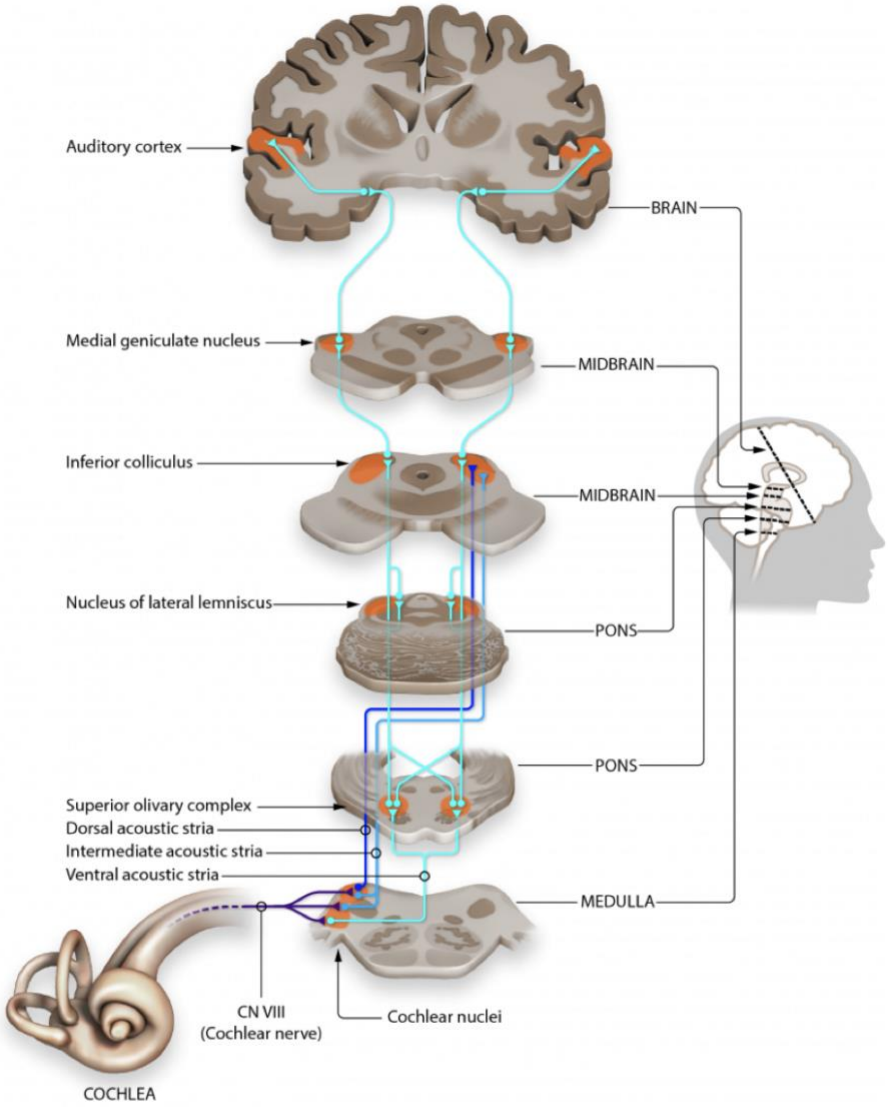

Figure 3 Schematic of the human auditory neural pathway

Sound waves transduced into electrical signals move from the start of the auditory pathway at the cochlea, then ascend to the different relay stations in the brain. The signal is sent from the cochlea through the vestibulocochlear nerve to the superior olivary complex, the inferior colliculus, and the medial geniculate nucleus, ultimately projecting to the auditory cortex (adapted from Olman, Cheryl, editor, 2022. Fig.6.10.1 by Jonathan E. Peelle, License: CC BY 4.0).

The mammalian central auditory pathway is the primary signal transduction pathway of the auditory system. It consists of various ipsilateral and commissural connections between the multiple brain regions involved in the processing of sound (**Fig. 3**). Cell bodies of the spiral ganglion in the cochlea serve as the primary afferents in the auditory system. These axons travel through the vestibulocochlear nerve enter the brainstem and diverge; these axons terminate in the dorsal and ventral cochlear nuclei located in the medulla, which is the inferior-most region of the brainstem. The dorsal cochlear nucleus in the medulla projects to the contralateral inferior colliculus in the midbrain via the dorsal acoustic stria between the medulla and the pons regions of the brainstem. In contrast, the central cochlear nucleus projects to either the superior olivary complex, the lateral lemniscus, or the inferior colliculus via the intermediate acoustic stria between the pons and the medulla. The medial superior olivary nucleus receives direct input from the ventral cochlear nucleus bilaterally; it is responsible for measuring the difference in time between afferent impulses received by each ear to aid in the localization of the sound stimulus detected by the peripheral auditory system. Nerve fibers ascend through the lateral lemniscus, located in the pons, and converge in the central nucleus of the lateral lemniscus. These fibers have multiple regions to which they send their projections; these brain regions include the ventral medial geniculate nucleus of the thalamus, the superior olivary complex, and to cells located in the cochlear nucleus (**Fig. 4**). The dorsal medial geniculate body receives projections from dorsal cortex of the inferior colliculus. Projections are also sent from the lateral lemniscus to the deeper layers of

the superior colliculus, which use this auditory information collected to integrate visual and auditory responses together, allowing for the localization of sound stimuli in the environment. Although the ascending central auditory pathway has various points at which projections cross the midline, projections sent to the central nucleus of the inferior colliculus have a strong preference for contralateral dominance, meaning that each hemisphere has the strongest response to information collected from the ear that lies contralateral to the respective hemisphere. In the posterior portion of superior temporal gyrus in the temporal lobe lies the primary auditory cortex, which receives projections from the medial geniculate body, which connects reciprocally to the primary auditory cortex (Standring, Susan., et al, editor, 2008); the primary auditory cortex contains precise tonotopic maps and gradients. In addition, most of the structures in the ascending auditory pathway, from the cochlea to the primary auditory cortex, demonstrate at least some tonotopic mapping; for instance, the cochlea can dissolve a sound stimulus and arrange it tonotopically along the surface of the basilar membrane within the organ of corti in the cochlea located in the inner ear (Purves D, Augustine GJ, Fitzpatrick D, et al., editors, 2001). Together, these different brain regions form this complex system to serve as relay stations for these signaling mechanisms, ultimately making up the central auditory pathway.

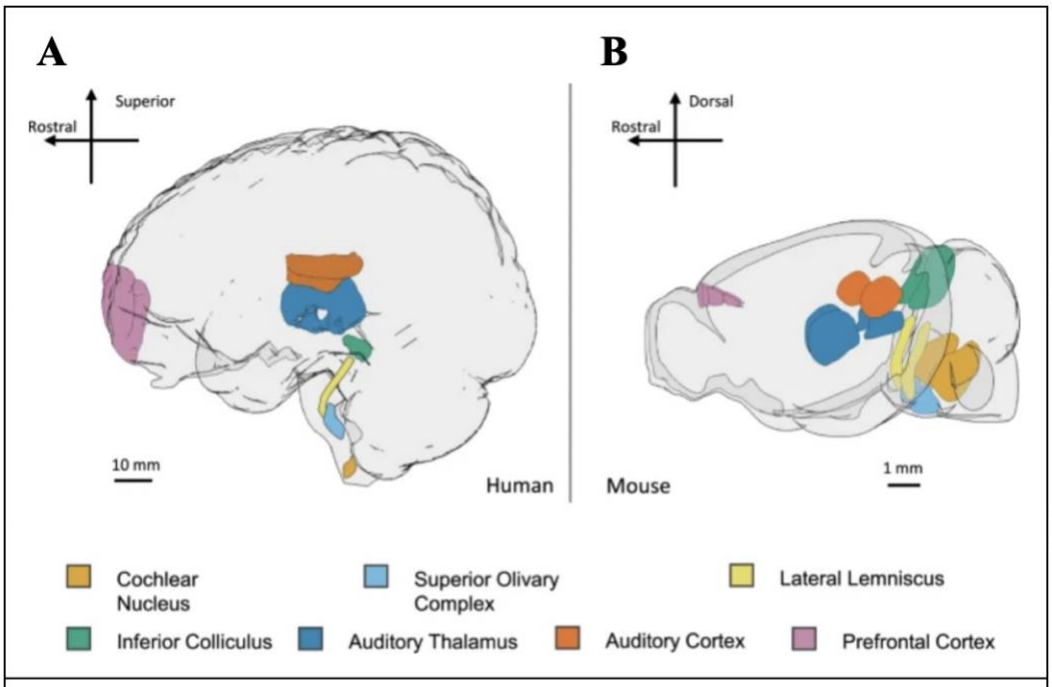

Figure 4 Schematic comparing human auditory brain topography to mouse auditory brain topography

(A) The human auditory brain regions and their topography **(B)** Mouse auditory brain regions and their topography. (Schematics (A) and (B) created by Wilde, M., Constantin, L., Thorne, P.R. et al., 2022)

1.1d The Brainstem and the Superior colliculus

The brainstem serves as one of the fundamental processing regions of the ascending central auditory system; it is involved in the integration of visual, somatosensory, and auditory information. The uppermost component of the brainstem, called the midbrain, plays an important role in sound detection, multisensory integration, and orienting the animal's head and eye movements in the direction from which the sound was detected. The ability to localize sound using coordinated head and eye movements is the result of a visual-auditory map organized by neuronal projections from the inferior colliculus, to other midbrain regions, such

as the superior colliculus (Hudspeth, A. J, and Masakazu Konishi, 2000). The superior colliculus is a layered structure located in the rostral midbrain region of the brainstem (**Fig. 5**). It has a laminated structure consisting of seven layers; the first three layers, also called the superficial layers, receive mainly visual inputs; the remaining four layers, also called the deeper layers, receive both auditory and somatosensory inputs (Kaas, Jon H, editor., 2017). The superior colliculus receives afferents from multiple sensory modalities: visual, auditory, and somatosensory; the various projections from the lateral lemniscus to the deeper layers of the superior colliculus allow for the integration of both visual and auditory information and responses together (Hudspeth, A. J, and Masakazu Konishi, 2000). The superior colliculus facilitates the localization of a sound stimulus in the environment by coordinating contralateral head and eye movements (Standring, Susan., et al, editor, 2008); these coordinated head and eye movements are the result of multisensory integration that occurs within the superior colliculus.

To better study and understand the properties that define auditory-responsive neuron populations, I needed to find a way to molecularly label active neurons. In the Feldheim lab, various experiments have been performed to record the activity of auditory responsive neurons in the superior colliculus. Electrophysiological experiments that were performed were able to record active neurons to construct a topographic map of auditory space in the superior colliculus of a mouse (Si, Yufei, et al., 2022); additionally, the Feldheim Lab has recorded active neurons to investigate nonlinear visuoauditory integration in the mouse superior colliculus (Ito, Shinya, et

al., 2021). Despite these novel and important findings discovered by the Feldheim Lab, we have yet to determine an effective way to fluorescently label the auditory-responsive that have been recorded in previous experiments, such as those aforementioned, to further investigate how auditory information is integrated in the superior colliculus. By molecularly labeling auditory-responsive neurons, these active populations can be visualized and further analyzed using neuronal markers, such as parvalbumin, to identify neuron types; we could identify whether these neurons are excitatory or inhibitory and the neurotransmitters that affect the function of auditory-responsive neuron populations. Additionally, molecularly labeling auditory-responsive neurons would allow for identification of genes that are expressed in these active neuron populations that are responsible for defining their auditory response properties. Using this information, the Feldheim lab can gain further insight into the complex process of multisensory integration that occurs within the superior colliculus and its role in the central auditory pathway.

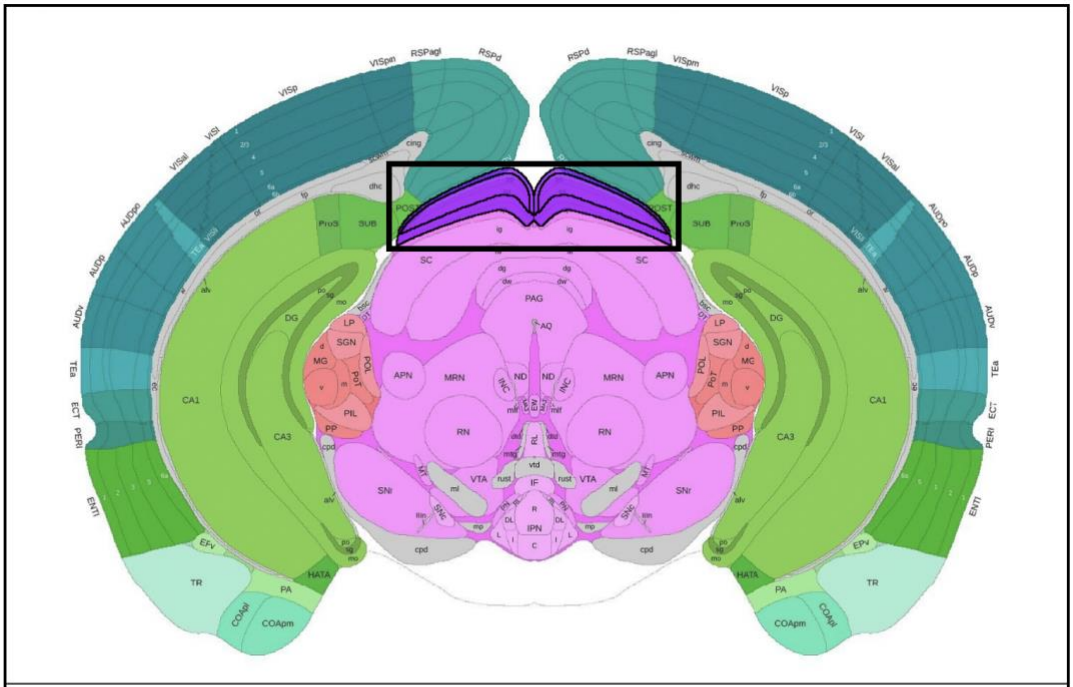

Figure 5 Mouse brain section showing the superior colliculus

The superior colliculus of the mouse (shown in a black box). (Image from the Allen Brain Atlas)

1.1e Genes of interest and their functions

Immediate early genes are transiently and rapidly induced during states of neuronal activity in response to a specific stimulus and serves as a marker of neuronal activation; one of these immediate early genes that is activated in response to a stimulus is the *fos* gene. The products of many immediate early genes, such as *fos*, are transcription factors, which use downstream transcriptional mechanisms to regulate cellular functions (Guenthner, Casey J, et al., 2013). *Fos* is a protein encoded by the *c-fos* gene (Dragunow M., Faull R., 1989).; *Fos* encodes a protein containing leucine zipper domains that can dimerize to form a transcription factor complex called AP-1; the *Fos* gene is translated in the cytoplasm and migrates to the nucleus where it forms

dimers with other co-induced transcription factors. The dimers formed by the *Fos* gene can then bind to the promoter regions to allow for the induction of the expression of target genes; the *c-fos* promoter was also found to respond to changes in calcium concentrations in neurons, indicating that *c-fos* expression is correlated to neuron activation (T. Herdegen, et al., 1998). In mice, the *fos* gene can be used to form a *Fos-Cre*^{ER} knock-in/knockout allele which abolishes the expression of the *fos* gene and induces expression of the *Cre*^{ER} fusion protein by using the promoters present in the *fos* immediate early gene (Guenthner, Casey J, et al., 2013). The *fos* gene is expressed in active neurons, therefore, its enhancer and promoter elements are utilized in genetic manipulation experiments, such as the *fos*/TRAP2 method, as *fos* serves as a molecular marker for neuronal activity (Chung L., 2015).

1.1f The *Fos*/TRAP2 Method to target active neuron populations

In order to gain genetic access to neuronal populations that have been activated in response to a stimulus detected in the environment, a novel method to fluorescently label active neurons was developed. The *fos*/TRAP2 method, or "Targeted Recombination of Active Populations" (TRAP), is a method used to fluorescently label populations of neurons based on their functional criteria, which is whether they produce synaptic activity as a result of exposure to a given stimulus (**Fig. 6**). This method allows for genetic access to neurons that were activated in response to a given stimulus using the immediate early gene *fos* and the effector gene *R26tdTomato* in a *cre*-dependent system in *fos-cre*^{ER} transgenic mice (**Fig. 6A**). *Cre* is expressed in the immediate early gene *fos* when neuronal activity is present; *Cre*^{ER}

requires 4-Hydroxytamoxifen in order be localized to the nucleus, where it is directed to the *loxp* sites that flank the "stop" codon that would have inhibited the expression of the *R26tdTomato* reporter gene (Guenthner, Casey J, et al., 2013). This results in the neurons that were activated in response to a given stimulus within twelve hours of exposure to 4-hydroxytamoxifen to become fluorescently labeled with *R26tdTomato* (**Fig. 6C**). I chose to use the effector gene *R26tdTomato*, which is a bright red fluorescent protein that is used as an alternative to the green fluorescent protein (GFP) tag, for cell labeling as it produces a stronger fluorescence as a result of its tandem dimer structure (*Takara Bio*). Without the use of 4-Hydroxytamoxifen, Cre^{ER} remains in the cytoplasm of the neurons that were activated in response to a stimulus and, therefore, does not result in genetic recombination with the effector gene (Guenthner, Casey J, et al., 2013). Furthermore, cells that were not activated in response to a given stimulus also will not undergo genetic recombination, as Cre^{ER} was not expressed in these cells (**Fig. 6B**) (Guenthner, Casey J, et al., 2013).

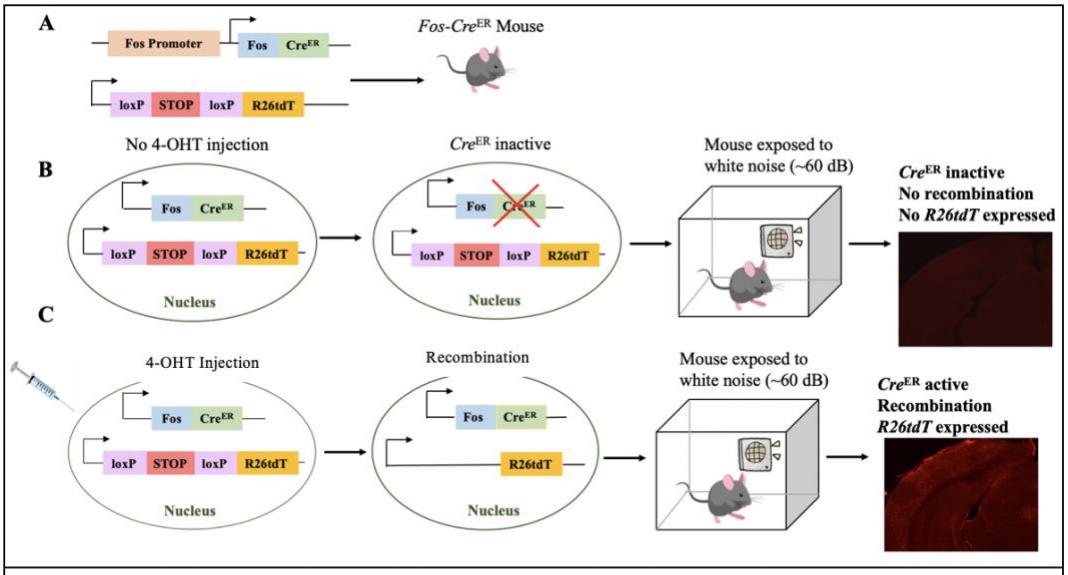

Figure 6 Schematic depicting the *Fos*/TRAP2 method

(A) Gene construct **(B)** Mouse exposed to sound without 4-hydroxytamoxifen injection, *Cre*^{ER} is inactive in neurons activated by sound. **(C)** Mouse exposed to sound after 4-hydroxytamoxifen (4-OHT) injection, allows for recombination and activates *Cre*^{ER}, neurons activated by sound express *R26tdTomato*.

1.1g The *Cre-loxp* System for genetic manipulation

The *Cre-loxp* system is used for mammalian gene manipulation experiments; it uses a single enzyme called *cre* recombinase that is able to recognize two repeated *loxp* sites, or splice sites, and excises the DNA located between these two sites. The "stop" codon located between these two *loxp* sites inhibits the expression of the effector gene; removing the "stop" codon flanked by the two *loxp* sites induces the expression of an effector gene, such as *R26tdTomato*, resulting in fluorescent labeling of active neurons which can then be used to identify the cells of interest (**Fig. 6C**). The inducible-*cre* system uses a *cre*^{ER} fusion protein, which is *cre* recombinase bound to an estrogen receptor (Donocoff, R.S., Teteloshvili, N., et al. (2020) ; *cre*^{ER} activity can be induced by using 4-Hydroxytamoxifen to drive transcription of the effector

gene in the *fos*/TRAP2 method to target and fluorescently label neuronal populations that had been activated in response to a given stimulus detected from the environment (Kim H, Kim M, et al., 2018). The *cre-loxP* system has been used to breed inducible-*cre* transgenic mouse lines that can then be used for genetic manipulation experiments, such as the *fos*/TRAP2 method.

1.2 Materials/Methods

1.2a Mice acquisition and breeding

The transgenic line of $Fos^{2A\text{-}iCreERT2}$, also named TRAP2, knock-in C57/BL6 mice used for the auditory experiment were acquired from Dr. Yi Zuo (University of California, Santa Cruz, Department of Molecular, Cellular, and Developmental Biology, Zuo Lab); Dr. Yi Zuo acquired the $Fos^{2A\text{-}iCreERT2}$ knock-in mice from the Jackson Laboratory (JAX strain #030323).

The mice were bred in the vivarium by pairing a male mouse and a female mouse to be housed together in the same cage; both the male mouse and the female mouse acquired from Dr. Yi Zuo were heterozygous for *fos-cre* (+/-) and *R26tdTomato* (+/-). The offspring that were homozygous for *cre* (+/+), *R26tdTomato* (+/+), or both were then paired together and bred in the same manner to produce a mouse line homozygous for these two genes.

Male and female mice of approximately two to three months of age were used in the auditory experiments and no significant differences between sexes were reported. The sample size(n) is 4 mice for each condition; it is important to note that

for the IC region analyses, the sample size is n=3, as the IC had been damaged while sectioning the brain using a microtome in one of the 50mg/kg experimental trials.

All of the experimental procedures conducted on the mice were performed in accordance with the protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, Santa Cruz.

1.2b Tail snipping procedure and genotyping

To determine the genotype of the mice, a series of experiments were conducted in both the vivarium and in the laboratory. The genotyping process began by conducting a tail snipping procedure in the vivarium. At approximately three weeks of age, the mice were weaned from their mother and were anesthetized using isoflurane. The scissors used were sterilized using heat in a bead sterilizer at a temperature of at least 100°C, for approximately five minutes. The mouse was held manually and approximately three to four millimeters of its tail was cut using sharp, sterilized scissors; this small tail snip was then collected in a 1.5mL eppendorf tube and was brought back to the lab for further processing. Once the tail has been snipped, the open wound on the mouse tail was dipped into a chemical cauterizing agent called Kwik Stop®, which contains antibiotics to prevent infection, has pain relieving properties, and stops blood flow from the surgical site to allow the wound to close. After the procedure, the mouse is returned to the cage; I monitor the mouse until they have woken up. Once the mouse is awake and alert, I relocate the mice back to their usual housing rack in the vivarium. The remaining biohazardous material, such as blood, fur, etc., is wiped off of the scissors using a paper towel or

kim wipe with 70% ethanol, and the scissors were then re-sterilized using heat; this process is repeated for each tail snipping procedure.

1.2c Tail digest procedure

To digest the tail snips in order to extract the mouse DNA, the three to four millimeters of

the tails collected from each mouse were incubated in 0.5mL of tail buffer in a 1.5mL eppendorf tube. The tail buffer used to digest the tissue is made up of 17mM Tris pH 7.5, 17mM EDTA, 170mM NaCl, 0.85% SDS, and 0.2% Proteinase K; the 0.2% of proteinase K is added to the buffer immediately before use, while the rest of the buffer and its components were prepared at an earlier time. The tails were incubated in 0.5% of tail buffer at 55°C for approximately 24 hours, or overnight, to digest completely; when fully digested, a pile of fur and bones had collected at the bottom of the eppendorf tube. Once the tails had fully digested, 0.25mL of saturated NaCl was added to each tube, which were then placed between two eppendorf tube racks and shaken manually for approximately three to four minutes. The eppendorf tubes were then left on ice to chill for ten minutes before they were spun in a microcentrifuge for five

minutes at 6800rpm. Then, I took 0.5mL of the supernatant from the eppendorf tube containing the DNA sample, and transferred this into a new 1.5mL eppendorf tube containing one milliliter of 95% ethanol; the tube was then inverted a few times and a small string of the DNA precipitate will have formed, which I then spooled gently onto a glass capillary tube. The glass capillary tube containing the spooled DNA was

rinsed gently in 70% ethanol for a few seconds; the tube was then inverted, DNA-side up, in a clean 1.5mL eppendorf tube until the DNA had dried, which took approximately five to ten minutes. Once the samples have air dried completely, the capillary tubes were inverted, DNA-side down, and the DNA was resuspended in 0.1mL of milliq H_2O. If the DNA did not precipitate when the eppendorf tube was inverted, the eppendorf tube was placed in the microcentrifuge for ten minutes and was spun at the maximum rpm of the microcentrifuge. The supernatant was removed from the eppendorf tube, then, the remaining pellet at the bottom of the tube was rinsed with 70% ethanol and left to air dry. Once the samples have air dried completely, the DNA pellet that had formed while in the microcentrifuge was resuspended in 0.1mL of milliq H_2O. The samples were placed in an incubator at 37°C and left overnight; if the samples were unable to be left overnight, they were left at room temperature on the laboratory bench for approximately 48 hours. Once the samples were fully incubated, three microliters of each sample were used for polymerase chain reaction analyses.

1.2d Polymerase chain reaction

To identify the genotype of each sample, a polymerase chain reaction (PCR), was performed. The 10x PCR buffer was prepared using 500mM KCl, 100mM Tris pH 9.0, and 1% Triton X-100. Each PCR contained three microliters of DNA and 47uL of the PCR mix that I prepared according to the gene being tested. For *R26tdTomato*, the PCR buffer contained 36.6μL milliq H_2O, 5 μL 10x PCR buffer, 1 μL 10 mM dNTPs, 0.2 μL of each primer, 3 μL 25mM $MgCl_2$, and 1 μL of Taq

polymerase; taq polymerase was added last in this reaction. For the *R26tdTomato* wild type PCR test, the forward primer sequence used was 5'-GCACTTGCTCTCCCAAAGTC- 3' and the reverse primer sequence was 5' -CCGACAAAACCGAAAATCTG- 3'. For the *R26tdTomato* mutant PCR test, the forward primer sequence used was 5'- AGCAAGGGCGAGGAGGTCATC - 3' and the reverse primer sequence was 5' - CCTTGGAGCCGTACATGAACTGG - 3'. For the *fos-cre* PCR test, the PCR buffer contained 39.4 μL milliq H_2O, 10 μL 5x HF buffer, 1 μL 10mM dNTPs, 0.2 μL each primer, and 0.5 μL phusion polymerase; the phusion polymerase was added last to the PCR buffer mix. For the *fos-cre* PCR test, the *fos* wild type forward primer sequence used was 5'- GTCCGGTTCCTTCTATGCAG - 3', the *fos-cre* forward primer sequence was 5' - CCTTGCAAAAGTATTACATCACG - 3', and the *fos* reverse primer sequence used was 5'- GAACCTTCGAGGGAAGACG - 3'. The PCR tests were then placed into the thermocycler and were run in their respective programs; it is important to note that the *R26tdTomato* gene was tested in two different reactions, one wild type PCR and one mutant PCR, but were run together in the same program. These results were analyzed using gel electrophoresis on a 1.5% agarose gel containing ethidium bromide.

1.2e 4-Hydroxytamoxifen Preparation

The auditory experiment procedure utilizes the *fos*/TRAP2 method to identify neuronal populations that have been activated in response to a sound stimulus. To prepare a 20mg/mL dilution of 4-Hydroxytamoxifen (4-OHT) (Sigma Aldrich

#H6278), I began by dissolving 50mg of 4-Hydroxytamoxifen in 2.5mL of 95% ethanol. It is important to note that extreme caution must be used when working with 4-Hydroxytamoxifen; the required PPE stated in the SOP for 4-OHT was worn during the drug preparation, IP injection procedures, and waste disposal; 4-OHT waste was disposed of according to the SOP and all surfaces in contact with 4-OHT were cleaned using 10% bleach. As an additional measure of precaution, a small piece of Parafilm was wrapped tightly around the lid of the 4-OHT bottle to prevent any spilling during the next step of preparation. After the 95% ethanol (EtOH) was added to the 4-OHT, the bottle was placed in a rotator into an incubator set to 37°C; the solution rotated in the incubator until the 4-Hydroxytamoxifen was fully dissolved, which took approximately 15 minutes. Once the drug had fully dissolved, the 4-Hydroxytamoxifen 20mg/mL dilution was aliquoted into 1.5mL eppendorf tubes and stored at -20°C. Prior to the administration of the IP injections, the aliquots were warmed in a rotator in an incubator set to 37°C for 15 minutes; as an additional measure of caution, the aliquots were wrapped with a small piece of Parafilm to prevent spillage. Once warmed, the 4-OHT 20mg/mL aliquots were diluted to 10mg/mL using corn oil (1:2 ratio of 4-OHT in 95% EtOH to corn oil); this mixture was vortexed until opaque and was then placed in a vacuum desiccator to evaporate the 95% ethanol from the mixture, which took approximately three hours. Once the ethanol has completely evaporated, the 4-OHT preparations was ready for administration via IP injection. The 10mg/mL 4-OHT dilutions were protected from degradation from light exposure by covering the lid of the vacuum desiccator with

aluminum foil; a small eppendorf box, also wrapped in aluminum foil, was used to transport the 4-OHT preparations to the vivarium procedure room.

1.2f Ear plug insertion

The auditory experiments were conducted on TRAP2 knock-in C57/BL6 transgenic mice that were of approximately two to three months of age. On day zero, the control mouse, which is the mouse that does not receive the sound stimulus, was taken into the procedure room in the vivarium. The mouse was anesthetized using isoflurane and placed under a microscope with a nose cone attached to administer a continuous flow of isoflurane to keep the mouse under anesthesia for the full duration of the procedure. I then took sterilized surgical forceps to gently expand the ear canal, and then inserted a small bead of Fun-tak mounting putty into the ear canal. The ear plug was secured by using a small drop of liquid super glue; any excess glue outside of the ear canal was removed using a cotton swab by gently swabbing the external ear. Once the glue dried, which took approximately one minute, the process was repeated for the other ear. After the procedure, the mouse was returned to its cage and monitored until it awoke from the anesthesia. The mouse was then checked daily throughout the course of the rest of the experiment to ensure that the ear plugs remained securely in place; if an ear plug was found to be missing, this same process of inserting the ear plug was repeated. The control mouse with ear plugs, along with a mouse without ear plugs that will serve as the experimental mouse by later receiving the sound stimulus, are brought into a separate experiment room in the vivarium for auditory deprivation for approximately 24 hours, or overnight.

1.2g Auditory experiment procedure and IP injection

The auditory experiment timeline occurred over the course of six days (**Fig. 7**). On day zero of the auditory experiment, the control mouse was fitted with ear plugs according to the ear plugging procedure (**1.2f**); a control mouse with ear plugs and a mouse without ear plugs that will serve as the experimental mouse by later receiving the sound stimulus, are brought into a separate experiment room in the vivarium for auditory deprivation for approximately 24 hours or overnight. On day one, the control mouse with ear plugs is removed from the experiment room and brought to its normal housing room in the vivarium. In the experiment room, the experimental mouse received two hours of auditory stimulation in the form of white noise at approximately 60 decibels in increments of ten seconds "on" and ten seconds "off". After two hours, the sound stimulus was turned off and the mouse was removed from the room; both the control mouse and the experimental mouse were then brought to the procedure room. In the vivarium procedure room, the mice were anesthetized using isoflurane and were administered an intraperitoneal (IP) injection of 4-Hydroxytamofixen at a dosage of 50mg/kg of body weight; the mice were weighed on a scale before 4-OHT administration to calculate the correct dosage for each mouse. For one of the experiment trials, I administered 4-OHT at a dosage of 30mg/kg of body weight to test whether a lower dose would be as effective in activating *cre*-inducible recombination; the experimental trial receiving a 4-OHT dosage of 30mg/kg is noted in the figure legends for **Fig. 9** and **Fig. 10**. After the IP injection was administered, the mice were monitored until they have awoken from the

anesthesia; the control mouse was returned to its original vivarium housing room, while the experimental mouse was brought back to the experiment room where it received an additional two hours of auditory stimulation at a volume of approximately 60 decibels in the form of white noise, which occurred in increments of ten seconds "on", meaning the sound was playing, and ten seconds "off" again, meaning the sound had stopped playing. After the two hours of sound stimulation had been complete, the sound was turned off and the control mouse was brought back into the experiment room; both the control mouse and the experimental mouse remained in the experiment room for 24 hours, or overnight, to undergo auditory deprivation. On day two of the experiment, both the control mouse and the experimental mouse were returned to their original housing room in the vivarium, and remained there for an additional four days. On day six of the experiment, the mice were removed from the vivarium to be perfused and dissected in the laboratory fume hood.

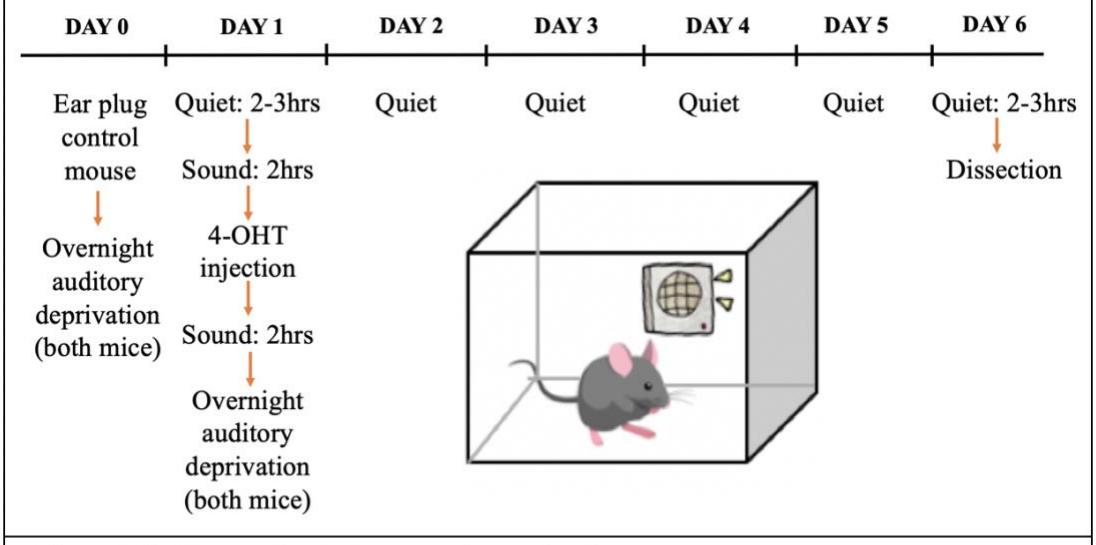

Figure 7 Auditory stimulation experiment timeline

Daily timeline starting at day 0 until day 6 of the auditory stimulation experiment (experiment timeline modified from Guenthner, Casey J, et al, 2013, Figure 5).

1.2h Brain harvesting and Tissue processing

The mice were euthanized prior to dissection in the chemical fume hood using isoflurane (VetOne Fluriso). Once the mouse was unresponsive to a tail pinch, the mouse was inter-cardially perfused with 1x PBS, which was then immediately followed by a perfusion with 4% paraformaldehyde in 1x PBS. Once the perfusion was complete, the brain was harvested and then fixed in 4% paraformaldehyde for approximately 24 hours, or overnight, at 4°C. The next day, the brain was transferred to a 30% sucrose in 1x PBS solution until the brain had sunk to the bottom of the 12 well plate, which took approximately 24 hours, or overnight, at 4°C. After the brain had sunk in the 30% sucrose in 1x PBS solution, the brain was sectioned along the coronal plane at a thickness of 100µm using a microtome.

1.2i Immunohistochemistry

I performed an immunohistochemistry assay on these brain sections to stain for the fluorescent label *R26tdTomato*; although *R26tdTomato* emits a strong fluorescent signal without antibody staining, I chose to continue with the immunohistochemistry analysis as it produced a stronger fluorescent signal and labeled active neurons more clearly. The coronal brain sections of 100μm thickness were first incubated in 0.6% TritonX-100 in 1xPBS at room temperature for one hour on a shaker at a low speed to prevent tissue damage. The blocking solution was prepared using 0.25% TritonX-100, 1X PBS, and 5% heat inactivated donkey serum (Sigma Aldrich #S30); the sections were then blocked in blocking solution for at least two hours at room temperature on a shaker at a low speed. The sections were first stained with a goat anti-*R26tdTomato* primary antibody (Origene #AB8181-200) at a dilution of 1:1000 in blocking solution; the sections were incubated overnight at 4°C on a shaker at a low speed to prevent tissue damage; the 24 well plates containing the sections were covered in aluminum foil to protect the tissue from light exposure throughout the immunohistochemistry experiments and analyses. The brain sections were then washed three times, with each wash lasting approximately 45 minutes to one hour, in 1x PBS on a shaker at a low speed. Once the sections had been washed, the were stained with AlexaFluor 546 donkey anti-goat secondary antibody IgG (Life Technologies #A11056 [H + L]) at a dilution of 1:1000 in blocking solution; they were incubated overnight, or for two to three hours at room temperature, at 4°C on a shaker while covered with aluminum foil. The sections were then washed for

approximately 45 minutes to one hour in 1x PBS on a shaker; during the second wash, a DAPI stain was added at a 1:1,000 dilution in 1xPBS and the sections were incubated on a shaker at room temperature for fifteen minutes. The sections were washed in 1x PBS an additional two more times, with each wash lasting approximately 30 minutes; then, the brain sections were ready to mount onto microscope slides.

1.2h Microscopy

The brain sections were delicately mounted onto microscope slides using a small paintbrush; the microscope slides were left to air-dry until the sections were transparent, which took approximately ten minutes. The sections were then sealed onto the microscope slide with a coverslip using Floromount mounting medium. Once the Floromount had dried, the sections were ready to be viewed and imaged using fluorescent microscopy.

1.2k Analysis

The fluorescent images were taken from 2x (**Fig. 8** and **Fig. 9A**) and 10x (**Fig. 9A**) objectives on an Olympus fluorescent microscope; the images were processed using ImageJ software and FIJI software. To ensure that the correct brain regions were being imaged, I used the Allen Brain Atlas as a reference for the mouse brain topography. For the 10x objective images (one trial shown in **Fig. 9A**), cellular density was determined by manually counting cells using FIJI's plug-in "cell counter" tool; I used GraphPad's Prism 9 software to quantify the results of one trial (**Fig. 9B**). For statistical analyses, I performed an unpaired t-test with Welch's correction to

compare the two experimental conditions across all trials using GraphPad's Prism 9 software; the results are shown as a bar graph and as a scatter plot (**Fig. 10**) that were made in GraphPad's Prism 9 software.

1.3 Results

To identify and molecularly label neuron populations that were activated in response to a sound stimulus, I used a transgenic mouse line of *Fos*$^{2A\text{-}iCreERT2}$, or TRAP2, knock-in mice that express *R26tdTomato*. This genotype allows for *cre*-inducible genetic recombination in cells that express *fos* when the mice are injected with 4-hydroxytamoxifen (**Fig. 6**). I conducted an auditory experiment over the course of six days that consisted of inserting ear plugs bilaterally into mice serving as controls, and exposing mice in the experimental condition to an auditory stimulus in the form of white noise at approximately 60 decibels for four hours. After two hours of sound stimulation, mice in both the control and auditory stimulation conditions received IP-injections of 4-Hydroxytamoxifen to activate *cre*^{ER} and induce genetic recombination. The mouse in the auditory stimulation condition was exposed to another two hours of sound before both the ear plugged mouse and the mouse receiving auditory stimulation were returned to their standard housing, where they remained for 4 days to allow for the genetic recombination, or "TRAPing", of the active neurons to occur before their brains were harvested.

I identified which neurons were activated during the auditory experiment using immunohistochemistry to stain for the fluorescent label *R26tdTomato* in the brain sections that correspond to the primary auditory cortex (A1), superior colliculus

(SC), inferior colliculus (IC), and primary somatosensory cortex (S1) (**Fig. 9A**). I manually counted the fluorescently labeled active neurons to determine their cell densities (# of active cells/FOV) in the brain regions A1, SC, IC, and S1 for each auditory experiment trial (**Fig. 9B** and **Fig. 10A**). The primary auditory cortex (A1), superior colliculus (SC), and inferior colliculus (IC) all receive auditory inputs and are part of the auditory pathway (Standring, Susan., et al, editor, 2008); the primary somatosensory cortex (S1) serves as a control for the auditory stimulation experiment as it does not receive auditory inputs and is not considered to be part of the auditory pathway (Standring, Susan., et al, editor, 2008). Additionally, I performed an experimental trial without the administration of 4-OHT to serve as a control; since Cre^{ER} expression is induced by 4-OHT, there should be no fluorescence detected due to the lack of 4-OHT present. The results of this trial did not show any labeled cells in the A1 and SC regions (**Fig. 8**); this demonstrates how the *Fos*/TRAP2 method requires 4-OHT to induce cre^{ER} (Guenthner, Casey J, et al., 2013). I hypothesized that a significant difference in the number of active neurons will not exist between the ear plug condition and the auditory stimulation condition in the S1 region, as it is not involved in audition; furthermore, a significant difference in the number of active neurons will be present between conditions for regions A1, SC, and IC, as they are involved in audition. I performed an unpaired t-test with Welch's correction to analyze these statistics; the results showed that there was a significant difference in the number of labeled active cells, or "TRAPed" cells, between the mice in the ear plug condition and the mice in the auditory stimulation condition for all experiment

trials in regions of anterior A1, posterior A1, anterior SC, posterior SC, and in the IC (**Fig. 10A**). The greatest significance was found in the anterior A1 region (n=4, **p<0.01, p=0.0017), followed by posterior A1 (n=4, *p<0.05, p=0.0153) (**Fig. 10A**). The results depicting A1 as having significance differences in the number of active neurons are consistent with A1 being a known auditory-responsive region in the brain (**Fig. 10A**). The data also showed a significant difference in the number of active neurons between the ear plug condition and the auditory stimulation condition for anterior SC (n=4, *p<0.05, p=0.0361), posterior SC (n=4, *p<0.05, p=0.0421), and IC (n=3, *p<0.05, p=0.0493) (**Fig. 10A**); these findings are consistent with what is known about the SC's function of sound localization and multisensory integration, and the IC's function in auditory processing. The results also demonstrated that there was no significant difference (n=4, ns = not significant, p>0.05, p=0.6691) in the number of active neurons between the ear plug condition and the auditory stimulation condition for S1 (**Fig. 10A)**; this result is explained by the S1 region not being involved in audition or the auditory pathway, therefore, neuron activation in S1 was unaffected by sound stimulation or sound inhibition.

To account for possible variation in 4-OHT dosage between mice due to difficulties with drug administration due to the viscous nature of the corn oil, I wanted calculate the ratio between the number of active cells in each auditory brain region, A1, SC, and IC, and the number of active cells in S1 within the same mouse for each trial ((# of active cells/FOV) / # of active cells in S1) (**Fig. 10B**). If the active neurons during the auditory experiment conditions successfully underwent

genetic recombination, or were "TRAPed", the mice exposed to the auditory stimulus should have a greater ratio between the number of active cells in auditory brain regions, A1, SC, and IC, and the number of active cells in S1 than the mice that had worn ear plugs. I performed an unpaired t-test with Welch's correction and found that there was a significant difference in the ratio between the number of active neurons in anterior A1 (n=4, **p<0.01, p=0.0062) and posterior A1 (n=4, **p<0.001, p=0.0031) compared to the number of active neurons in S1 for both the ear plug condition and the auditory stimulation condition across all trials (**Fig. 10B**). The finding of A1 having the greatest significance in the ratio between the number active neurons in both anterior and posterior regions of A1 and the number of active neurons in S1 suggests that A1 has a larger population of auditory-responsive neurons compared to the SC and IC, which also receive auditory inputs. Furthermore, I found that there were no significant differences in the ratios between the number of active neurons in the anterior SC (n=4, ns = not significant, p>0.05, p=0.0738), posterior SC (n=4, ns = not significant, p>0.05, p=0.0764), or in the IC (n=3, ns = not significant, p>0.05, p=0.0635) in comparison to the number of active neurons in S1 for both the ear plug condition and the auditory stimulation condition across all trials (**Fig. 10B**). These insignificant findings are inconsistent with the known responsive properties of neurons in the SC and IC (Hudspeth, A. J, and Masakazu Konishi, 2000); this could be the result of high background of labeled cells in these regions, even when deprived of auditory stimuli.

In conclusion, the results of the auditory stimulation experiment found that the

fos/TRAP2 method could serve as a tool for genetic recombination experiments that can be used to target and fluorescently label neuron populations that were active in response to the presence of an auditory stimulus.

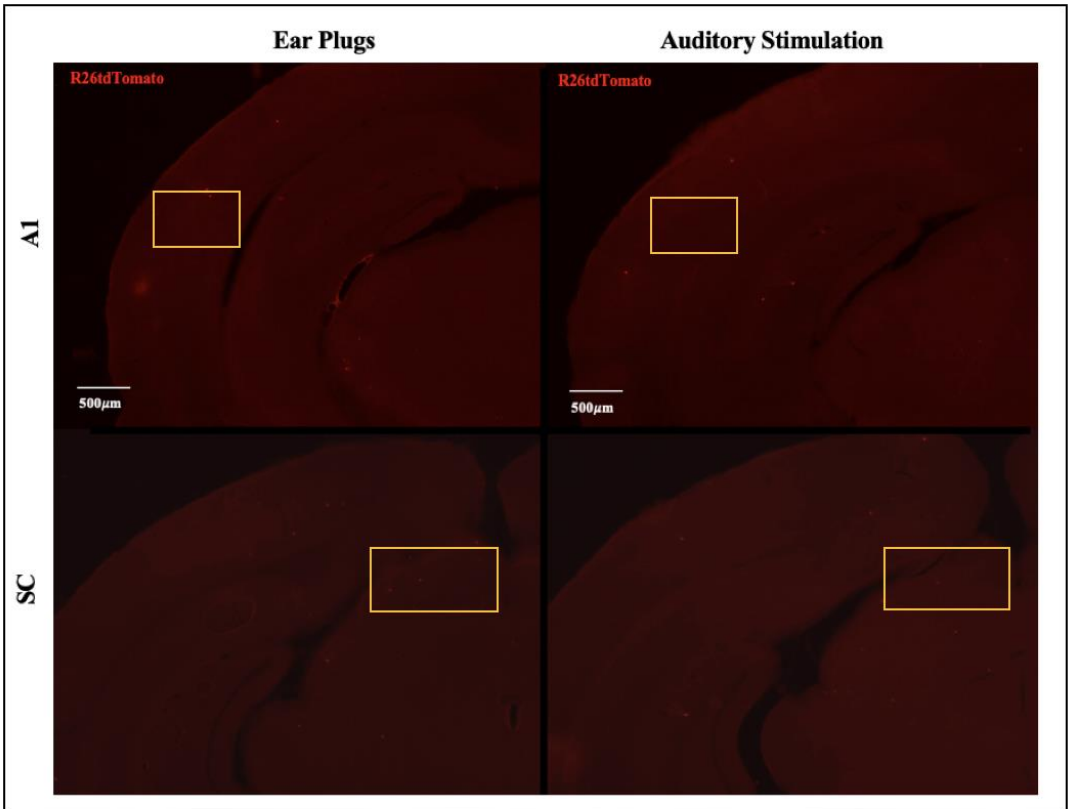

Figure 8 *Cre^{ERT}* is inactive without the presence of 4-hydroxytamoxifen

Fluorescent images of A1 (top) and SC (bottom) regions depicting the results of an auditory stimulation experiment trial conducted without the administration of 4-hydroxytamoxifen (4-OHT). The brain sections were stained for *R26tdTomato*. The left images show the results from a mouse with ear plugs; the right images show the results of a mouse that received auditory stimulation (2x objective with 500μm scale bar). A1 and SC regions are boxed in yellow.

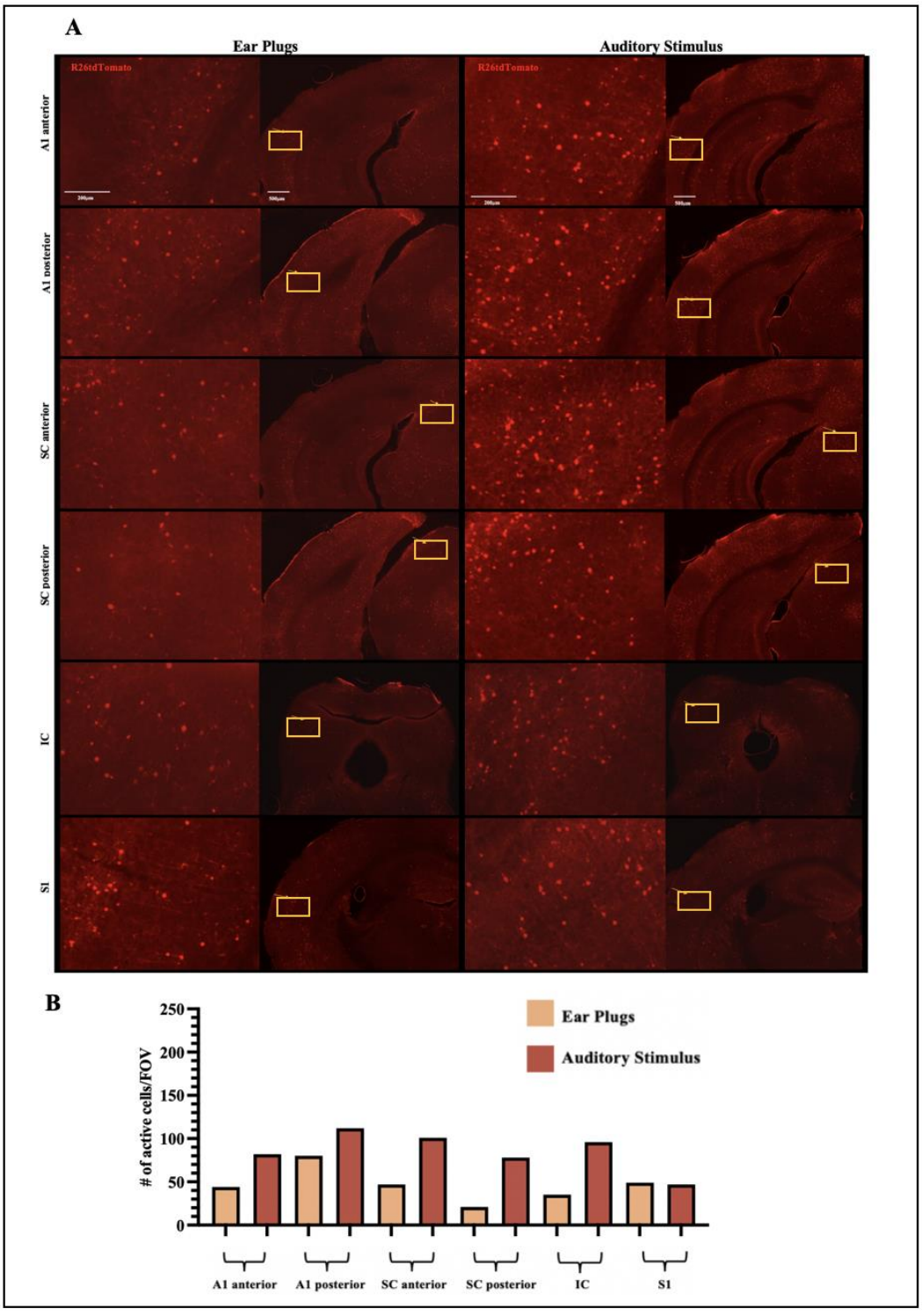
A
Ear Plugs
Auditory Stimulus
R26tdTomato
R26tdTomato
A1 anterior
A1 posterior
SC anterior
SC posterior
IC
S1
B
Ear Plugs
Auditory Stimulus
# of active cells/FOV
250
200
150
100
50
0
A1 anterior
A1 posterior
SC anterior
SC posterior
IC
S1

Figure 9 Mice exposed to auditory stimuli express greater numbers of TRAPed cells in auditory brain regions

(A) Fluorescent images depicting results of one auditory experiment trial (mice were injected with 4-OHT at 50mg/kg dosage). The brain sections were stained for *R26tdTomato*. The left panels show the results of a mouse with ear plugs, the right panels show the results a mouse that received auditory stimulation (2x objective images with 500μm scale bar, 10x objective images with 200μm scale bar). The brain regions shown (from top to bottom) are the anterior primary auditory cortex (A1 anterior), posterior primary auditory cortex (A1 posterior), anterior superior collicus (SC anterior), posterior superior colliculus (SC posterior), inferior colliculus (IC), and primary somatosensory cortex (S1). The boxed regions in the 2x images correspond to the approximate brain region for each respective 10x image. **(B)** Bar graph depicting number of active cells for each brain region in a mouse with ear plugs (orange) and a mouse receiving auditory stimulation (red) per FOV (field of view) (from fig. 9A)).

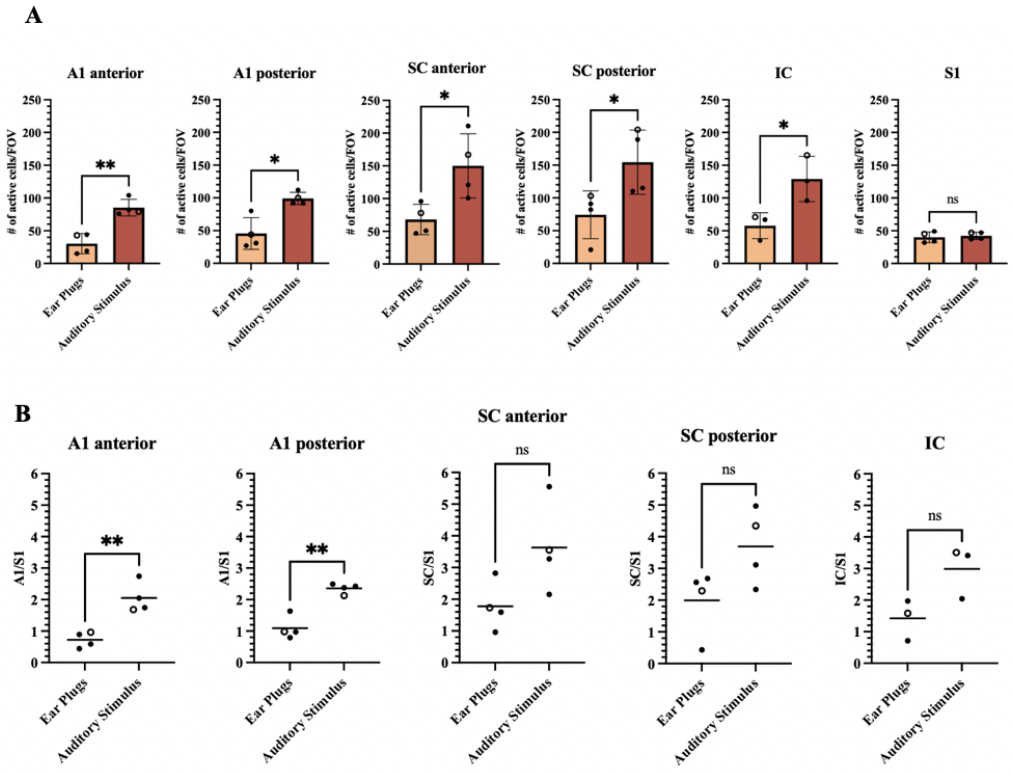

Figure 10 Statistical analyses across all auditory experiment trials

(A) Bar graphs depicting the mean numbers of active (TRAPed) cells per FOV for each auditory brain region and primary somatosensory cortex across all auditory experiment trials (n=4 for each region except for IC (n=3)). The orange bar shows the mice with ear plugs, the red bar shows the mice exposed to auditory stimuli. One circle represents one mouse; the filled in circles are mice that were injected with 4-OHT at a dosage of 50mg/kg, the open circles are mice that received a 4-OHT dosage of 30mg/kg (unpaired t-test with Welch's correction, ** = $p<0.01$, * = $p<0.05$, ns = not significant). **(B)** Scatter plots depicting the ratio between the number of active (TRAPed) cells per FOV for each auditory brain region across all auditory experiment trials, and the number of active (TRAPed) cells per FOV in S1 across all auditory experiment trials (n=4 for each region except for IC (n=3)). One circle represents one mouse; the filled in circles are mice that were injected with 4-OHT at a dosage of 50mg/kg, the open circles are mice that received a 4-OHT dosage of 30mg/kg. The bar shows the mean value of each condition (unpaired t-test with Welch's correction, ** = $p<0.01$, ns = not significant).

1.4 Discussion

I used a transgenic mouse line of *Fos*$^{2A\text{-}iCreERT2}$, or TRAP2, knock-in mice that can undergo *cre*-inducible genetic recombination in cells that express *fos* in the presence of 4-Hydroxytamoxifen to identify and locate the neurons that were activated in response to auditory stimulation. Previous literature has shown that the newly developed *fos*/TRAP2 method for *cre*-inducible genetic manipulation serves an effective tool to molecularly label active neuron populations in auditory, somatosensory, and visual stimulation experiments (Guenthner, Casey J, et al., 2013). I used the *fos*/TRAP2 method to conduct an auditory stimulation experiment to molecularly label auditory-responsive neurons in a mouse model; I analyzed the primary auditory cortex (A1), the superior colliculus (SC), the inferior colliculus (IC), and the primary somatosensory cortex (S1).

I found a significant difference in the number of labeled cells in the mice that had their ears plugged compared to the mice that were exposed to a sound stimulus in A1, SC, and IC regions (**Fig. 9** and **Fig. 10A**), which are known to receive auditory inputs and are part of the auditory pathway; the A1 regions showed the greatest difference in the number of labeled neurons (**Fig. 10**). This finding this consistent with the results reported by Guenthner, Casey J, et al., (2013) in their Figure 5 where they showed how cells in the cochlear nucleus of a mouse that were activated within the twelve hour TRAPing window were successfully labeled by the effector gene when the mouse was exposed to a sound stimulus. I found no significant difference in the number of active neurons in S1 for mice in the ear plug condition compared to

mice in the auditory stimulation condition, suggesting that the neurons that were active are not auditory-responsive, as neuron activity was unaffected by the presence or absence of sound stimulation (**Fig. 10A**). Furthermore, I found that there was no significant difference reported for the ratio between the number active neurons in the SC and the number of active neurons in S1 (**Fig. 9B**); this could indicate that there is a smaller auditory-responsive neuron population in this region compared to A1. Although there was a greater number of active neurons in the auditory stimulation condition than in the ear plug condition for each trial, I did not find a significant difference in the ratio between the number active neurons in the IC and the number of active neurons in S1 (**Fig. 9B**); this could have been the result of a smaller auditory-responsive neuron population present in this region in comparison to the SC and A1. Additionally, the insignificant results for the ratios of active cells in the SC and IC regions compared to those in S1 could also be the result of high background in these areas, even when deprived of auditory stimuli. The variation in the number of labeled cells for each region across trials could be the result of leakage of the drug during administration via IP injection due to the high viscosity of the 4-OHT dilution in corn oil. Moreover, I tested a lower dose of 4-OHT for one of the auditory experimental trials (trial noted in figure legend of **Fig. 9**) and found that a 30mg/kg dose of 4-OHT was as effective as a dose of 50mg/kg; this could be beneficial as it would reduce the amount of drug used during the experiment while still fluorescently labeling auditory-responsive neurons.

Ultimately, these findings demonstrate that the *fos*/TRAP2 method could serve as a useful tool to target and molecularly label neurons that are activated in response to auditory stimulation.

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