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Anna Sotirescu April 10, 2024

Development of Surgical Techniques and Behavioral Methods to Explore Adult Rat Vocal Production

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Abstract

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Similar to how humans show emotions through different vocalizations, rats and other rodents have different vocal responses based on varying affective states. In addition to producing audible squeaks and calls, rats can communicate by producing ultrasonic vocalizations (USVs). These vocalizations are inaudible to humans and occur in the 20 - 100 kHz frequency range. Adult rats produce USVs divided into two general frequency classes, 50 kHz or 22 kHz, which generally correlate to different emotional states. Fifty kHz vocalizations are often associated with appetitive/rewarding emotions evoked by positive situations, whereas 22 kHz vocalizations often correlate to aversive emotions and environments. Analysis of USVs serves an important role in many laboratory studies across multiple scientific areas and is used to address questions related to the internal states of the rat, vocal motor output, and social behavior. The mechanism the larynx uses to produce USV in rats is just beginning to be understood. To aid in completing our own studies and those in other labs, we are developing a general protocol to measure the muscle activity of the larynx during USV production. To study how muscle movements in the larynx elicit different frequencies of USVs in rats, behavioral and surgical techniques are crucial to perform and understand this goal. To this end, we have taken two major steps: developing behavioral paradigms that optimize the elicitation of USVs (Aim 1) and performing surgical techniques to access different intrinsic laryngeal muscles in rats to perform EMG recording (Aim 2). The development of Aim 1 allows experimenters to successfully produce USVs in rats with highly variable vocalizations based on the specific personality of the animal. Aim 2 specifically targets intrinsic laryngeal muscles of interest that aid in the production of USVs: cricothyroid (CT), posterior cricoarytenoid (PCA), and thyroarytenoid (TA).

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Introduction:

A. Intraspecies Vocalization

Many vertebrates rely on vocalizations as a fundamental component for intraspecies communication. Vocalizations are one of the oldest characteristics found in vertebrates and are highly unique to individuals. For hundreds of millions of years, vocalizations have been remarkably adaptive and biologically significant for shaping animal behavior (Brudzynski, 2021). Vocalizations used for intraspecies communication are critical for various aspects of mammalian life, such as adjustment to new environments, expression of emotions, reproduction, survival, and socialization (Brudzynski, 2021). These aspects of behavior often indicate different internal states, including affective, motivational, and arousal components (Nieder & Mooney, 2020). Communicative vocalizations have information-baring parameters such as sound frequency and duration, which can translate to the conditions of the individuals listed above. Different species produce vocalizations at different frequencies and have different ranges of frequencies that their auditory system can detect. For example, humans typically can hear frequencies ranging from 20 Hz to 20 kHz (Purves *et al*., 2020). Understanding these aspects of vocalizations, which are emitted in various environments and physical conditions, can serve as an important indicator of the internal state and sentiments of the mammal.

B. Ultrasonic Vocalizations

Similar to how humans show emotions through vocalizations such as laughing, crying, or moaning, small rodents, including rats and mice, have different vocal responses based on varying situations and affective states. In addition to producing audible squeaks and calls, rats and mice communicate by producing ultrasonic vocalizations (USVs). USVs are inaudible to humans, with

a frequency ranging from 20 - 100 kHz (Schwarting & Whor, 2012). Based on varying conditions and emotional states, juvenile and adult rats emit USVs at frequencies that fall into two general ranges centered around either 22 kHz or 50 kHz. However, rats do produce vocalization in other frequency ranges. For example, rat pups produce 40 kHz calls when separated from their mothers or siblings (Schwarting, 2023). For this study, we focused on adult USV production of 22 and 50 kHz.

Generally, when rats emit USVs around 22 kHz, these calls are associated with adverse environments or situations that often cause negative emotions. Some factors that can induce 22 kHz calls include loud noises, rapid changes in temperature, and unfavorable encounters with predators or other more dominant rats. This 'class' of vocalizations can have durations (length of vocalization) between 300 to 4,000 ms (Portfors, 2007) (Figure 1A). Conversely, when rats emit USVs around 50 kHz, these calls are generally associated with positive environments or situations that cause appetitive emotions. Factors that induce 50 kHz vocalizations include juvenile play, the anticipation of mating and reward, novel environments, socialization, and tickling (Portfors, 2007). In addition, 50 kHz calls are generally emitted in locomotion activities (Brudzynski, 2021). This class of vocalizations has narrow bandwidths (span of a specific frequency range) from one to seven kHz and much shorter durations lasting 10 to 150 ms (Portfors, 2007; Berz, 2022) (Figure 1B).

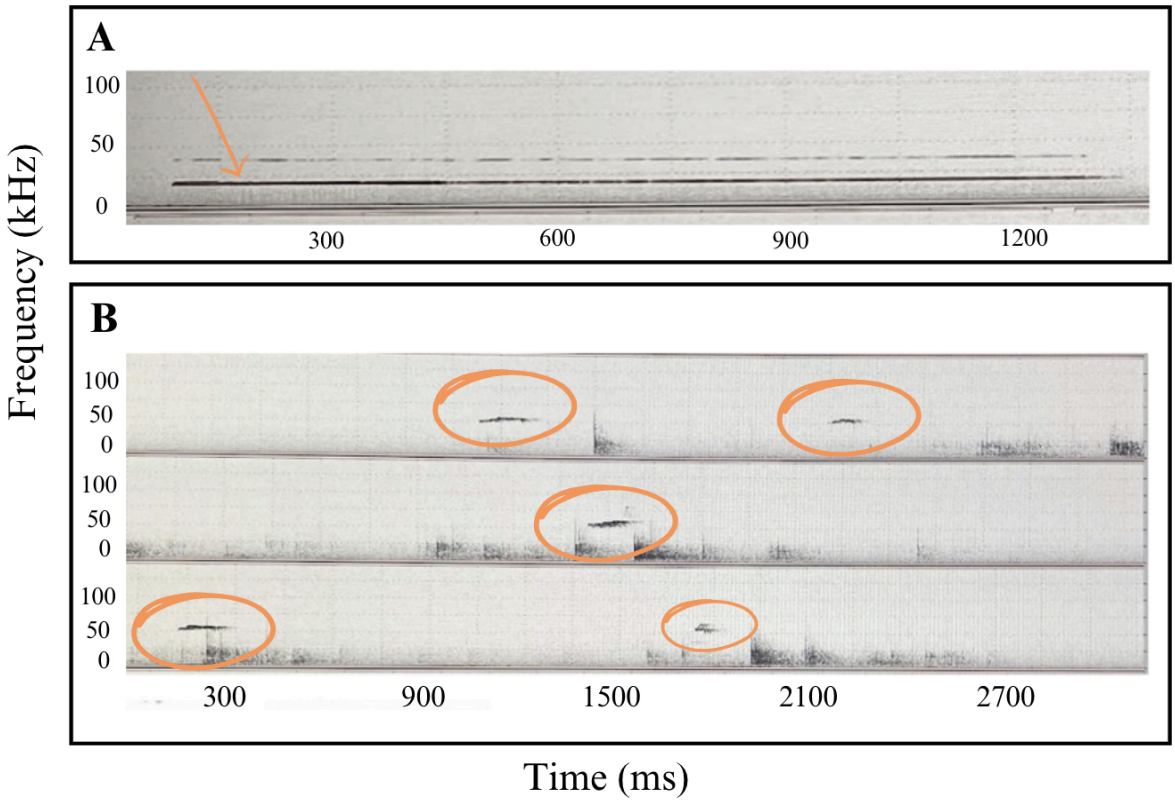

Figure 1: Two frequency classes of USVs recorded on a spectrogram. The scale of the left corresponds to the frequency (kHz) and the bottom axis to time (ms). External noise picked up by the microphone unrelated to the USVs is shown as a lighter shade of gray or in larger, darker clusters.

A. Observed USV in the 22 kHz range. This call was measured to be about 1,350 ms (2.2 s).
B. Observed USVs in the 50 kHz range. Each call is estimated to be about 150 - 200 ms.

It is important to note that the correlation between frequency ranges and emotional states is often variable; calls around 22 kHz do not always associate with adverse conditions, and likewise for the 50 kHz class for appetitive environments. For instance, after copulation, male rats emit USVs around 22 kHz, which is not typically regarded as adverse (Brudzynski, 2021).

Additionally, it has been found that rats have also emitted USVs around 50 kHz when restrained by researchers in states that looked like submissive postures (Wohr *et al*., 2008). Since these USVs cannot be detected without specialized equipment, many researchers who work with these common animal models may be unaware of their affective states, which can alter data collection results. Although not audible to humans, rats can both emit and perceive these calls through their auditory system, which can also affect the receiver's behavior (Schwarting**,** 2023).

C. Vocal Production Mechanism

The larynx is the primary organ for producing vocalizations for most vertebrates and is found right below the base of the tongue and the top of the trachea (tubular passageway to the lungs). In humans, sounds are produced by the vibration of vocal folds. Vocal folds are two bands of layered tissue that stretch across the larynx in the anterior-posterior direction, positioned opposite to each other. The layers of the vocal folds include an intermuscular layer composed of the thyroarytenoid muscle, a soft tissue layer of lamina propria, and an outmost epithelium layer. The vocal folds attach on the anterior sides of the thyroid cartilage and the posterior side of the arytenoid cartilage. Both of these cartilages are found on top of the cricoid cartilage, which interacts with the arytenoid cartilage through the cricoarytenoid joint and the thyroid cartilage through the cricothyroid joint (Zhang, 2016) (Figure 2A, 2C).

Vocalizations are produced through the flow of air from the lungs, which causes pressure under the larynx to increase. Eventually, the pressure causes the air to flow through the vocal folds, which vibrate and push apart or together as the air travels through the glottis (the airspace between the two opposite vocal folds). The vibrations of the vocal folds can change the airflow through the glottis, creating the source of vocalization before going through the vocal tract to be

developed for output. The vocal tract starts with the vocal cords and continues through the pharynx, oral cavity (mouth), and nasal cavity; this system creates diverse speech sounds by modifying and adjusting the sound emanating from the vocal cords. During breathing, the vocal folds are spread apart (abducted state), and the glottis is open; during the start of voice production, the vocal folds narrow (adducted state), and the glottis closes (Figure 3).

After the vocal folds vibrate, the airflow within the glottis becomes a circulating jet flow of self-sustaining vibrations that travels upwards to be modified before output (Zhang, 2016). Specifically in mammals, the means of laryngeal vocal production can be explained by a myoelastic-aerodynamic (MEAD) mechanism that sustains the oscillations of the vocal folds. This mechanism is caused when the vocal folds are fully adducted (closed), so the subglottal pressure accumulates. When the subglottal pressure goes past a certain threshold, the vocal folds are pushed apart and thus cause a negative intraglottal pressure, which closes the glottis again in a cycle. This cycle's repetition allows restoring forces to occur within the tissues and thus become self-sustaining vibrations and cause the production of vocalizations (Van den Berg, 1958, Elemans *et al*., 2015).

Multiple intrinsic muscles in the larynx are involved in vocal production, including the cricothyroid (CT), posterior cricoarytenoid (PCA), and thyroarytenoid (TA) (Hakansson *et al*., 2021) (Figure 2D). As opposed to extrinsic muscles, whose primary function is to move the larynx, intrinsic muscles mainly function to produce movements that lead to vocalizations. The CT muscle consists of two concave surfaces: the superior and the inferior (Suarez-Quintanilla *et al*., 2023). This muscle is found between the cricoid arch and the lower lamina of the thyroid. The CT muscle elevates and depresses the cricoid cartilage of the posterior section of the thyroid cartilage, resulting in the elongation of the vocal folds (Vashishta, 2017) (Figure 2A). This

movement causes high-pitch phonation. The PCA muscle is attached to the posterior cricoid cartilage to the arytenoid cartilage (Suarez-Quintanilla *et al*., 2023). The PCA muscles depress the cricoid cartilage on each side to rotate the arytenoid cartilage laterally, lengthening/abducting the vocal cords (Vashishta, 2017) (Figure 2B). Finally, the TA muscles are found from the thyroid cartilage to the central of the cricothyroid ligaments and then go into the arytenoid cartilage (Suarez-Quintanilla *et al*., 2023). The TA muscles bring the arytenoid cartilages inward to create less tension and adduct the vocal cords (Vashishta, 2017) (Figure 2C).

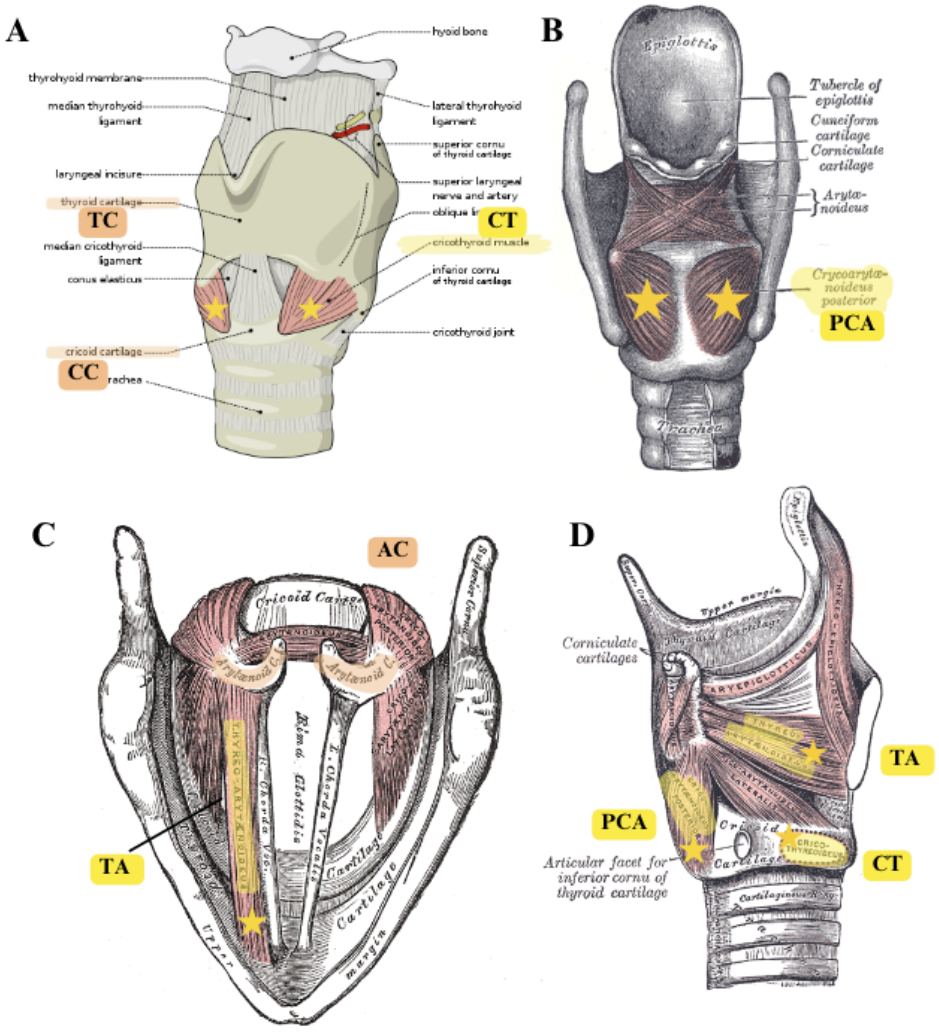

Figure 2: Anatomical Images of the Human Larynx.

A. Anterolateral view of the larynx with visible cricothyroid (CT) muscle, thyroid cartilage (TC), and cricoid cartilage (CC) (Remesz, 2008).
B. Posterior view of the larynx muscles, revealing the posterior cricoarytenoid (PCA) muscle (Gray, 1918).
C. Superior view of the larynx, exposing the thyroarytenoid (TA) muscle and arytenoid cartilage (AC) (Gray, 1918).
D. Side view of the larynx with the right lamina of thyroid cartilage removed, revealing the PCA, TA, and where the CT muscle would appear (Gray, 1918).

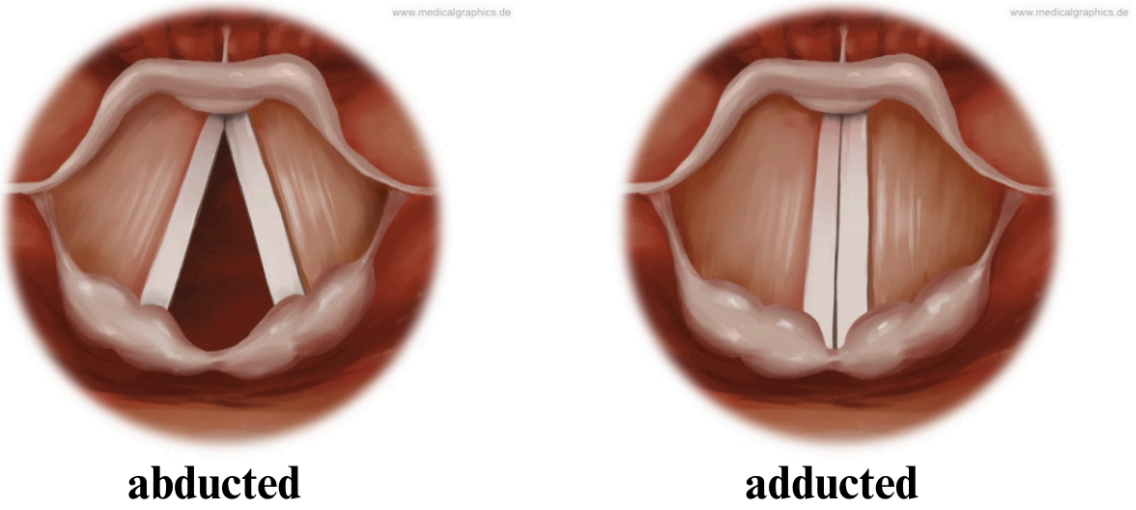

Figure 3: Vocal folds in an abducted (open) and adducted (closed) state (MedicalGraphics)**.** The vocal folds are abducted during breathing and adducted during the start of voice production. USVs are produced when the vocal folds are fully adducted.

D. USV Production Mechanism

In addition to vocalizations audible to humans, the larynx produces USVs in rats (Hakansson *et al*. 2021). However, rats elicit USVs through a mechanism different from human vocalization (Figure 4A). Murine larynxes produce USVs using a whistle mechanism from a jet of air impinging on the structure in the larynx when their vocal folds are fully adducted. The adducted vocal folds leave a narrow opening in the glottis dorsally between the arytenoid cartilages through which respiratory air travels. Specifically, the glottal air jet impinges on the inner wall of the thyroid, which causes slight fluctuations in the air jet due to developed feedback loops (Figure 4B). This mechanism has important parameters, including the size of the glottal area, which determines jet exit speed. In the small nature of the rat larynx, glottal jet speeds can reach up to 10% of the speed of sound. This mechanism causes steady high-frequency tones from the air jet of the range where USVs occur: from 20 to 100 kHz (Hakansson *et al*., 2021).

The CT, PCA, and TA muscles each have detailed and specific controls that contribute to USV production. The CT muscle controls the length of impingement while the TA and PCA muscles work together to set the adduction of the vocal folds and, therefore, the glottal area. Both of these movements are critical to the mechanism of USV production (Hakansson *et al*., 2021). When the CT muscles contract, the thyroid wall rotates away from the glottal opening, increasing the jet impingement length. The contraction and shortening of the CT causes the the frequency of the vocalizations to increase (Hakansson *et al*., 2021). The TA and PCA muscles are important for the movement of the vocal folds. The TA muscle must be activated for the glottis to be closed entirely for the jet impingement mechanism to occur. The TA muscle can also slightly counteract the rotation of the thyroid wall, which decreases the impingement length due to the shortening of the muscle. In addition, the TA strongly contributes to whistle stability

(Hakansson *et al*., 2021). Oppositely, the PCA muscle abducts the vocal folds. Thus, while this movement is the opposite position of the state of the vocal folds when USVs are produced, its function is necessary to help determine the duration of time between calls. The PCA and TA work jointly to determine the glottal area and jet exit speed.

Since different internal states determine these features of vocalizations, their mechanism is essential to understand. The precise controls of these muscles in the larynx are critical in shaping and determining the frequencies of USVs, and their mechanisms in connection to the action of motor neurons require further exploration. Although it is known that neural spikes contain information that directs muscles to move and result in complex behaviors like the elicitation of USVs, the precise signals that brains send to muscles are only beginning to be understood.

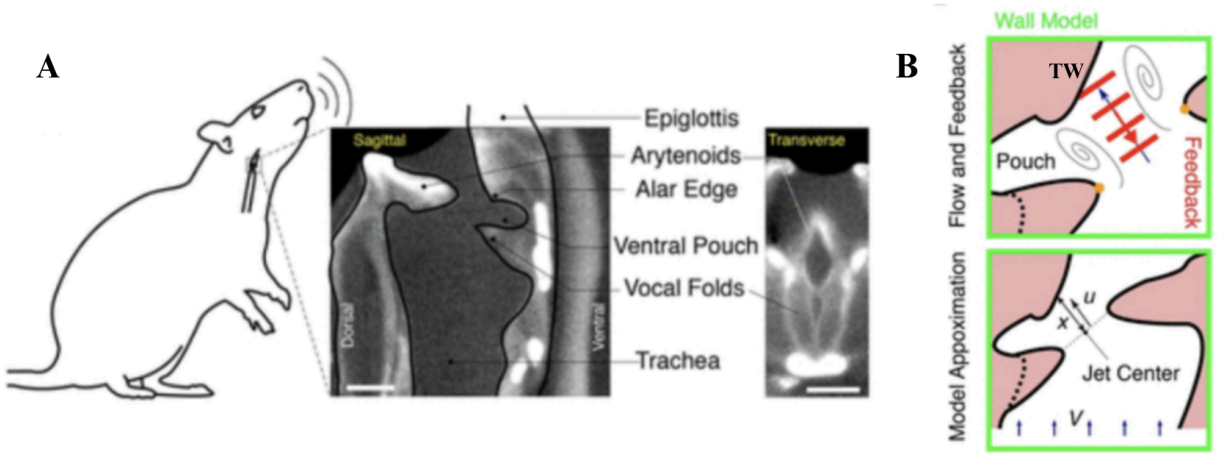

Figure 4: USV production mechanism (Hakansson *et al*., 2021).

A. The location of the USV production mechanism in the larynx shows a sagittal and transverse view of the larynx parallel to the vocal folds.
B. The wall model of USV production is caused by a glottal air jet impinging on the inner wall of the thyroid (TW), initiating a feedback mechanism. The black lines represent the airflow, and the red lines represent the feedback mechanism. The model parameters are labeled *x* for impingement length, *u* for jet exit speed, and *V* for tracheal flow.

E. Study Aims

The overarching goal of this project is to simultaneously record neuronal signals the larynx receives and correlate these signals to USVs produced by rats. This activity can be measured using electromyography, or EMG. EMG is a technique to assess the electrical activity produced in muscles. Specifically, EMGs measure muscle response to a nerve's stimulation of the muscle. To do so, Myomatrix arrays are used as a tool to record muscle activity for the EMG recordings (see *Methods)*. Thus, these arrays have to be surgically implanted on the muscles of interest to record data. This study focuses on two significant steps to achieving this goal: *Aim 1* is to perform behavioral experiments to optimize the elicitation of USVs, and *Aim 2* is to develop surgical techniques to access different intrinsic laryngeal muscles (specifically CT, PCA, and TA) in rats and secure arrays which will read the muscle activity onto them to perform EMG recordings.

In pursuit of recording EMG, these two techniques are necessary to create a systemic approach to collecting optimal data. Exposure of the larynx to place the electrodes is a highly invasive surgery. Therefore, a steadfast method to elicit USVs from the rats after the surgery is critical for collecting data. The production of USVs in rats is highly variable between individuals

but can be elicited through behavioral training. A reliable procedure to ensure the production of USVs is necessary to ensure vocalization from all rats being studied; additionally, the careful placement and technique of the electrodes to muscles in the larynx is crucial to effectively collecting the electrical signals.

Methods

A. Subjects and Housing:

Four cohorts of four to six 70–to 90-day-old male Sprague Dawley Rats were received from Charles River Laboratory. The rats were housed together in pairs in the Division of Animal Resources in the Woodruff Memorial Research Building at Emory University, and each rat was given identifying names. Cohort A included four rats that were named Rat 1, Rat 2, Rat 3, Rat 4; Cohort B included six rats that were named A1, A2, B1, B2, C1, C2; Cohort C included six rats and were named Heart 1, Heart 2, Smile 1, Smile 1, Star 1, and Star 2; Cohort D included four rats and were named Snow 1, Snow 2, White 1, White 2. For Cohorts B-D, similar names relate to cage mates. The rats underwent a handling procedure to familiarize themselves with human touch. We modified standard handling procedures to acclimate the rats to our experimental setup to fit our experimental goals (please see Results). All procedures were approved by the Emory University Institutional Animal Care and Use Committee.

B. Recording USVs and EMG

We recorded USVs produced by rats in a clear novel cage within a soundbox. The soundbox provided sound insulation and allowed us to create a controlled environment for our behavioral experiments (Figure 5). To record the USVs, an Avisoft UltraSoundGate Condenser Microphone CM16 was arranged to hang above where the rats were placed above the cage floor (Avisoft Bioacoustics). This specialized microphone is sensitive to frequency responses spanning from 15 to 200 kHz. The microphone was connected to a computer through an UltraSoundGate USG416H Audio device (Avisoft Bioacoustics). The USVs were displayed on a spectrogram in real-time and on the accompanying Avisoft-RECORDER USGH software. The software also

provided statistics on the USVs, including the number of events, total duration (s), mean duration (ms), max duration (ms), min duration (ms), variance of durations (ms2), and standard deviation of durations (ms) for up a recording session based on parameters that were set. For automated counting of USVs, the parameters were set to limit peak search to 15 - 70 kHz (to include USVs for both frequency ranges), the minimum whistle duration to 5 ms (to reject noise), and the hold time to be 10 ms (to merge close USVs). The number of events corresponds to the number of detected USVs (in both the 22 and 50 kHz frequency ranges), and the total duration corresponds to all detected USVs in seconds. The recording spans were saved as .wav files.

An Intan RHD recording system was used to record EMG signals. An SPI interface cable connected the headcap connector to the controller. The Intan software was used to record EMG signals simultaneously with USV recordings in real-time (Intan Technologies).

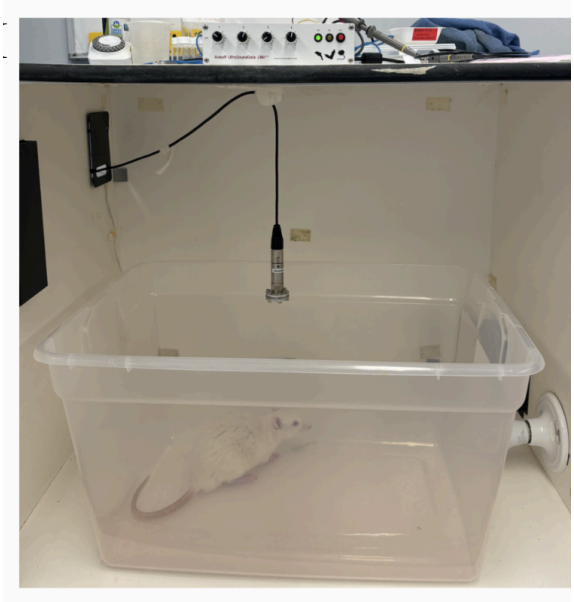

Figure 5: Experimental design for USV recording sessions. Animals were individually placed in a clear novel cage inside a soundbox. A specialized microphone overhead recorded their USVs, which were processed through the Avisoft UltraSoundGate before being visualized on a computer.

C. Electrode Array Preparation

Unique electrode technology developed by the CAMBER center at Emory University and the Georgia Institute of Technology was used to record EMG. Myomatrix arrays are made of layers of plastic insulators and gold electrode contacts treated with PEDOT to enhance recording (CAMBER). These high-density electrode arrays have low impedance while remaining both lightweight and flexible; these features allow for high-resolution EMG recordings of motor unit action potentials caused by the spiking of motor neurons. Motor units combine an individual motor neuron and all the muscle fibers it innervates, and traditional EMG usually records the "bulk" EMG signal where multiple single units are combined. Unlike surface EMG, our arrays are surgically placed intramuscularly or on top of a muscle.

The specific array has features to help measure high-resolution EMG. The array is divided into two main parts: the connector and the array "threads" (Figure 6). The connector is 10 mm long and is placed on the top of the rat's head to be plugged into the recording system. The four "threads" stem from the connector and bear eight electrode contacts each (for 32 electrodes in total), which is the area of the array that records activity from a muscle of interest. The threads also include designated areas proximally and distally from the electrodes for suture holes to anchor the array to the muscle. The flexibility of the threads allows for recordings of muscle activity during free movement. The ends of the threads contain circular pull-through tabs, which allow surgical instruments to maneuver the array without harming the electrodes (CAMBER).

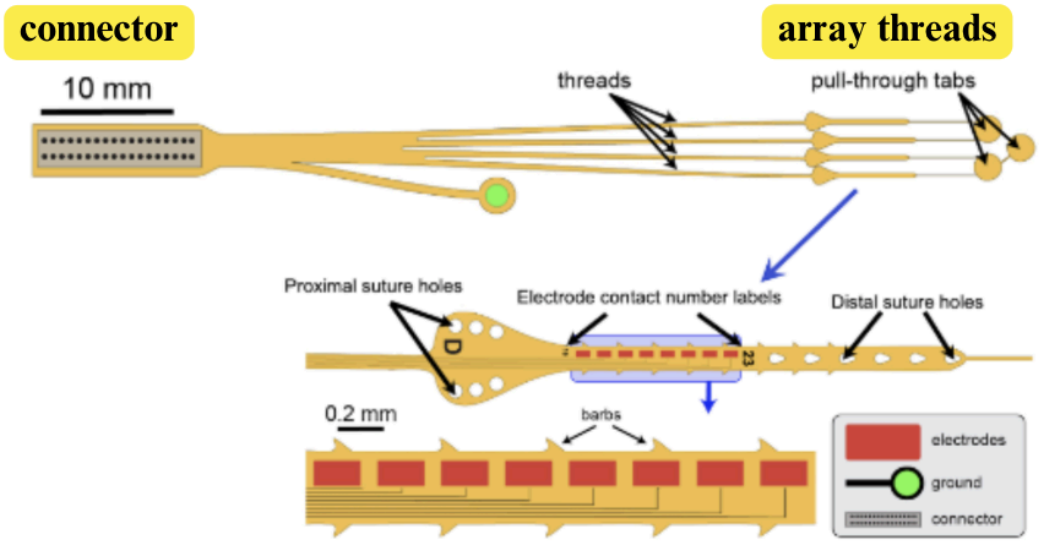

Figure 6: Myomatrix arrays are used to record electrical activity in muscles (CAMBER). During surgery, the connector is placed on the head of the rat, and the electrode contacts are placed on specific muscles. Arrays have several features that ease surgical implantation, including suture holes that hold the electrodes in place and barbs that secure arrays into muscles when implanted intramuscularly. The "neck" of the electrode (spanning from the connector to the most proximal electrode contact) is approximately eight cm long.

D. Dissection

Rats used for exploratory surgery for anatomy were euthanized with an intraperitoneal injection of Pentobarbital (50 mg/kg). After exposing the trachea, iris scissors were used to cut from the middle of the trachea to the epiglottis. The dissected larynx was placed in Phosphate-Buffered Saline (PBS 0.1 M) for preservation before analysis. Forceps were used to maneuver the dissected larynx under the microscope (please see *Results* of further dissection discussion).

E. General Surgical Procedure

Each array placement surgery had two major steps. First was implanting the head cap (the connector of the array), which is a fairly standard surgical procedure used in neuroscience research. The second was the placement of the arrays on the intrinsic laryngeal muscles of interest, requiring a novel procedure we developed to expose the larynx and secure the arrays (see *Results*).

I. *Pre-Operational Procedure*

Before surgery, we sterilized our surgical area to create a controlled environment to ensure animals experienced minimal discomfort from surgery. First, surgical tools were sterilized using an autoclave; next, pre-and post-surgical drugs for pain management (Meloxicam, Lidocaine, and Buprenorphine; 1 mg/kg each), were measured at the necessary dosage after weighting the surgical animal. Isoflurane was used as our general anesthesia. The valves on the oxygen tank, which is used to deliver the isoflurane, were opened and checked to ensure they were full enough to last the duration of surgery. The amount of isoflurane within the anesthesia machine was checked and filled to maximum volume. Finally, we ensured the nose cone (plastic tubing that delivers the isoflurane to the rat) was placed correctly and the heating pad was turned on. Lastly, the surgical areas were prepared (over the heating pad, two square pieces of lab mats were placed on top of each other (one for non-sterile preparation, one for sterile procedure)). Animals were food and water-deprived 30 minutes before surgery.

II. Commencement of Surgery

To prepare to induce the animal, we placed a rat using our specialized handling grip (please see *Results*) into an induction chamber connected to an anesthesia machine providing 3% isoflurane/97% oxygen. After the rat was placed in the box, the box was covered with a cloth to allow the rat to fall unconscious with minimal stress. To check if the animal was fully induced, sensitive distal areas were pinched, such as the end of the tail or between the toes, and if the rat gave no reaction to the pressure, the animal was ready to be transferred to the surgical set-up. The induction step generally took between three to five minutes.

Next, the isoflurane tubing was switched from the induction chamber to the appropriate nosecone in the surgical area. The rat was quickly transferred from the induction box into the nosecone on top of the top lab mat. When the rat was securely attached to the nosecone, the hair was removed from the surgical area of interest with an electric razor and Nair. These areas included the top of the head and the area on the underside of the animal directly over the larynx from the mandible to the manubrium. After the hair was removed, these areas were rinsed with saline. A generous amount of eye lubricant was placed on top of each eye to cover them fully. Lastly, for pain management, Meloxicam was injected subcutaneously into pinched skin near the incision sites. During surgery, vitals were continuously monitored by checking for breathing and heartbeat. Eye lubricant was reapplied if needed.

III. Headcap Placement

Following the transfer of the rat from the induction box to the surgical area, we laid the rat with its dorsal side facing up. Ear bars from the sterotax (Kopf) were secured horizontally on both sides of the rat's head. A drape was added, and holes were cut above the planned incision sites to cover all unshaven skin. The planned incision sites were sterilized through a sterile scrub with the alternating application of 3x Ethanol and 3x Iodine wipes. This process concluded with a final application of iodine. We injected lidocaine into the incision site five minutes before creating the incision. The crest on the caudal end of the skull was located, and an incision was made approximately 0.5 cm rostral from the crest. After sterilization, the skin was removed using iris scissors in a rugby ball shape (2-3 cm long and 1 cm wide), starting 0.5 cm rostral from the skull crest and extending up to between the eyes to reveal the skull (Figure 7B). During the incisions, it is important to avoid cutting caudally from the bone crest to avoid excessive bleeding. The skull tissue was then cleared by cutting around the perimeter of the incision using a scythe-shaped, curved scalpel (#12). The tissue was peeled with forceps, and many rough strokes with cotton applicators were used to clear all connective tissue. The bone was lightly scored with a scalpel, and any debris was removed until it appeared white and blood-free. The lambda and bregma (two points on the skull's surface helping the stereotactic localization of specific brain regions) of the skull were visible at this point (Figure 7A). A waiting period was observed until the area was completely dry before proceeding.

Next, four small holes were drilled into the skull to help secure the headcap in a rectangular formation. Adequate room was left for the metal handle and connector in between. The holes were about 900 um deep and were created using the skull burr bit (0.9mm). Following this, the dust from the skull was cleared using puffs from a large pipette. Subsequently, four

surgical screws were carefully screwed into the holes using a surgical screwdriver (Figure 7C). A mixture of two-part Orthojet dental cement and one-part sterile water was created and applied as an acrylic base coat onto the skull and screws. The metal handle was inserted first, followed by the connector (Figure 7D). It was important that the cement did not enter the connector holes, and the cement encapsulated the screws and ground lead (flatter bottom of connector). More cement was used to fill the walled area. The structure was secured once completely dry (Figure 7E, 7F) (adapted from unpublished protocol written by Sean O'Connell).

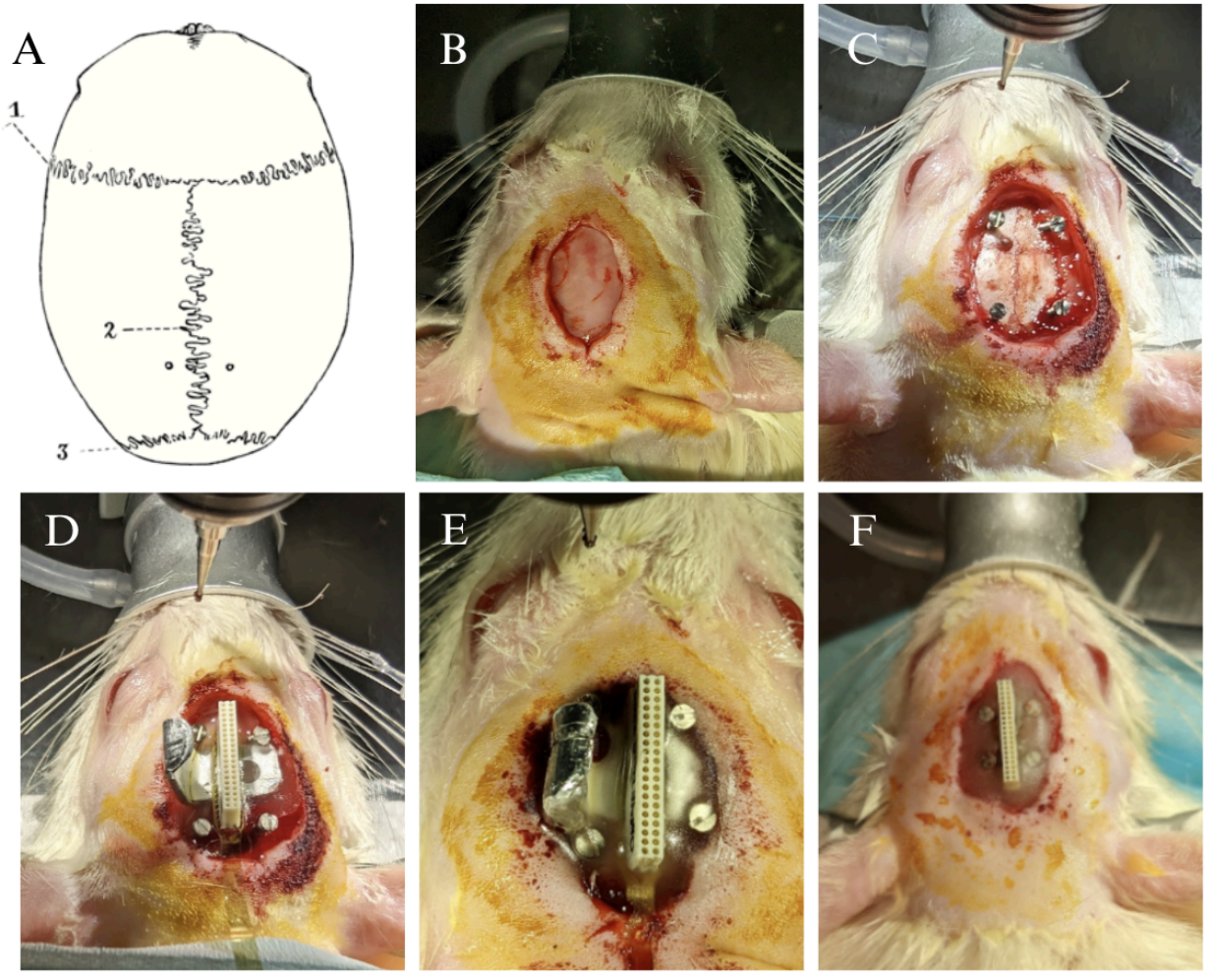

Figure 7: Headcap placement procedure.

A. Superior view of the skull showing the bregma (1) and lambda (2). (Kollmann, 1901).
B. The skin was removed in a rugby ball shape starting rostral from the skull crest.
C. Four surgical screws were placed into drilled holes after excess tissue was removed to reveal the skull.
D. Metal handle (used to help plug the connector into the EMG acquisition system) and connector were placed in-between the screws with cement used as an acrylic base coat.
E. More cement was used to secure the metal handle and connector and left to dry.
F. Final headcap procedure product.

IV. Tunneling of the Array

Following the placement of the head cap and the exposure of the larynx, there was an additional procedure to tunnel the threads of the array from the head through the superficial cervical fascia (SCF) to the neck. Two sets of forceps were used simultaneously: one held the pull tabs of the arrays from the head cap, and the other cut through the SCF.

V. Post-Operative Care

After surgical procedures were completed, buprenorphine (1mg/kg) was injected subcutaneously into two locations near the incision sight for pain management. The isoflurane was turned off, and the rat was placed alone into a clean cage with the heating pad. The activity of the rat was logged every 15 minutes until the rat recovered and was ambulatory. The surgical area and tools were cleaned and sterilized. Animals were monitored daily for five days following surgery and assessed for any signs of distress. Some signs of poor health or infection include abnormal postures, blisters or excessive discharge near the incision site, or extreme lethargy.

Results

A. Aim 1 (Behavioral Results)

I. Handling Procedure

We developed a handling procedure to familiarize animals with the experimental setup and to prepare the animals to undergo USV recordings; this procedure was developed from observational notes working with rats from each cohort. USVs communicate the emotional state of the rats; thus, we wanted the rats to be as familiar as possible with the laboratory environment to lower stress and encourage the rats to be comfortable and produce a variety of vocalizations. Rats, like other social animals, including humans, display a variety of personalities and produce different classes of USVs based on the rat's unique emotional state. Thus, we cannot expect all rats to behave as we want and produce the USVs at the frequencies we desire – we had to think creatively and take innovative routes to ensure vocalizations to collect the EMG data.

Each newly arrived rat was handled for 10 minutes daily for two weeks to familiarize them with the human touch. Movements in the handling procedure included rubbing their bodies and heads and blowing on their faces gently to build a tolerance to discomfort. To prepare the rats for the installation of the head cap and eventual data collection (see surgery section of *Methods* for more information), the handling sessions included stroking the heads of the rats to acclimate each rat to the pressure required for connecting wires to record data. Rats were also picked up from the cage with a specific grip, with the thumb and middle finger curled under the shoulders of the rats and the index finger on top of the head to prevent struggling or potential biting. From this handling procedure, we have observed that the rats become more docile, easier to work with, and comfortable with the researchers from the daily petting before preliminary data

collection. The grip has also allowed us to control the rats more efficiently by picking them up and avoiding picking them up from their tails, which could lead to stress and possible injury.

Connecting and removing data acquisition equipment to the connector, which was surgically installed on the head of each rat, can cause distress and sometimes require rats to receive a small amount of anesthesia. We wanted to avoid using anesthesia when performing this connection step since this could potentially change the behavior of the animals during our recording sessions. Throughout our study, we worked to attach and remove the data acquisition cable to our rats without the use of anesthesia, which resulted from this established handling procedure and works to benefit the overall health of the animal. By the end of the study, we were removing the data aquisition cable successfully during numerous experiments.

II. Initial Exploration of Eliciting USVs

After monitoring the USV production of our initial cohort, we investigated strategies for encouraging rats to produce USVs. Based on a literature review and observational evidence, we hypothesized several features would evoke USVs and performed experiments to see which factors most increased USV production. These factors included treats, such as banana and chocolate-flavored pellets; novel toys, such as tubes and hay balls; soiled female bedding; and touch/tickling (using a motion that attempted to mimic juvenile play–with dorsal contact and a ventral contact with a gentle pin). Through observational evidence, we found that touching the rats under the stomach and above the shoulders and adding female beddings showed the most vocalizations in the preliminary recordings (Figure 8A). We recorded four rats in Cohort D to see the effects of these factors on eliciting USVs. The rats were individually recorded for 15 minutes once a day for three sessions: the first five minutes recorded the rat with no stimulus, the next

five minutes recorded with the addition of soiled female bedding, and the last five minutes recorded the rats with touch (Figure 8B). Our findings showed that touching the rats significantly produced more USV events than no stimulus or the addition of female bedding. Without touch, the likeliness for the rats to vocalize was much lower to elicit USVs. Each rat consistently produced ample USVs for every trial while being touched (Figure 8C). We also observed individual variability in each of the rats; for example, *Snow 1* elicited 15 USVs without any stimulus, whereas *White 1* and *White 2* produced zero in one trial. While all the rats consistently elicited USVs while being touched, some had more total USV events than others by up to 100 events (Figure 8C).

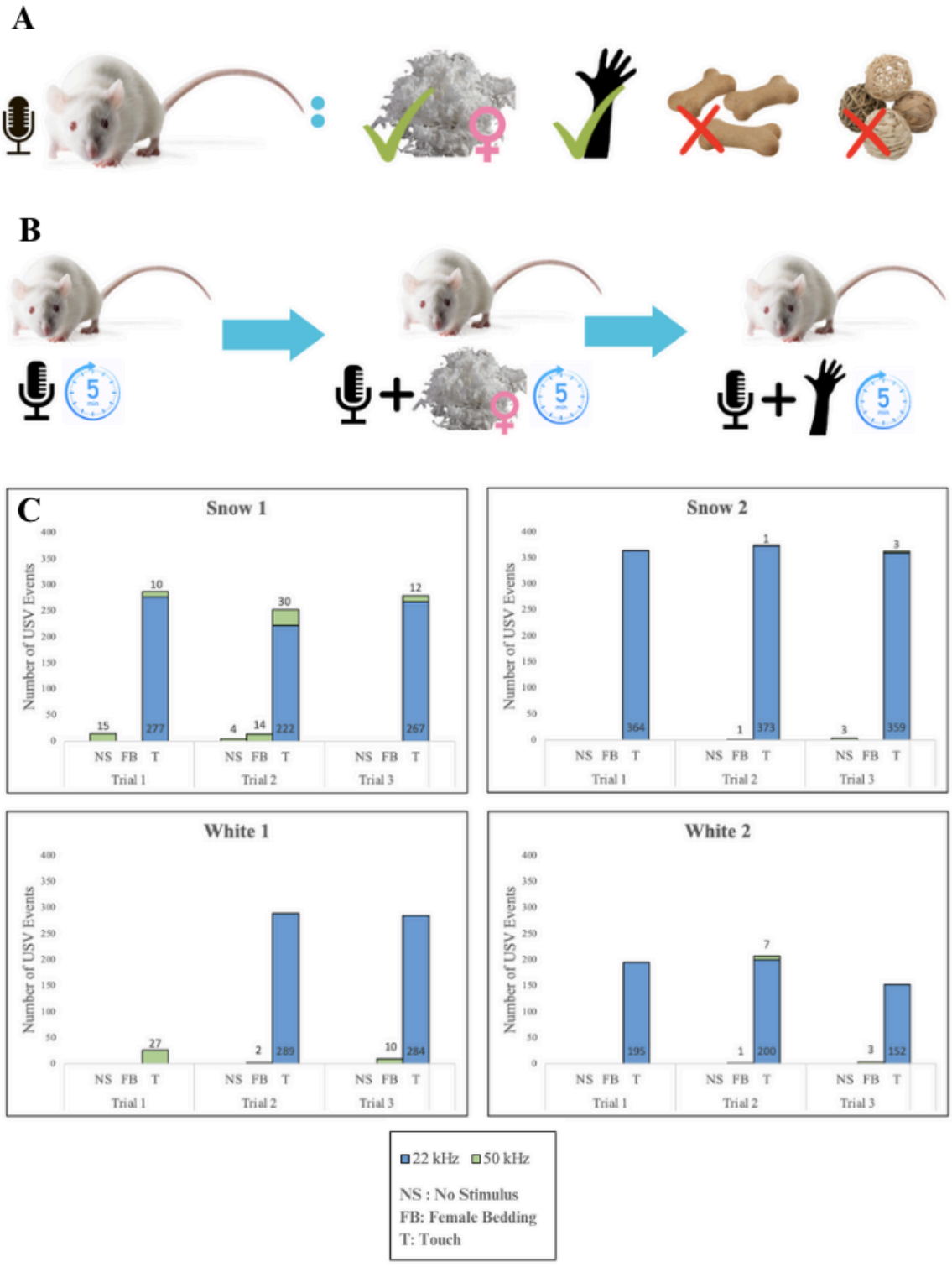

Figure 8: Initial experiments to explore USV production.

A. We tested four factors to encourage USV production: soiled female bedding, touch, treats, and novel toys. Of the factors tests, touch and female bedding evoked the most USVs.
B. An experimental procedure for fifteen-minute trials was used to measure USV elicitation from three factors: no stimulus, female bedding, and touch. Each factor was tested for five minutes.
C. Results from three trials of the experimental procedure explained above (II) showed that touch consistently elicited USVs from all rats. 22 kHz calls are shown in blue, and 50 kHz calls are shown in green; there were significantly more 22 kHz calls elicited. The addition of female bedding produced some USV production, but this factor was highly variable based on the individuals. With the stimulus of touch, the production of USVs was consistent and reliable for 22 kHz events.

III. Behavioral Procedure to Elicit USVs

Overall, a procedure to ensure that the rats produce USVs was critical. Our general experimental goal requires long, invasive surgeries to surgically implant arrays to reach targeted vocal muscles; due to experimental constraints, we aimed to collect both EMG and USV data within the first couple of days after performing surgery. Using findings from the initial exploration eliciting USVs, we established an experimental procedure for a 10-minute recording session (Figure 9). Many observations from the preliminary recording sessions allowed us to establish the time frames concerning the stimuli. The procedure started with one minute to allow the rats to acclimate to the novel, empty cage; we also noticed from previous recordings that rats who vocalized without stimulus did so within the first minute of exploring the new environment. Although touch caused the most consistent production of USVs, this stimulus caused some

distress to the rats who produced primarily 22 kHz calls; thus, there was time between the touch sessions to allow for relaxation. Our previous observations also showed that the initial contact and beginning of the touching period produced the most USVs in a timeframe as the rats became more accustomed to the stimulus; therefore, touch was placed into two set timeframes to maximize the USVs produced. Although female bedding did not produce as many USVs as touch in our preliminary recordings, all the rats who did vocalize to this stimulus produced 50 kHz calls; to potentially see some more variety in the types of calls produced, female bedding was added to a small portion of this procedure.

Time for 10-minute session	**Stimulus**
0 - 1 minute	No Stimulus - Allow rat to acclimate in empty cage
1 - 3 minutes	Touch
3 - 5 minutes	No Stimulus - Take away touch
5 - 7 minutes	Add Female Bedding - Scatter around one side of the cage
7 - 9 minutes	Touch
9 - 10 minute	No Stimulus

Figure 9: An established behavioral procedure to elicit USVs for a 10-minute recording session. This procedure consistently encouraged animals to produce many USVs, allowing us to correlate USV production with vocal muscle EMG (See *Discussion*).

IV. Assessing Best Vocalizing Rats for Electrode Implementation

As previously discussed, USV production varies based on individual rat personality; thus, we established a procedure to select the animals from the cohort that are best suited to use in our EMG studies. The surgeries to implant the electrodes are intensive, so we want to choose rats that will produce many USVs to maximize experimental resources. We recorded USVs from each animal in the cohort and measured the number of USVs produced using real-time recorder software. The real-time USV detection parameters were used to count USVs in both frequency ranges while blocking out audible background noises. We compared data from different rats' performances to assess which rats would be predicted to vocalize the most during our EMG recordings.

For example, for Cohort C, each rat was recorded for 10 minutes for three trials of the above behavioral procedure. The rats were ranked based on their ability to produce USVs from the trials to help decide which rats would proceed with the EMG experiments. Based on the average total duration of seconds that the rats produced USVs, we were able to "rank" them in order to choose the best candidate for surgery. From the figure below, *Heart 2* produced the longest average total duration of seconds of USVs and thus was the best candidate for surgery, whereas *Star 2* was the worst (Figure 10). As the figure shows, it is evident that there is a lot of variability in the amounts the rats vocalize, so this assessment is critical to ensure that the chosen rat for surgery will produce USVs for EMG data. Due to the high variability of vocalizations between individuals, we observed that not every rat was suitable for surgery and recording substantial EMG data. Thus, this assessment was necessary to sort our cohorts between which individuals would be sent to surgery and which would proceed with behavioral experiments.

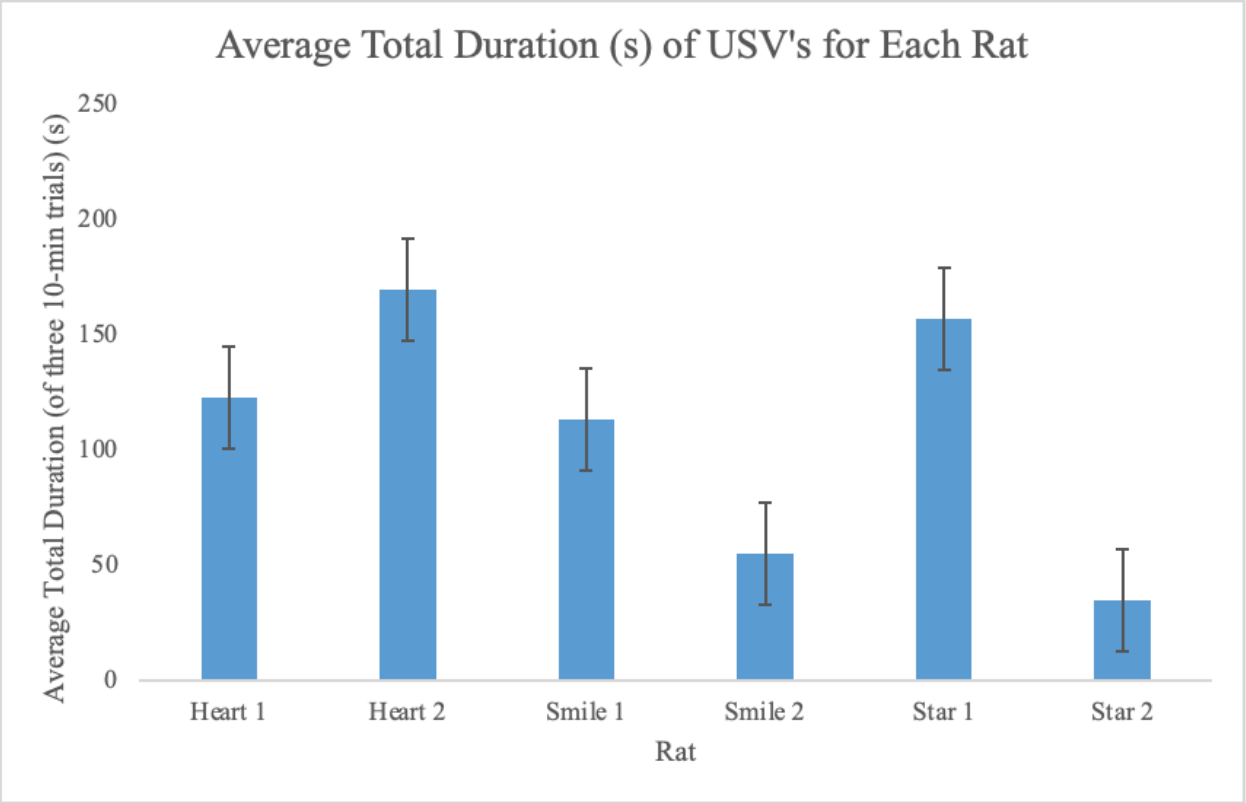

Figure 10: The average total duration of USVs for three 10-minute trials of the six rats in Cohort C with standard error. The total duration of USVs recorded contains both 22 and 50 kHz calls; we observed the majority of calls were 22 kHz. Since this vocal assessment is primarily interested in general USV output, we did not distinguish between different frequency events. *Heart 2* showed the most total duration of USVs, while *Star 2* vocalized the least. Quantifying the duration of USV allowed us to choose rats to undergo array implantation surgery and successfully record EMG while the animals vocalized.

B. Aim 2 (Surgical Results)

I. Larynx Dissection

We dissected a larynx and performed non-recovery surgeries to understand larynx anatomy better and guide our surgical techniques (Figure 11A). These procedures helped us better understand the intrinsic muscles of interest on the larynx of adult rats so we could perform surgeries to install our arrays. The size of the rat larynx is significantly smaller than a human larynx, making the muscles of interest more inaccessible; for scale, we measured the width of the adult rat cricoid cartilage to be approximately between two to four mm. Compared to human males, this width ranges from 11 to 21.5 mm (Randestad *et al.*, 2000). We identified the three muscles of interest and discussed the best surgical techniques to install the arrays to record muscle activity in awake, behaving animals. The CT muscle was easily identifiable once the larynx was exposed on both sides of the median cricothyroid ligament (Figure 11B). To reveal the TA and PCA muscles, the thyroid cartilage was cut through and peeled back to reveal the posterior side (Figure 11B, 11D). Additionally, we explored the esophagus (the tubular organ that connects the throat to the stomach) and its proximity to the trachea in relation to the PCA muscle (Figure 11F). The esophagus has three anatomical segments: cervical, thoracic, and abdominal. The cervical segment is situated directly posterior to the trachea, connected to the trachea by loose connective tissue (Chaudhry & Bordoni, 2023)**.** The separation of the esophagus and trachea was necessary to expose the PCA muscles.

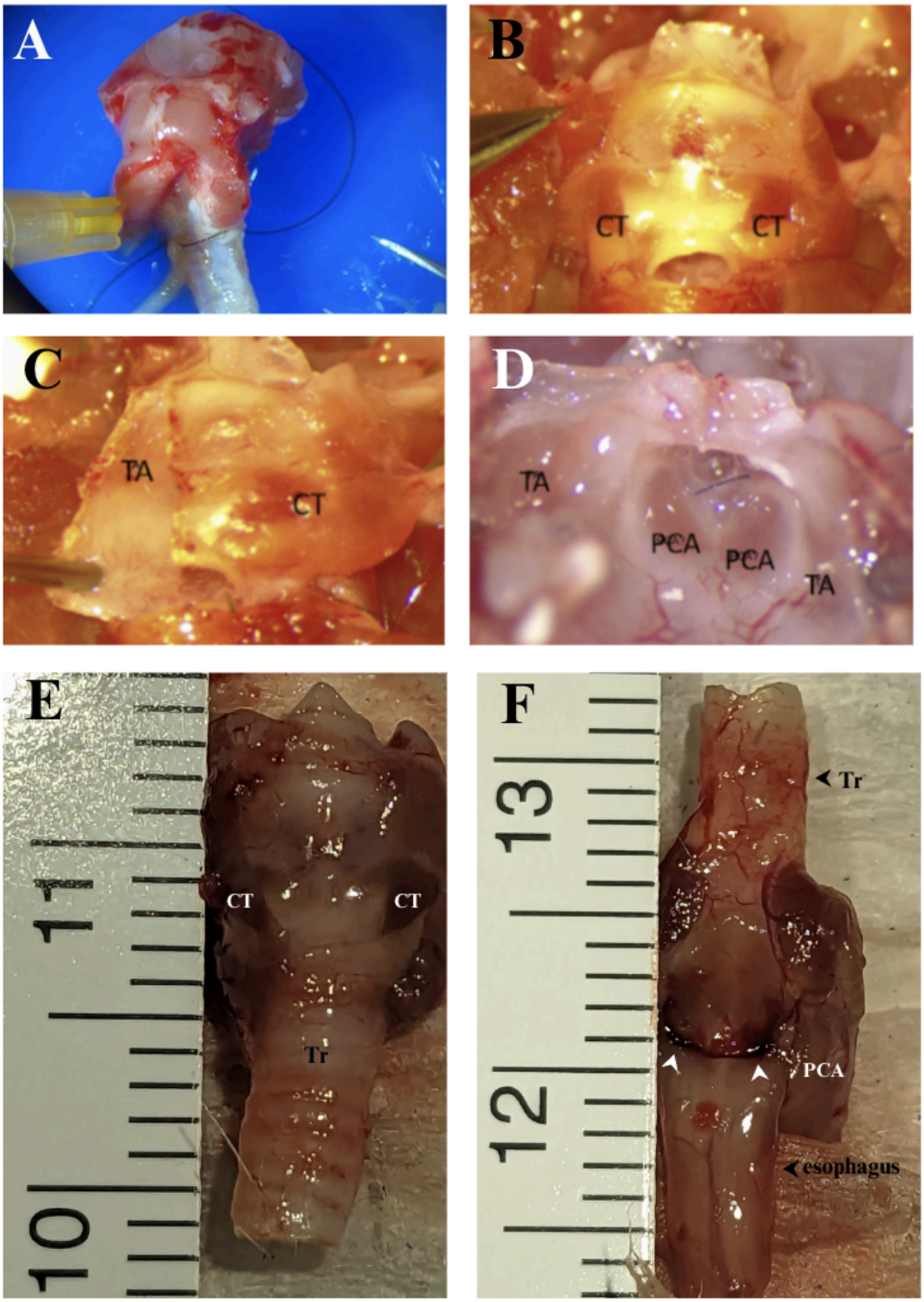

Figure 11: Larynx dissection.

A. A view of a dissected larynx of an adult rat.
B. Anterior view of a magnified image of the larynx. The two surfaces of the CT muscle are observed on the external surface of the larynx.
C. Anterior view of the larynx with the thyroid cartilage peeled back. The thyroid cartilage was cut through vertically down the middle and moved to the side to reveal the TA muscle.
D. Further inspection with the removed thyroid cartilage displays the PCA muscles on the posterior side.
E. Anterior view of a dissected larynx revealing the CT muscle. The structure measures to be about 1.7 cm from the middle of the trachea (Tr) to the epiglottis.
F. A posterior view of a dissected larynx reveals what we observed to be PCA muscle once the trachea was pulled upwards from the esophagus.

II. Surgical Technique to Reach the Larynx

To place our electrode array on the larynx, we developed a surgical procedure to expose muscles of interest on the larynx in a living rat. Following the completion of the general surgical procedure and headcap procedure (found the *Methods)*, the ear bars were unscrewed, and the surgical rat was turned ventral side facing upwards. The appropriate nosecone tube was secured over the nose, ensuring the continued delivery of isoflurane. Using iris scissors, an incision on the vertical midline over the ventral side of the neck was cut to expose the superficial cervical fascia (SCF) (Figure 12A). The SCF was pulled away using two tweezers in a tearing motion, where the tips of the tools meet in the middle of the exposed region. Structures such as lymph nodes, external jugular veins, and sternomastoid muscles were carefully avoided. We retracted

the exposed region with four retractors on the tops and bottoms of the mandibular glands. After the facia and connective tissue were cleared away, the sternohyoid (SHM) was identified (Figure 12B). The SHM was separated at the vertical midline with the same tearing motion until the trachea was exposed. The SHM was retracted to expose the trachea, revealing the CT muscle below the thyroid cartilage (Figure 12C).

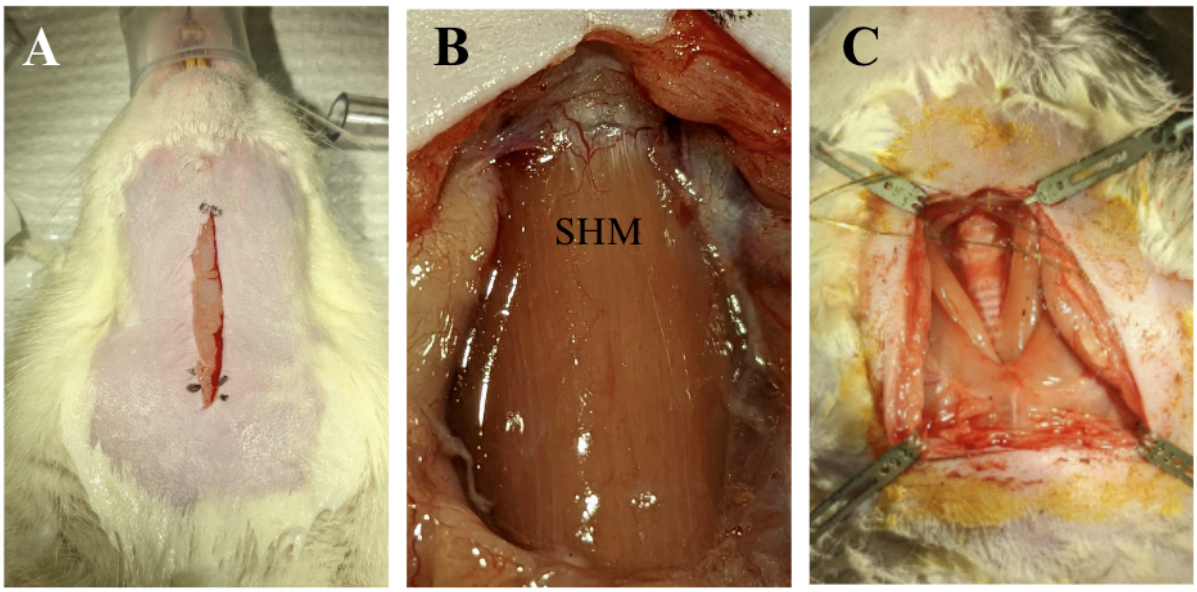

Figure 12: Procedure to expose the larynx.

A. An incision on the vertical midline over the ventral side of the neck.
B. Following the incision in I, the facia and connective tissue were cleared to reveal the sternohyoid muscle (SHM).
C. The exposed larynx is revealed by holding back surrounding tissue with four retractors. Please note the thin, visible gold filaments laying over the larynx are array threads.

III. Surgical Techniques to Reach Target Laryngeal Muscles

A. CT Muscle Procedure

After the trachea region was exposed, the two concave surfaces of the CT muscles were evident below the exposed thyroid cartilage (Figure 13A). Initially, we explored placing the array intramuscularly by puncturing a small hole through the muscle as a way to suture the array directly; however, this invasive procedure did not allow for the array to stay in place and could damage the muscle, resulting in disrupted vocalization (Figure 13B). We were challenged to establish another way to secure the electrodes on this small muscle–in fact, the CT muscle is smaller than the span of the eight electrode contacts (Figure 13D). To secure the arrays on the muscle, we used SCF along the trachea to tie the four array threads on both surfaces of CT with small 10-0 sutures (Figure 13C). We successfully used this technique to record EMG and USVs from the CT muscle on an awake, behaving rat (see *Discussion*). This procedure allowed the rats to vocalize post-surgery and cause minimal harm to the CT muscle.

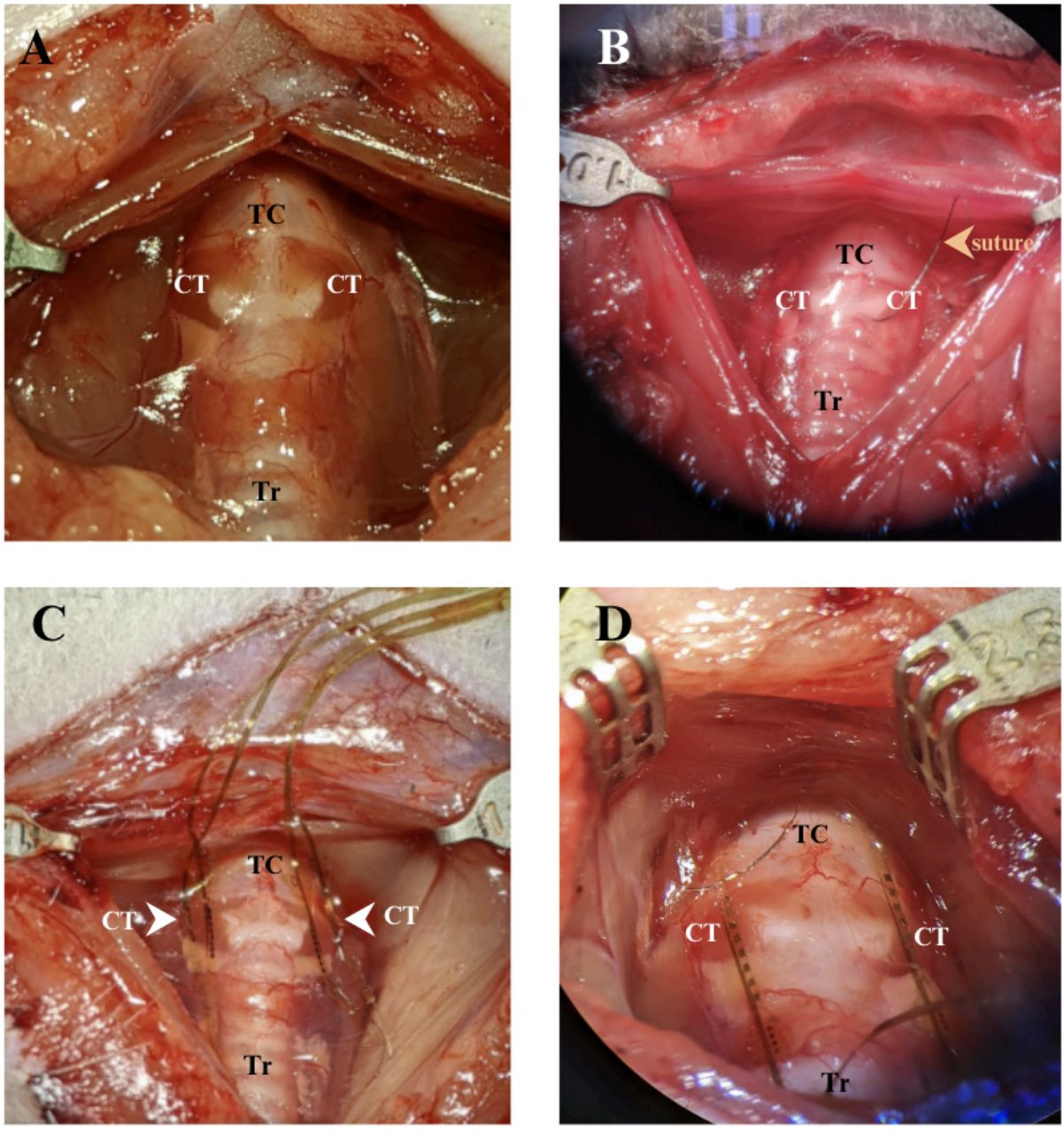

Figure 13: Exposure of CT muscle and placement of the array.

A. Two surfaces of the CT muscles were located after exposing the larynx under the thyroid cartilage (TC) and above the trachea (Tr).
B. Exploratory attempt to explore the feasibility of placing array threads intramuscularly within the CT muscle. A small 10-0 suture needle is visible, puncturing a small hole through the muscle. Due to the size of the CT muscle, we decided to place electrodes on the surface of the CT muscle.
C. Four array threads were secured on both surfaces of the CT muscle.
D. A closer view of the electrode contacts placed on top of the CT muscle. A small 10-0 suture needle is seen passing through a suture hole on the array. We use secure arrays to the muscle by suturing the array, using both the distal and proximal suture holes, over CT.

B. PCA Muscle Procedure

Since the PCA muscle is found on the back of the larynx (see Figure 11F), we needed to develop different surgical strategies to access the muscle. We decided to "lift" the larynx and trachea out of the chest cavity to expose the posterior side of the cricoid cartilage–where the muscle attaches (Figure 14C). Our first step was to separate the connective tissue between the esophagus from the back of the trachea. We used both vannas scissors and forceps to carefully separate the esophagus from the back of the trachea, being mindful not to rupture either structure (Figure 14B). Once separated, we investigated how best to lift the trachea. We used either 6-0 or 8-0 sutures to create a loose loop around the trachea at different locations, and helping hands were used to hold the trachea up at different angles. As shown in Figure 14B, this allowed us to reach the posterior side of the larynx, which we hope to record EMG soon.

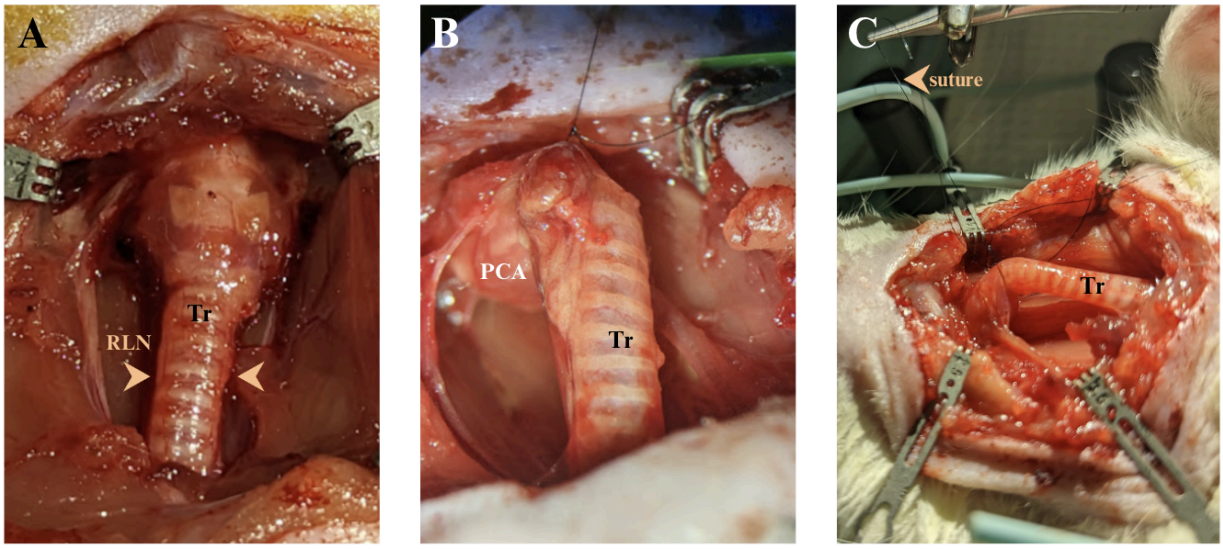

Figure 14: Exposure of PCA Procedure.

A. The surrounding superficial fascia was cleared away from both sides of the trachea to allow us to reach the ventral side of the larynx. The recurrent laryngeal nerve (RLN) was located lining the trachea (Tr) and carefully avoided in this process.

B. Using a 6-0 or 8-0 suture, a loose loop was tied around the larynx region and pulled upwards to reveal what we observed to be the PCA muscle on the posterior side.

C. Lateral view of the lifted trachea held upwards by a suture and helping hand. This step occurred after the trachea was separated from the esophagus.

Discussion:

A. EMG Recordings after Array Implementation

The two aims of this study are part of a larger project to record and correlate EMG received from target laryngeal intrinsic muscles to USVs produced by rats. While the recording of EMG is not the center of this paper, the recordings are crucial to measuring the success of the techniques developed for this study. We successfully recorded EMG in CT muscle during USV production in post-surgically recovered rats (Figure 15). The correlation of these signals displays a triumph for this study to display the elicitation of reliable USV production after an intensive and invasive surgery.

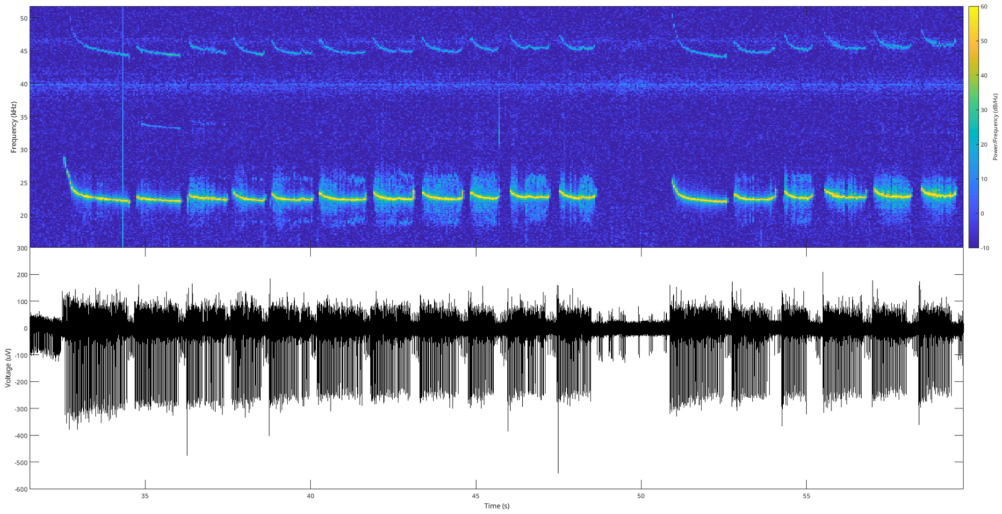

Figure 15: **EMG recordings in CT muscle during USV production.** The top graph is a frequency spectrogram (y-axis: frequency, x-axis: time) showing USVs at the 22 kHz range, with brief pauses during the calls. The color on the spectrogram indicates the power of the calls; the bottom graph shows the correlating electric signal (y-axis: voltage, x-axis: time) received by the CT muscle. The success of our array placement on the CT muscle reveals a clear correlation between USVs and EMG recording.

B. Social Dominance Observations

Through our behavioral observations, we noticed patterns of social dominance between cage-mates and correlations to USV production. It is known that rats are social creatures; in the wild, they have naturally occurring hierarchies, with certain rats overpowering others (Fulenwider, 2022). In almost every pairing of rats between all cohorts, we have been able to identify dominant and submissive behaviors, which has helped us predict USV patterns; we observed that dominant rats in relation to their cage-mate were more likely to produce 50 kHz USVs. From our observations, dominant rats displayed patterns of weighing more and being more docile when handled. Preliminary USV recordings showed that these rats produced more 50 kHz calls when exploring a novel cage and were more likely to react to the introduction of female bedding. More submissive rats were noted to be skinner and skittish when being handled, often trying to burrow into the laboratory coat of the handler. When being touched for USV recordings, dominantly acting rats were much more active in the cage and would chase the hand when touch was released. Oppositely, more subordinate rats stood still when touched or ran away from the stimulus to avoid more contact.

A future study exploring the correlation of behavior, social ranks, and USVs would be beneficial to test preliminary vocalization rankings further. We hypothesize that behavioral observation of the interactions of rats can predict which rats would elicit more variety of USVs and be the best candidates for data collection. Examining agonistic behaviors is a commonly employed approach to evaluate rodent social hierarchy and could be a future direction in this effort. Agonistic interactions encompass both offensive and defensive behaviors; the development of an experiment to observe cage-mates and their interactions with these categories could help determine social rank. Offensive behaviors commonly involve lateral attacks, chasing,

biting, and barbering, while defensive behaviors typically include flight, freezing, and the display of submissive/defensive postures (Fulenwider, 2022). From our observations, the determination of social rank correlates to USV patterns and should be further explored.

C. Study Limitations

This study had several limitations. We, including my advisor, had minimal experience performing behavioral studies and survival surgeries with rats before this project; thus, much guidance from more experienced researchers and ample literature review went into learning about behavioral and surgical practices. We learned much about behavioral tendencies throughout the project as we developed procedures. Additionally, we observed significant variability per individual through recording USVs–especially within the event number and category of vocalizations. We noticed some rats were more vocal than others, even with stimuli included. Similarly, some rats were observed to be more likely to produce calls solely in the 22 kHz category, 50 kHz category, or a mix of both.

We regularly successfully elicited USVs from around 22 kHz; however, further behavioral training is required to elicit 50 kHz more effectively. Only a small number of rats we worked with reacted with 50 kHz calls when touched. From Cohort B, on average, 33% of animals (two out of six rats) produced only 50 kHz calls instead of 22 kHz calls when touched. However, we observed from this study that our developed touching technique made the rats produce more of the 22 kHz calls. Since the surgery to place the electrodes is highly invasive and will inherently cause stress to the animals when recovering, we are trying to steer away from causing more perceived distress. Although this technique has been reliably successful in eliciting

vocalizations, future directions must be taken (see *Future Directions below)* to test more behavioral perimeters to encourage more 50 kHz calls.

D. Future Directions

I. Aim I (Behavioral Related)

Currently, we have been successful in eliciting multiple 22 kHz calls with touch, but further behavioral training is required to elicit 50 kHz more effectively. We observe 50 kHz vocal production (see Figure 1B) but do not robustly elicit these vocalizations. Additional exploration of factors should be investigated. Some ideas of novelties to test include the introduction of drugs, specifically amphetamines, which could elicit anticipatory 50-kHz USV responses (Kuchniak *et al.*, 2019). Additionally, since we have had some success eliciting 50 kHz calls with female bedding, we hypothesize that exploring more with female interaction may be successful. Specifically, techniques that could be used could include placing the subject male rat with the female rat for a short period of time, then removing the female rat, or placing the rat in an entirely soiled cage of a female for the experimental set-up. Another future direction could include altering the technique of our touch stimulus. In other laboratory cases exploring USVs, rat tickling causes mostly 50 kHz calls rather than what we have observed. Thus, exploring more about tickling techniques to mimic "rough-and-tumble" juvenile play could be beneficial to receive a variety of calls.

Additionally, this study would benefit from further behavioral tracking of rats. For example, we could add video cameras inside the soundbox. We have observed that closing the soundbox entirely makes the rats more comfortable in the clear, novel cage; we hypothesize this aspect is because rats are nocturnal. However, closing the soundbox does not allow us to observe

the activity of the rat inside. A video system would be beneficial to witness exactly when the rats make the calls and the timing of their interaction with specific stimuli. Another future direction would also be to record the rats before handling them every day when they first arrive in the lab. While our handling technique makes them more comfortable with us and allows the preparation of EMG recordings to reduce the use of isoflurane, the handling may affect USV production.

II. Aim 2 (Surgical Related)

We have successfully placed the electrodes on the CT muscle during surgery; currently, we are working to develop surgical techniques to place electrodes on the TA and PCA muscles efficiently. While our method to expose the PCA muscle effectively reveals the surface, the current array design used for the CT muscle lies poorly on the posterior surface of the larynx. With the inaccessibility of these muscles, different array designs are necessary to record EMG reliably from these muscles. Thus, we are working with engineers to create new array designs to test, including the one below (Figure 16).

For this study, we also explored reaching the TA muscle, but we require further investigation to reveal this specific muscle. To attempt to reach the TA muscle, we attempted to bore a small hole with a dental drill (the same one used for the head screws explained in the methods, *Headcap Placement)* through the lateral thyroid cartilage. We tried this technique with the expectation that a new design array could be created to fit into this small hole to be placed on the muscle. However, further techniques will be explored to expose and place an array on the TA muscle more reliably. Overall, future exploratory surgeries are necessary to investigate the least invasive but most efficient way to implement the array on the PCA and TA muscles.

Prior studies have successfully recorded EMG from CT and TA muscles in rats during ultrasonic vocalizations (Riede, 2011). However, these studies used fine wire electrodes, which have substantially different surgical procedures to place on the muscles than the flexible Myomatrix arrays that we use. The fine wire electrodes also collect much lower-resolution EMG data. Thus, developing surgical techniques to place our specific, high-resolution electrodes will lay the groundwork for procedures to collect meaningful EMG data in correlation to USVs. With this study, we are closer to closing the gap in understanding just exactly how the brain sends signals to muscles to create complex movements and add to our growing understanding of how biological systems produce different vocalizations.

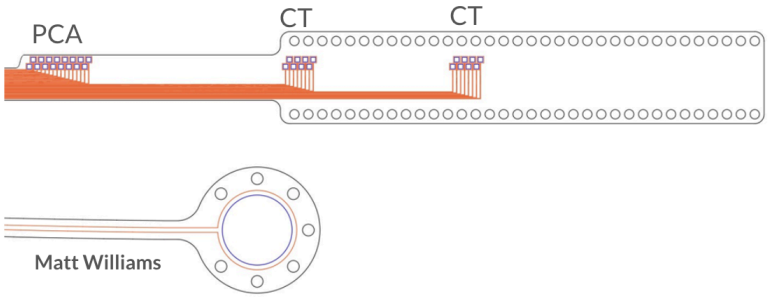

Figure 16: A new array design for future surgical procedures is being developed. This design is intended to wrap around the larynx and reach both PCA and CT muscles.

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