

## Video Article

# Targeted Training of Ultrasonic Vocalizations in Aged and Parkinsonian Rats

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## Abstract

Voice deficits are a common complication of both Parkinson disease (PD) and aging; they can significantly diminish quality of life by impacting communication abilities.<sup>1,2</sup> Targeted training (speech/voice therapy) can improve specific voice deficits,<sup>3,4</sup> although the underlying mechanisms of behavioral interventions are not well understood. Systematic investigation of voice deficits and therapy should consider many factors that are difficult to control in humans, such as age, home environment, age post-onset of disease, severity of disease, and medications. The method presented here uses an animal model of vocalization that allows for systematic study of how underlying sensorimotor mechanisms change with targeted voice training. The ultrasonic recording and analysis procedures outlined in this protocol are applicable to any investigation of rodent ultrasonic vocalizations.

The ultrasonic vocalizations of rodents are emerging as a valuable model to investigate the neural substrates of behavior.<sup>5-8</sup> Both rodent and human vocalizations carry semiotic value and are produced by modifying an egressive airflow with a laryngeal constriction.<sup>9,10</sup> Thus, rodent vocalizations may be a useful model to study voice deficits in a sensorimotor context. Further, rat models allow us to study the neurobiological underpinnings of recovery from deficits with targeted training.

To model PD we use Long-Evans rats (Charles River Laboratories International, Inc.) and induce parkinsonism by a unilateral infusion of 7 µg of 6-hydroxydopamine (6-OHDA) into the medial forebrain bundle which causes moderate to severe degeneration of presynaptic striatal neurons (for details see Ciucci, 2010).<sup>11,12</sup> For our aging model we use the Fischer 344/Brown Norway F1 (National Institute on Aging).

Our primary method for eliciting vocalizations is to expose sexually-experienced male rats to sexually receptive female rats. When the male becomes interested in the female, the female is removed and the male continues to vocalize. By rewarding complex vocalizations with food or water, both the number of complex vocalizations and the rate of vocalizations can be increased (Figure 1).

An ultrasonic microphone mounted above the male's home cage records the vocalizations. Recording begins after the female rat is removed to isolate the male calls. Vocalizations can be viewed in real time for training or recorded and analyzed offline. By recording and acoustically analyzing vocalizations before and after vocal training, the effects of disease and restoration of normal function with training can be assessed. This model also allows us to relate the observed behavioral (vocal) improvements to changes in the brain and neuromuscular system.

## Video Link

The video component of this article can be found at <https://www.jove.com/video/2835/>

## Protocol

### 1. Pre/Post-training Recordings

1. Begin ultrasonic monitoring with Avisoft Recorder using the configuration shown in Figure 2.
2. Ultrasonic vocalizations can be monitored either visually by watching the real-time spectrogram, or aurally with headphones plugged into the ultrasound recording interface.
3. Place a sexually receptive (in estrus) female rat into the home cage of the subject male rat. Signs of estrus include lordosis, ear wiggling, and rapid darting. Females in estrus elicit more vocalizations from males than non-sexually receptive females.
4. After the male has shown interest in the female (e.g., sniffing, chasing, and/or mounting), remove the female and record the male's vocalizations for 1-2 minutes.
5. If the male does not vocalize immediately, briefly return the female rat to the male's home cage to stimulate vocalization.

## 2. Vocal Training

1. Begin ultrasonic monitoring with Avisoft Recorder using the same configuration shown in Figure 2.
2. Place the male rat in his home cage under the microphone.
3. Put a female rat into the male's home cage. As stated previously, the female rat should be in estrus to maximize the vocalization response from the male.
4. Once the male has shown interest in the female remove the female.
5. Immediately after the male produces the target vocalization, reward simultaneously with a pen click and a brief presentation of the water bottle or food treat. Initially, any 50-kHz frequency modulated (Figure 5B) call is rewarded. As training progresses only increasingly complex strings of calls, such as strings of 5 to 10 calls in rapid succession, are reinforced.
6. Continue the training session until you have given 30 reinforcements. This typically takes between 5 – 10 minutes.

## 3. Preparing for Acoustic Analysis

1. Always archive the original sound files and make edits and measurements on a working copy, as the analysis will require filtering and erasing noise from each file.
2. Using the batch mode of Avisoft SASLab Pro, high pass filter all the sound files to be analyzed at 25 kHz to remove unwanted noise below the ultrasonic vocalizations.
3. In order to use the automatic detection feature of SASLab Pro, the noise threshold must first be determined. To do this, open a sound file and create a spectrogram using the settings shown in Figure 3.
4. The Automatic Parameter Measurements display should be setup according to the settings shown in Figure 4.
5. Find an area of the spectrogram where there are no calls or noises; this is the background noise.
6. Using the "automatic (single threshold)" option, set the threshold in the Element separation section of the Automatic Parameter Measurements setup dialog box to just above the background noise by adjusting the black line in the power spectrum window above the spectrogram. In Figure 4, the threshold has been set to -55 dB.
7. At the bottom of the dialog box, set the "Reject if peak amplitude is less than" value to 1 dB below the threshold found in the previous step. These two values should now be used for all sound files analyzed, assuming all recording settings were the same.

## 4. Vocalization Identification

1. To label the vocalizations using the automatic threshold set above, first create a spectrogram with the above settings.
2. Manually erase any noise that meets the following criteria: 1) has been wrongly identified as a vocalization, 2) is affecting the discrimination of the beginning and/or end of the vocalization, and/or 3) has been wrongly identified as the highest or lowest frequency in the vocalization (indicated by the upper and lower red lines, respectively). Alternatively, the start/stop times of the vocalization labels can be adjusted manually after creating permanent labels in step 4.5 below. Noise affecting the high and low frequency measurements, however, must be manually erased.
3. In some cases a vocalization will not be able to be separated from the surrounding noises, such as the sound of bedding during locomotion. In these cases, the call cannot be accurately analyzed and should be erased.
4. Once all vocalizations have been identified, permanently erase the deleted sections of the spectrogram from the sound file by selecting "Remove erased spectrogram sections from waveform..." from the Tools drop-down menu. If this command is not executed before the spectrogram is closed, all erasing work will be lost.
5. Create permanent labels in the sound file by clicking the 'edit' button in the Element Separation section of the Automatic Parameter Measurements setup window.
6. Visually and aurally review each labeled vocalization to determine its category: simple, frequency modulated (FM), or harmonic. Assign the appropriate label using the pre-defined text-modules. Examples of each vocalization category are shown in Figure 5.
7. Close the spectrogram, save the sound file, and continue with the next sound file to be analyzed.

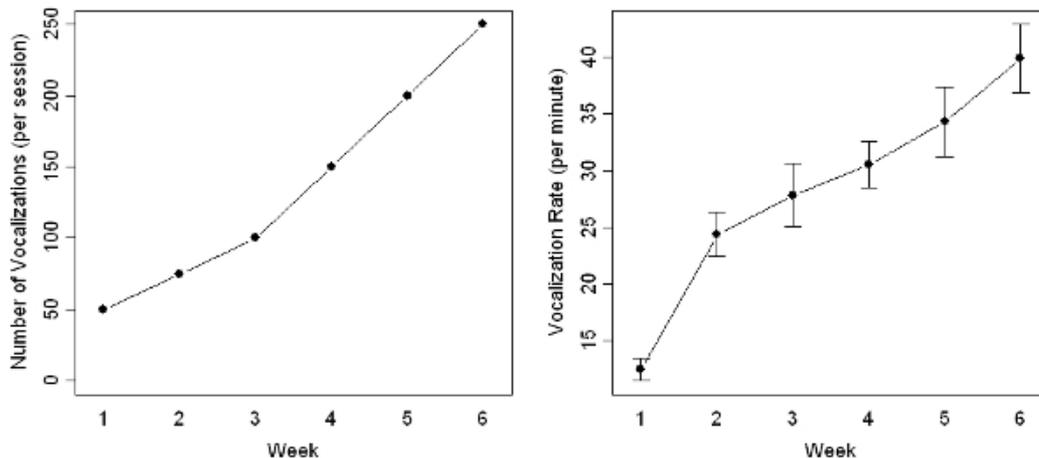
## 5. Acoustic Measurements

1. After vocalizations in all the sound files have been identified, use the batch processing tool (Tools: Batch Processing) to automatically measure the acoustic parameters of all the files.
2. In the Batch processing dialogue box, select "Automatic Parameter Measurements" from the drop-down menu.
3. Select the "process all files in the selected folder" checkbox then click the "folder" button and select the folder where the sound files are saved.
4. Click "Start" and each file will be automatically analyzed according to the settings last used in the Automatic Parameter Measurement setup dialogue box.
5. The measurements will be saved in a text file that can be defined using the DDE Parameters/Log File Setting command. This text file can then be imported into a program for statistical analysis.

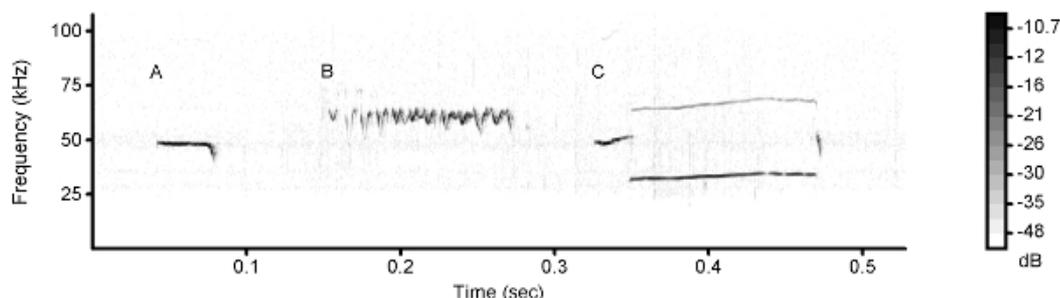
## 6. Representative Results:

Ultrasonic vocalizations of rats are affected by physiological changes associated with aging and 6-OHDA Parkinson disease models.<sup>13</sup> Typically, we see a reduction in quality of the ultrasonic vocalizations characterized by a reduction of the following quantitative acoustic parameters: bandwidth, peak frequency, duration, and intensity. Figure 6 shows the frequency modulated vocalizations of a rat in the PD model at three different time points: baseline, after induction of PD, and after vocalization training. In the PD condition, the call shows a reduction in bandwidth, duration, and intensity. Additionally, the frequency modulation has become irregular.

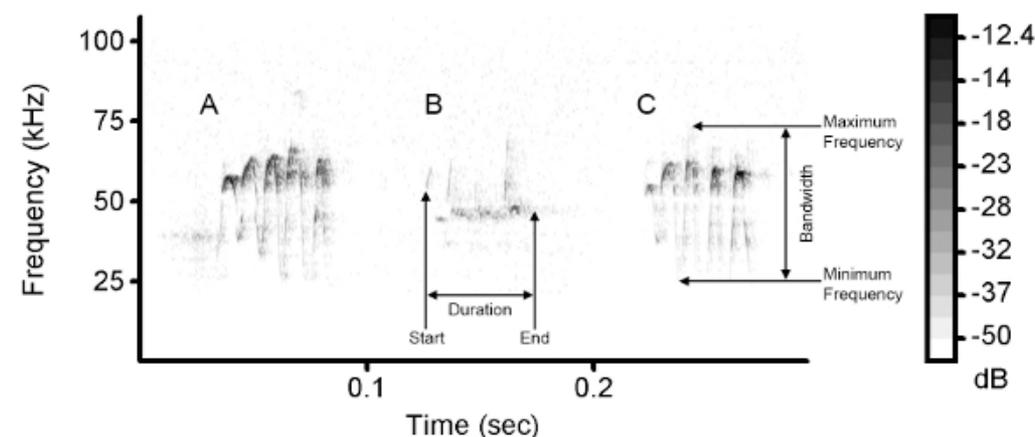
In addition to degradation of specific acoustic parameters, we also observe less overall complexity in the types of vocalizations produced in the aged and parkinsonism rats. For example, after induction of PD a rat produces a greater number of simple calls and fewer frequency modulated calls. Following vocalization training, many acoustic parameters, such as duration and intensity, approach baseline levels and the number of complex calls increases (Figure 6). Aged rats manifest similar degradation of acoustic parameters in their ultrasonic vocalizations. We are currently investigating the effects of vocal training in aged rats.



**Figure 1.** Number of vocalizations per session (target met by all rats each week) and vocalization rate (mean and standard error shown) both increase over a 6-week training period in a group of both young (9 mo) and old (32 mo) Fischer 344/Brown Norway F1 rats.



**Figure 2.** Representative vocalizations of each of the three categories of 50 kHz vocalizations; (A) simple, (B) frequency-modulated, and (C) harmonic.



**Figure 3.** Representative frequency-modulated vocalizations from a single rat at (A) baseline, (B) post-PD induction, and (C) after vocalization training.

## Discussion

Ultrasonic vocalizations in rats appear to be vulnerable to aging and disease processes, such as Parkinson disease. These ultrasonic vocalizations provide a behavioral model of vocal function, including deficits that accompany physiological changes due to disease and aging. In addition, acoustic changes in ultrasonic vocalizations are easily measured and quantified. Further, ultrasonic vocalizations can be modified

through behavioral interventions. Therefore, rat ultrasonic vocalizations provide a useful model to study the impact of disease, aging, and therapeutic interventions on both behavior and underlying neurobiological mechanisms.

Our method of vocal training uses a conditioned stimulus (a click) associated with a positive reinforcement (a water or food treat reward). The click followed by the reward is used to reinforce vocalizations of increasing complexity. We define complex vocalizations as those containing a "trill" (frequency modulated) component (Figure 5B) versus those that are "flat" and maintain a single frequency (Figure 5A). This frequency modulated component is easily identified by its sinusoidal appearance. While 50-kHz vocalizations may be entirely sinusoidal (Figure 5B), it is common to encounter more complex 50-kHz vocalizations that consist partly of this frequency modulated component as well as either large jumps in frequency or a brief flat component. These more complex vocalizations may have short breaks of less than 10 ms between components but are considered a single vocalization. Although further detailed sub-classification of these vocalizations is possible offline,<sup>14</sup> we classify all 50-kHz vocalizations containing a frequency modulated component as complex to reduce the need for online subjective classification and reduce reinforcement variability. In your own lab it is important to choose how data is classified and analyzed according to a study design that is appropriate for the purpose of the study.

There are many factors to consider when using this protocol. First of all, despite genetic homogeneity and procedural consistency, rats have a variable response to training. Some rats naturally vocalize more than others,<sup>5</sup> or are motivated by exposure to a female more than by a food or water reward. One way we account for this variability is by examining the change in acoustic parameters from individual rats' baseline vocalizations to their post-lesion and post-training vocalizations. Although not discussed in this protocol, we also have behavioral assays to quantify interest in the female, such as latency to mount. Additionally, depending on the age and activity level of an individual rat, a wire top may be needed on the home cage during training to prevent escape. Despite individual variability, we have successfully used this protocol on a variety of strains and ages to improve vocalizations relative to each individual rat's baseline.

Rats are sensitive to seemingly small changes in the environment, such as scents, temperature, and personnel. An effort should be made to avoid scented personal care products, such as lotions, hair products, and perfumes. Before attempting training, an individual must be comfortable interacting with and handling rats. Furthermore, any inconsistencies in personnel or environment may affect the rats' vocalization response.

Because rats are nocturnal, they should be kept on a reversed light cycle to allow for training to take place during the dark portion of their cycle when they are most active. We illuminate our training room with red lights. Additionally, if a water reward is used during training, rats should have restricted access to water. Our rats are water restricted for 21 hours prior to the training session. Three hours of water exposure was determined by our Institutional Animal Care and Use Committee to be the least restrictive time period that allowed water to be useful as reinforcement in our behavioral studies without presenting a substantial compromise to animal health or well being. Rats are given access to water at random times (10 minutes to 30 minutes) after training and subsequently have free access to water for three hours during the dark portion of their light cycle. Rats should be inspected every day for signs of dehydration and weighed every other day to ensure there is no significant (greater than 10%) weight loss. A food restriction is not necessary if using a food treat as a reward. Treats specific to rats are commercially available, but we have found a small piece of a sugary cereal to be an effective reward.

As mentioned above, female rats in estrus elicit a higher vocalization response from males than non-sexually receptive females. By maintaining a large number of females in the colony, there is a high likelihood that at least one female will be in estrus on any given day. However, estrus can also be pharmacologically induced in female rats by subcutaneously injecting 10 µg of β-estradiol in 0.1 cc sterile sesame oil 48 hours prior to training followed by 500 µg of progesterone in 0.1 cc sterile sesame oil 4 hours before training. Additionally, male rats that are naive to female rats must be sexually experienced prior to vocal training. This is accomplished by placing a female rat that is exhibiting signs of sexual receptivity (darting, lordosis, ear wiggling) into the male's home cage until the male mounts and ejaculates. Males may not mount during the first few sessions and the female may be removed after the male shows interest in the female by sniffing and/or chasing the female around the cage. Sexually experiencing is done once a day for two weeks. This time period also provides an opportunity for rats to habituate to the experimenter(s) and recording room.

One limitation to this method is the subjective determination of the vocalization type. In addition, this factor limits the speed of data collection, as each call must be independently examined and manually classified. Related to this is the importance of having experienced raters who have been consistently trained in vocalization identification. Both intra and inter-rater reliability should be assessed within your own lab.

In summary, ultrasonic vocalizations in rodents are used in many different models to study brain-behavior relationships, including emotional states, reward and addiction, and disease states, such as autism.<sup>6,14-16</sup> This novel method is a way to study the mechanisms underlying targeted vocal training for the treatment of voice deficits in aging and Parkinson Disease. This method has the potential to be applied to other disease models which affect communication and voice.

## Disclosures

Experiments on animals were performed in accordance with the guidelines and regulations set forth by the Public Health Service Policy on Humane Care and Use of Laboratory Animals, the NIH guide for care and use of laboratory animals, and the animal welfare act. The animal use protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Wisconsin-Madison School of Medicine and Public Health.

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