

Correspondence

Mice produce ultrasonic vocalizations by intra-laryngeal planar impinging jets

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Rodent ultrasonic vocalizations (USVs) are a vital tool for linking gene mutations to behavior in mouse models of communication disorders, such as autism [1]. However, we currently lack an understanding of how physiological and physical mechanisms combine to generate acoustic features of the vocalizations, and thus cannot meaningfully relate those features to experimental treatments. Here we test and provide evidence against the two leading hypotheses explaining USV production: superficial vocal fold vibrations [2], and a hole-tone whistle [3]. Instead, we propose and provide theoretical and experimental evidence for an alternative and novel vocal production mechanism: a glottal jet impinging onto the laryngeal inner planar wall. Our data provide a framework for future research on the neuromuscular control of mouse vocal production and for interpreting mouse vocal behavior phenotypes.

Murine rodents produce USVs with complex acoustic song-like structure that play important social roles, such as mating and territory defense [4]. USVs are emitted between 30 and 100 kHz peak frequency and often contain instantaneous peak frequency jumps separated by 15–35 kHz. USVs are hypothetically produced by a hole-tone whistle mechanism (Hypothesis 1), in which two spaced circular orifices generate a whistle [3] akin to the teakettle whistle [5]. These sounds are produced without structural motion, only aerodynamic and acoustic feedback. In rats, the peak frequency of USVs shifted upwards in lower density heliox atmosphere,

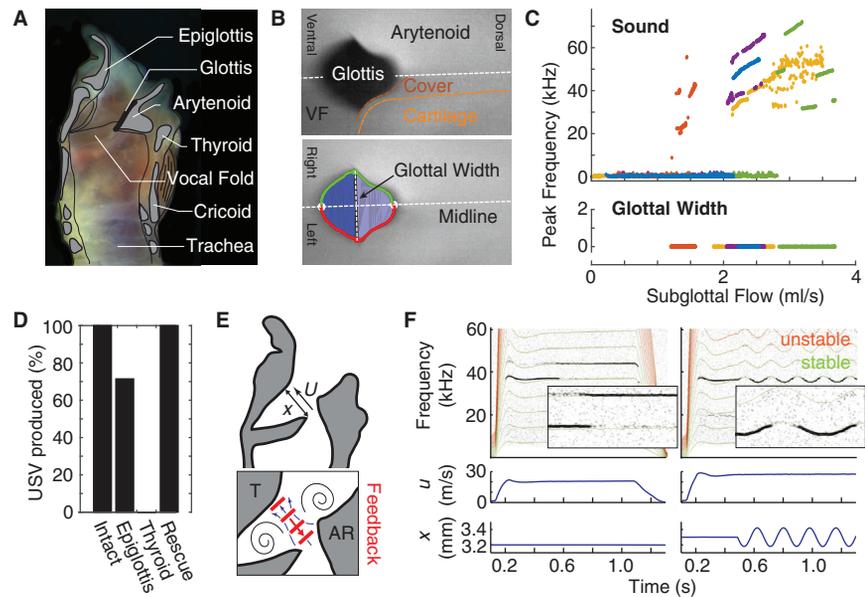


Figure 1. Mouse USVs are produced within the larynx by a planar impinging air jet.

(A) Sagittally sectioned isolated mouse larynx. Bar = 1 mm. (B) Extracted glottal dimensions from video still. The glottis consisted of the cartilaginous glottis between the arytenoids only. (C) Elicited USVs in excised mouse larynges (N = 5, color coded) show a clear increase of peak frequency with subglottal flow (top). The extracted glottal width (bottom) does not contain the frequency components found in the sound waveform and therefore does not vibrate at those frequencies during USV production. (D) Epiglottis and thyroid removal reduced USV production to 77% and 0%, respectively, in 7 larynges. USVs were rescued 100% by a metal plate replacement of the thyroid (N = 5). (E) Planar impinging jet model of mouse USV production with jet exit speed u and impingement length x . The feedback (red) travels back in the jet to the glottis. (F) The planar impinging jet model accurately predicts observed frequency modes during constant (left) and modulated impingement length x (right). Predicted stable (green; $d/x \leq St < 1$) and unstable (orange) modes plotted on top of a spectrogram of the experimental recording. (Glottal area = 0.375 mm². Power spectral density spectrogram; FFT length: 1024, overlap: 75%, Hamming window, dynamic range: 50 dB in units²/Hz.)

supporting this mechanism [3]. The first orifice would be the glottis, and the location of the second orifice could be one of three possibilities: the oropharyngeal opening leading to the mouth, the epiglottis in semiclosed position, or superior vocal folds. Identification of the production mechanism and these constrictions is crucial for understanding the (constraints on) neural control of peak frequency and other acoustic parameters [6]. Alternatively, USVs could be produced by oscillation of only superficial vocal fold layers (Hypothesis 2), akin to the human ‘whistle’ falsetto mode [2] in which the effective oscillating mass is reduced compared to normal chest voice allowing higher oscillation frequencies.

To test these hypotheses, we studied sound production in excised mouse larynges using high-speed imaging (100,000 frs/sec) of the vocal

folds. With adducted vocal folds, a glottal opening was located between the arytenoid cartilages on the dorsal side of the larynx (Figure 1A,B). Above subglottal air flow of 1–2 ml/s, USVs were readily elicited in the larynges of 15 animals (7 females and 8 males). The frequencies of *in vitro* USVs corresponded well to *in vivo* USVs (Figure S1 in the Supplemental Information). In five animals, we extracted glottal shape parameters from high-speed video (Figure 1B). Glottal width did not oscillate at the frequencies observed in the emitted sound (Figure 1C), arguing against an oscillating vocal fold origin of USVs (contra Hypothesis 2). These data suggest that if mice use a hole-tone whistle mechanism to produce USVs, both constrictions must be confined within the larynx.

To test if USVs are produced by a hole-tone whistle mechanism

and determine the location of the second constriction, we subsequently removed the epiglottis and the thyroid cartilage to just above the vocal folds. Superior vocal folds were not observed. Removal of the epiglottis silenced the larynx in 2 out of 7 animals, but upper thyroid removal silenced 7 out of 7 preparations (Figure 1D), which suggests the upper thyroid is essential to USV production. To further test this hypothesis, we replaced the upper thyroid with a metal plate (N = 5); USVs recovered 100% after rescue. Because USVs are generated without a second constriction present, these observations are inconsistent with the hole-tone mechanism (contra Hypothesis 1).

Self-sustained whistles can occur when an air jet impinges on an object, a phenomenon that has predominantly received attention for supersonic and high-speed subsonic flows [7–9]. The tones are caused by a feedback loop between coherent flow structures travelling downstream and acoustic waves travelling upstream in the flow [7]. Feedback could also happen in reverberating acoustic conditions. Both rat and mouse *in vivo* USVs and our *in vitro* data clearly show stable frequency modes and jumps between modes, indicating this system is resonance driven.

We propose that rodent USVs are produced by feedback between downstream convecting coherent flow structures from the glottis and upstream-propagating acoustic waves. The downstream convecting flow structures are generated by instabilities in the jet formed at the glottal opening. The upstream-propagating acoustic waves are generated by impingement of the coherent flow structures on the planar inner laryngeal wall, consisting of the thyroid, and perhaps partially the epiglottis. The resonance frequencies, f_n , require an integer number (n) of waves be present: $n = f_n(x/c + x/u)$, where x is the distance between the jet exit (glottis) and planar wall (the impingement length, Figure 1E), u is the mean convection speed of the coherent structures, approximated as jet exit speed, and c is the sound speed [7]. Because $u/c \ll 1$, the whistling frequency is given by:

$f_n = n * u/x$. This model predicts multiple discrete whistling frequencies and can explain the different density gas observations in [3] (see Experimental Procedures). Furthermore, this model produces stable whistle frequencies only within a narrow range of flow conditions, approximately when $d/x \leq St < 1$, where St is the Strouhal number $St = f_n * d/u$, with d the effective jet diameter.

To test this model we systematically changed jet speed u , by altering subglottal pressure, and impingement length x with a metal plate after thyroid removal in four larynges. We could induce whistles that exhibited stable whistling modes, jumps between modes, and concurrent modes in all four larynges. The modal frequencies were accurately predicted by our model (Figure 1F), providing strong support for our hypothesis that USVs are produced by a jet impinging on a planar wall.

What exact mechanism constitutes the feedback remains unknown; it could be either upstream propagating acoustic disturbances through the core of the jet, or outside of the jet, or some edge effect [7,8]. Furthermore, we currently cannot accurately predict which modes are stable and what triggers jumps to occur; the exact St values where modes are stable depends on the shear boundary layer properties at the jet exit [8,9]. We speculate that jumps occur with sudden changes in jet speed as potentially found during higher states of attention/arousal [10].

Our model predicts that stable modes are set by jet speed, effective jet diameter and distance from glottis to thyroid, parameters controlled by combinations of intrinsic laryngeal anatomy, and respiratory and laryngeal motor programs [6]. Our data therefore imply that the categorization of USV syllables requires careful consideration, because current classifications in mice may represent random jumps between stable modes that do not necessarily reflect specific motor commands. Furthermore, our results suggest that strain-specific USVs or USV changes in mouse models of genetically linked human communication disorders, such as autism, stuttering, and dyspraxia,

may be the result of altered laryngeal geometry as well as motor programs.

SUPPLEMENTAL INFORMATION

Supplemental Information including experimental procedures and two figures can be found with this article online at <http://dx.doi.org/10.1016/j.cub.2016.08.032>.

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