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Ultrasonic vocalizations during male–female interaction in the mouse model of Down syndrome Ts65Dn

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HIGHLIGHTS

- We characterized ultrasonic vocalizations by the mouse model of Down syndrome Ts65Dn.
- Minimum and maximum peak frequencies of calls were generally lower than in controls.
- We found longer durations for many types of vocalizations compared to euploid mice.
- Ts65Dn produced a reduced number of complex vocalizations compared to euploid mice.

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ABSTRACT

Down syndrome (DS) is the leading cause of genetically defined intellectual disability. Although speech and language impairments are salient features of this disorder, the nature of these phenotypes and the degree to which they are exacerbated by concomitant oromotor dysfunction and/or hearing deficit are poorly understood. Mouse models like Ts65Dn, the most extensively used DS animal model, have been critical to understanding the genetic and developmental mechanisms that contribute to intellectual disability. In the present study, we characterized the properties of the ultrasonic vocalizations (USVs) emitted by Ts65Dn males during courtship episodes with female partners. USVs emitted by mice in this setting have been proposed to have some basic correlation to human speech. Data were collected and analyzed from 22 Ts65Dn mice and 22 of their euploid littermates. We found that both the minimum and maximum peak frequencies of Ts65Dn calls were lower than those produced by euploid mice, whereas the mean individual duration of "down" and "complex" syllable types was significantly longer. Peak, minimal and maximal, and the fundamental frequencies of short syllables generated by Ts65Dn mice were lower compared to those by euploid mice. Finally, Ts65Dn males made fewer multiple jumps calls during courtship and the mean total duration of their "arc", "u", and "complex" syllables was longer. We discuss the human correlates to these findings, their translational potential, and the limitations of this approach. To our knowledge, this is the first characterization of differences between adult Ts65Dn and euploid control mice with respect to USVs.

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1. Introduction

Down syndrome (DS), the phenotypic consequence of the triplication of human chromosome 21 (HSA21) [36], is the most prevalent genetically defined cause of intellectual disability, with an incidence of 1 in 732 live births [9,58]. While individuals with DS maintain relatively high levels of social intelligence and procedural learning, they often display disproportionally impaired declarative memory [45]. In addition, children show particular difficulties in acquiring language [12,56]. The lack of linguistic development in children with DS seems to coincide with the beginning of an apparent developmental quotient (DQ) or intellectual quotient (IQ) decline during the first few years of life [71], suggesting that language deficiencies may be closely related to early cognitive impairment associated to this genetic disorder [12,56].

Toddlers with DS exhibit problems in assimilating all four basic components of language, i.e., phonology [17,55,66,67]; semantics [5,10,40]; syntax [13,18,29,34]; and pragmatics [1]. Deficits in each of these linguistic domains become readily salient as children and adolescents

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with DS often have poor speech intelligibility during conversation [65], stunted vocabulary growth, and reduced word production [5,40]. These shortcomings ultimately result in poor conversational or narrative skills, in which children and adults with DS show deficits in expressive language disproportionate to what would be expected from their general cognitive abilities and mental age [31,41].

Although, at first glance, the mouse may seem an unlikely model for the more cognitive, and hence "human" qualities of language, these animals nevertheless are able to emit ultrasonic vocalizations (USVs) at particular frequency ranges that then elicit ear twitches and vibrissa movements in conspecifics [8,53,57], and that are ideally suited to trigger strong cochlear microphonic responses [6] and peak electrical responses within the inferior colliculus [7]. Upon reaching the auditory cortex in the mouse brain, the signals additionally function as potent stimuli that can entrain cortical subfields to their particular frequencies over repeated introduction [38,39]. That is, like in the human brain, they can lead to neuroadaptations that will facilitate future detection. By and large, humans and mice also appear to use similar psychoacoustical mechanisms for the breakdown and perception of species-specific communicative sounds [21,23], and in both species, the recognition of these utterances is lateralized to the left hemisphere [20]. Further studies suggest that mice not only have the substrate to emit and process USVs, but that they also use these signals in a purposeful sense as adults to influence the behavior of conspecifics in agonistic settings and during courtship [48,51].

In the field of animal models for developmental and intellectual disabilities, the reduced level of calling and unusual calling patterns have been reported in mouse models of autism spectrum disorders [22,60,61]; Wohr et al., 2011. Such work inspired us to investigate USVs in the most widely used murine model for DS, the Ts65Dn mouse. Ts65Dn mice are trisomic for contiguous segments of mouse chromosome 16 highly homologous to the long arm of HSA21 ([16]; recently reviewed by Ref. [14]), and reproduce some of the most fundamental characteristics of DS involving abnormalities of the brain, as well as those in the craniofacial skeleton and audition [24,70].

To determine whether Ts65Dn mice show phenotypic characteristics similar to the dysfunctional articulatory processing seen in persons with DS, we recorded USVs from male euploid and Ts65Dn mice during "courtship" episodes with euploid female mice. Female-elicited USVs from male rodents are a very robust phenomenon that has been intensively studied for almost four decades. During social exploration and courtship of female rodents, males will emit unusually rich USV sequences that display characteristics of song with several syllable types organized into phrases and motifs with undulating or shift-like pitch changes, or sharp punctuations [28]. Here, we present our findings and discuss their human correlates, potential translational value, and the limitations of this approach.

2. Material and methods

2.1. Mice

We have used 22 Ts65Dn mice and 22 euploid littermates in this study. Their handling and care were consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and all experimental methods were approved by the University of Colorado Denver's Animal Care and Use Committee.

Ts65Dn mice were generated by repeated back-crossing of Ts65Dn females to C57BL/6JEiJ × C3Sn.BLiA-Pde6b+/D F1 hybrid males (as described by Ref. [15]) in colonies at the University of Colorado Anschutz Medical Campus or at The Jackson Laboratory. All experiments were performed at the University of Colorado Denver, during the authors' respective stints at that institution. Animals from the same litter were housed together by gender, and maintained on a 12:12 h light/dark cycle (lights off at 7:00 pm) with free access to food and water. All the mice were sexually naïve at the time of testing at 8–10 weeks of age.

2.2. Acoustic data acquisition, analysis, and classification of ultrasonic vocalizations

USVs were recorded during the early part of the dark or "active" phase once the mice had an hour's opportunity to register the change between the light and dark cycles (between 8:00 and 11:00 PM). This time of day has proven optimal for measuring USVs during affiliative behavioral interactions [49,68], and allowed us to evaluate USVs without disturbing the animal's circadian rhythms or sleep schedule. In general, the courtship assay involved: 1) a habituation process by which the male mice could acclimate to the testing environment; and 2) a 5-min session where an individual male mouse was paired with an individual female. Habituation reduces anxiety and stress during the USV recording session, directs the male's attention to the female, which should now be the most salient feature of interest, and increases the probability that the males will emit ultrasonic songs. Per habituation, all of the mice were progressively conditioned to the testing procedures over 3 days. On each of the first two nights, euploid and Ts65Dn males and euploid females were conditioned to being wheeled into the behavioral facility 1 h before lights out in the colony room. After adapting to the transition from the light phase to the dark for 60 min, cagemates were placed together under the recording microphone in empty acrylic cages and given time to explore the restricted space around a sound-attenuating chamber for 5–10 min. These "test cages" were positioned within the chamber about 10 in. directly underneath the microphone. At the end of the exploration period, the animals were returned to their home cages, kept in the behavioral facility overnight, and returned to the colony room the next day when the lights were on again. On the third night, all of the mice were further conditioned to being placed alone in the test cages for 5 min in preparation for being paired with a male/female partner the next evening.

On the fourth night (test day), each male mouse was paired with a different randomly-selected euploid female for 5 min. Clean cages with fresh bedding were used for each new pairing. Female mice were never used more than once and were placed in the testing cage first for 1–2 min before introducing the male. Interactions between male and female animals were left to unfold naturally. No mating attempts or aggressive behaviors were observed during these sessions.

Vocalizations were recorded over a 5-minute period using a pressure-field ¼-inch microphone (Type-4938, Bruel and Kjaer, Nærum, Denmark) with a functional range of 10 Hz to 100 kHz and a sensitivity of 1.6 mV/Pascal. The resulting electrical signal was pre-amplified (Falcon Range® 1/4-inch Microphone Preamplifier — Type 2670, Bruel and Kjaer), then processed by an instrumentation amplifier (Model 440; Brownlee, San Jose, CA). It was collected using a 16 bit analog-to-digital converter at a 200 kHz sampling rate (Digidata 1322A; Molecular Devices Corporation, Sunnyvale, California) driven by pClamp 9.2 software (Molecular Devices). An acoustic calibration of the system was performed by positioning a 1 kHz ± 0.1%, 94.0 dB ±0.2 dB Sound Level Calibrator (Type 4231, Bruel and Kjaer) in a clean test cage at the same relative position to the microphone where the animals would be placed. This calibration resulted in a final gain of 0.14812 V/dB; i.e., a full range of 135 dB peak-to-peak and a resolution of 0.002 dB/bit. Acquisition files were converted into audible.wav files using QuB (http://www.qub.buffalo.edu/wiki/index.php/Main_Page).

USVs were analyzed and categorized into syllable types using sound spectrograms (Avisoft Bioacoustics SasLab Pro software, Berlin, Germany, Software Version 5.2.06). Spectrograms were generated with a Fast Fourier Transform (FFT)-length of 512 and a Hamming style time window overlap of 50% (100% Frame). The spectrogram was produced at a frequency resolution of 391 Hz and a time resolution of 1.28 ms. A low cut-off frequency of 25 kHz was used to reduce background noise outside the relevant frequency band.

Analysis of USVs was performed blind to male genotype. Each syllable was classified as one of 9 waveform categories (Fig. 1) based on internal pitch change, length and shape, according to previously reported

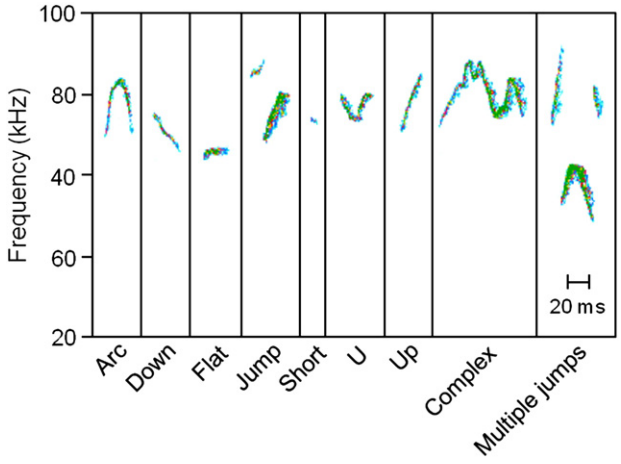

Fig. 1. Representative USVs for each of the 9 categories of calls emitted by adult male mice based on spectrograph parameters.

nomenclature [11,25,32,46]. The syllable categories are described below:

Arc syllables (also referred to as Chevron syllables) waveforms with increases and then decreases in frequency, with the highest frequency reaching >5 kHz above the beginning and end frequencies.

Down syllables (also referred to as Downward syllables) waveforms exhibiting a continual decrease in frequency, with a sweep >5 kHz.

Flat syllables waveforms with less than 5 kHz of modulation.

Jump syllables (also referred to as One-Jump syllables) waveforms containing one break in frequency, but without breaks in intensity.

Short syllables waveforms less than 10 ms in duration.

U syllables waveforms exhibiting decreases and then increases in frequency, with the lowest frequency reaching >5 kHz below the beginning and end frequencies.

Up syllables (also referred to as Upward syllables) waveforms showing a continual uniform increase in frequency, with a sweep >5 kHz.

Complex syllables waveforms containing two or more directional changes in frequency and >5 kHz modulation.

Multiple jumps syllables waveforms containing at least two breaks in frequency with no break in intensity.

2.3. Statistical analysis

The following discrete call measures were analyzed: (1) total number of calls; and (2) number of calls per syllable type. Several acoustic parameters were also quantified for each USV syllable category: (1) mean duration of calls; (2) energy; (3) peak-to-peak amplitude; (4) peak frequency; (5) amplitude; (6) peak or dominant frequency; (7) fundamental frequency; (8) maximal and minimal frequency; (9) bandwidth; and (10) entropy. Total calling times were computed by summing the duration of each call emitted by the subject. Differences between the groups (Ts65Dn vs. euploid) were analyzed by Mann Whitney tests for data not normally distributed. Comparisons were statistically analyzed using unpaired Student's t-tests with Welch's correction for normally distributed data. All statistical analyses were carried out by GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA). The results are presented as mean ± standard error of the mean (S.E.M.). For all comparisons, a "p" value of <0.05 was deemed to be statistically significant.

3. Results

3.1. Comparisons between USVs recorded from euploid and Ts65Dn mice: aggregate analysis

A total of 11,723 calls were produced by euploid mice (n = 20) and 7125 calls by Ts65Dn mice (n = 18). Data from two controls and four Ts65Dn mice were excluded from analysis because these animals failed to emit vocalizations during courtship. The loss of subjects was not statistically significant between groups (9% in the WT genotype, 18% in the Ts65Dn genotype, Fisher's exact test, p = 0.6640).

When all of the syllables produced by each genotype were combined, statistical comparisons between the mean values of the following standard USV properties revealed no significant differences: number of calls emitted during courtship (p = 0.1359), peak duration (p = 0.9185), amplitude (p = 0.1022), fundamental frequency (p = 0.2141), peak frequency (p = 0.1178), bandwidth (p = 0.9650), peak-to-peak amplitude (p = 0.1022), energy (p = 0.0634), and entropy (0.3420) (all of these measures were compared with the Mann Whitney test except for "peak amplitude," which passed the normality test and was analyzed with an unpaired *t*-test with Welch's correction). However, the maximum and minimum peak frequencies of the calls emitted by Ts65Dn mice were significantly lower on average than those produced by euploid siblings (unpaired *t*-test with Welch's correction; Ts65Dn mean: 70.69 ± 3.296 kHz, control mean: 78.11 ± 1.077 kHz, p = 0.0449; Ts65Dn mean: 67.88 ± 3.254 kHz, control mean: 75.18 ± 1.115 kHz, p = 0.0464; respectively) (Fig. 2).

3.2. Comparisons between USVs recorded from euploid and Ts65Dn mice: specific vocalization categories

When the vocalizations were broken down into different syllable motifs, we were still unable to detect a genotypic effect on the number of calls of most vocalization categories except for the mean number of multiple jumps calls (Mann Whitney test; control mean: 109.7 ± 47.47, Ts65Dn mean: 38.06 ± 18.74, p = 0.0438; Fig. 3-a). However, we detected significant genotypic effects for several spectral parameters. The mean individual duration of down and complex syllables was significantly longer in Ts65Dn mice relative to euploid animals (unpaired *t*-test with Welch's correction; control mean: 19.05 ± 0.8590 ms, Ts65Dn mean: 23.31 ± 1.674 ms, p = 0.04070; Fig. 3-b; and control mean: 34.47 ± 3.463 ms, Ts65Dn mean: 49.81 ± 3.642 ms, p = 0.0063; Fig. 3-c; respectively). The mean durations of Ts65Dn arc, "u", and complex syllables were also significantly longer (Mann Whitney test; control mean: 0.2064 ± 0.06413, Ts65Dn mean: 0.8796 ± 0.4176 s, p = 0.0498; Fig. 3-d; control mean: 0.2306 ± 0.1024 s, Ts65Dn mean: 0.3442 ± 0.1403 s, p = 0.0373; Fig. 3-e; control mean: 0.5724 ± 0.2602 s, Ts65Dn mean: 2.479 ± 0.9561 s, p = 0.0145; Fig. 3-f; respectively). Although the peak-to-peak frequencies were not significantly genotype-dependent for any vocalization category, for flat syllables this measure reached borderline statistical significance (unpaired *t*-test with Welch's correction, control mean: 0.09457 ± 0.009504, Ts65Dn mean: 0.1462 ± 0.02316, p = 0.0525).

Finally, the peak, minimal, maximal, and fundamental frequencies of short syllable USVs generated by euploid mice were higher compared to Ts65Dn littermates (unpaired *t*-test with Welch's correction, control mean: 79,303 ± 1069 kHz, Ts65Dn mean: 69,528 ± 3368 kHz; p = 0.0118; control mean: 78,404 ± 1091, Ts65Dn mean: 68,460 ± 3370, p = 0.0107; control mean: 81,075 ± 1059, Ts65Dn mean: 71,149 ± 3373, p = 0.0108; Mann Whitney test, control mean: 72,780 ± 2222, Ts65Dn mean: 65,174 ± 3241, p = 0.0409; respectively) (Fig. 4).

4. Discussion

In the present study, we have quantified the number and analyzed the characteristics of the USVs generated by Ts65Dn males during

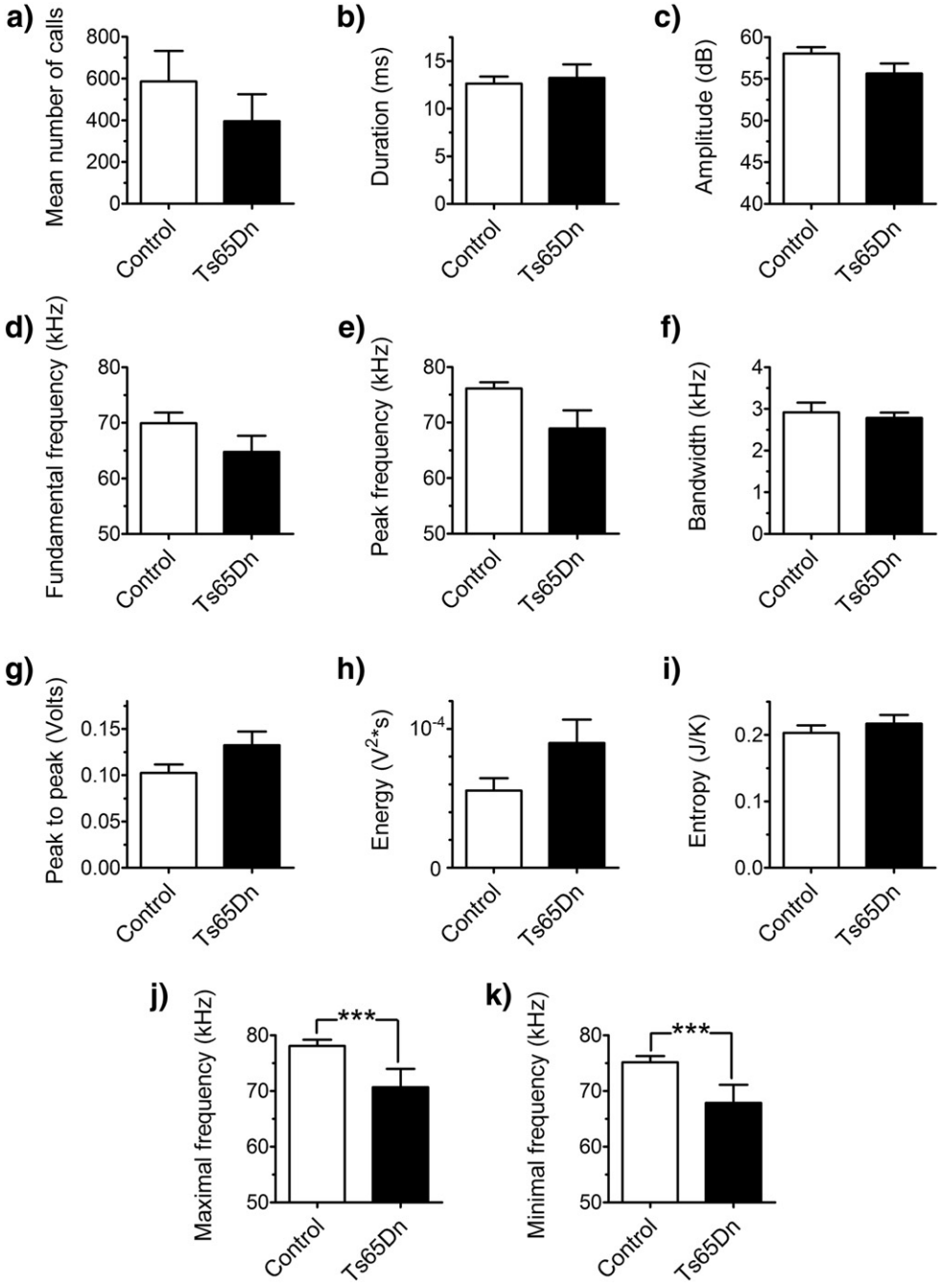

Fig. 2. Comparison between USVs recorded from euploid and Ts65Dn mice. For all syllables combined, we could not detect significant differences for any of the following measures: (a) total number of calls emitted by adult male euploid and Ts65Dn mice in the presence of a female, (b) peak amplitude, (c) duration, (d) fundamental frequency, (e) peak frequency, (f) bandwidth, (g) peak-to-peak amplitude, (h) energy, and (i) entropy. However, the (j) minimum and (k) maximum peak frequencies of the calls emitted by Ts65Dn mice were significantly lower than those produced by euploid animals (see text for respective "p" values). Data indicate means ± standard error of the mean.

courtship episodes with euploid female partners. We found that the minimum and maximum peak frequencies of Ts65Dn calls were generally lower than those produced by euploid mice, whereas the mean durations of "arc," "u", and "complex" syllable types were significantly longer. Peak, minimal & maximal, and the fundamental frequencies of short Ts65Dn syllables were lower compared to those of euploid mice. Finally, Ts65Dn USVs featured fewer multiple jumps calls during courtship, and the mean individual duration of their "complex" and "down" syllables was longer.

Research on the spectral features of the voice of individuals with DS not only has had a long history, but has also generated inconsistent results (reviewed recently by Ref. [33]). These studies have focused primarily on voice quality parameters, such as fundamental frequency, jitter, shimmer, and signal-to-noise ratio. The quantification of fundamental frequency of verbal utterances has been a necessary constant of all studies in the human literature. This measure can be seen as the frequency of the "sound carrier wave" and is expected to provide the listener with the basic perception of high and low voice pitches. Here, we observed a significantly lower mean fundamental frequency for the short syllable USVs emitted by the mouse model of DS Ts65Dn when compared to their euploid siblings. Older studies involving individuals with DS also reported lower fundamental frequencies compared to typically developing controls [37,42,43]. Yet, newer studies have been mixed with some describing lower or higher fundamental

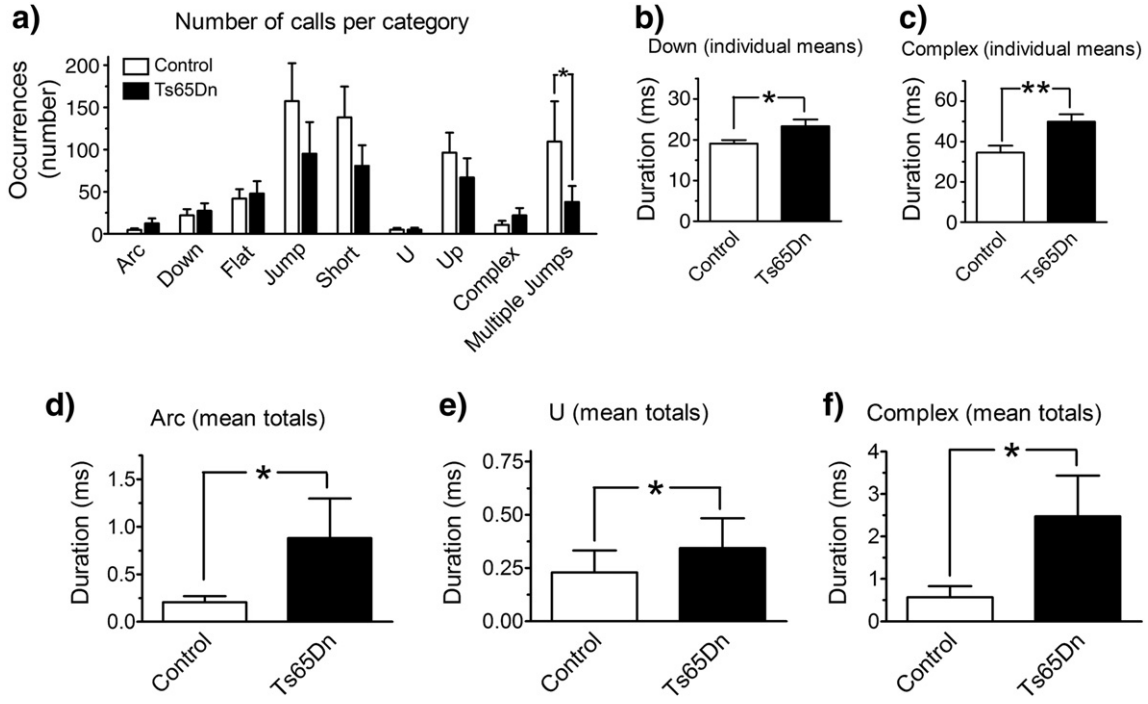

Fig. 3. Differences in the USVs emitted by adult male euploid and Ts65Dn mice according to call category. (a) There was no genotype effect on the number of calls of most categories of USV emitted by adult male euploid and Ts65Dn mice in the presence of a female, except for the mean number of multiple jumps calls. The mean duration of individual (b) down and (c) complex syllable types was significantly shorter in euploid control than in Ts65Dn mice. Also, the mean total duration of (d) arc, "u" (e), and (f) complex syllable types was significantly longer for Ts65Dn mice. Data indicate means ± standard error of the mean.

frequencies, or no significant difference at all [2,35,44,62]. The reasons behind this discrepancy are difficult to ascertain, but the results of the older studies seem to be in better agreement with clinical and naturalistic acoustic perceptual assessments of voice quality in persons with DS, which has been characterized as "low pitched and raucous" when compared to that of most typically developing individuals [42,47,64]. It is tempting to speculate that the older studies might have been a better representation of the "natural" voice characteristics of persons with DS, given that these studies were performed at a time when systematic and frequent sessions of speech therapy from infancy through adolescence (and many times into adult years) were not the standard of care for persons with DS. If that is truly the case, one could argue that the

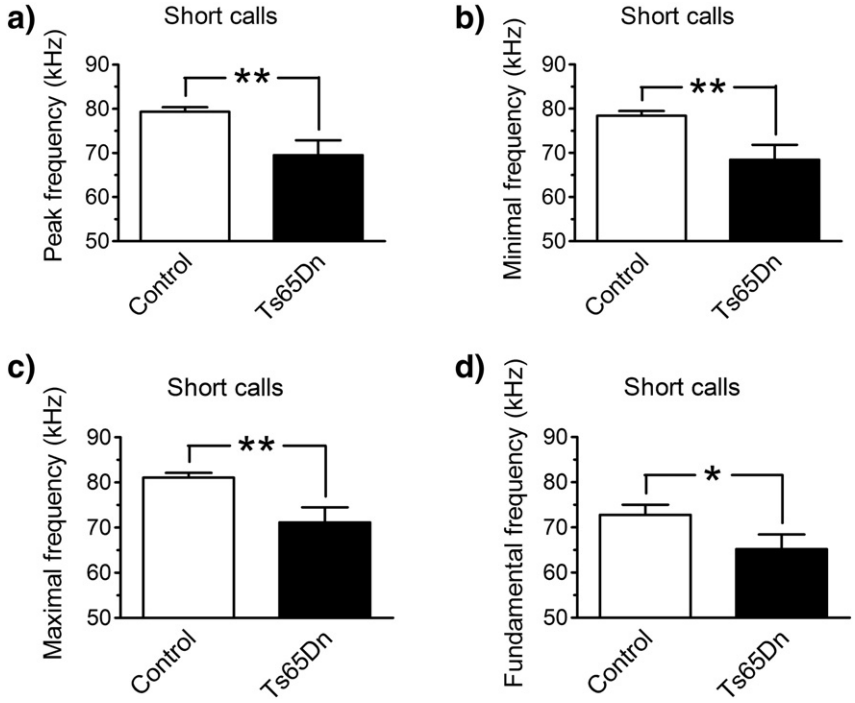

Fig. 4. Differences between short syllable USVs emitted by adult male euploid and Ts65Dn mice. (a) Peak, (b) minimal, (c) maximal and (d) fundamental frequencies of short syllable USVs in euploid control mice were higher compared to Ts65Dn littermates. Data indicate means ± standard error of the mean.

lower fundamental frequency recorded from the Ts65Dn mouse may be an apt model for the "natural voice characteristics" of individuals with DS. However, it is also not difficult to argue that the basic spectral characteristics of human speech and mouse USVs may not be directly comparable, given the different physical requirements for the anatomical apparatus necessary to produce audible sounds compared to vocalizations in the ultrasonic range.

In terms of the actual characteristics of the USVs produced by Ts65Dn mice, we found a significantly longer duration for many types of vocalizations and a reduced number of complex vocalizations (i.e., those consisting of multiple jumps) compared to those by euploid mice. The significantly longer vocalizations exhibited by Ts65Dn mice at lower fundamental frequencies can be envisioned as stretched out versions of the vocalizations generated by euploid control animals. Although potentially more interesting from a DS modeling point of view, the finding of a significantly lower occurrence of complex vocalizations by Ts65Dn mice can be interpreted in radically different ways, depending on one's assumptions about the nature and significance of the "syllabic structure" of rodent USVs. For example, if one interprets each syllable as being functionally analogous to a human word, then, the obvious and tempting interpretation is that we might be seeing the mouse equivalent to a shorter mean length of utterance. This is a robust and disproportionate developmental deficit in expressive language that is seen in persons with DS ([52]). On the other hand, if each of these multisyllabic structures is viewed as primitive versions of individual human "words", then, the less complex USVs produced by Ts65Dn mice in relation to their euploid siblings might represent an intriguing correlate to the fluency disorders seen in persons with DS, which occur at a rate of 10–45% [33].

Admittedly, one has to take any anthropocentric interpretation of USVs with a grain of salt, given the vast evolutionary distance between humans and rodents, as well as the uniquely complex and culture-dependent nature of human vocal communication. However, one also would be naïve in discounting the potential significance of findings involving alterations in the characteristic of USVs generated by mouse models of human disorders. Recent findings (e.g., [3]) demonstrate that male mice have some limited vocal modification abilities with at least some neuroanatomical features previously thought to be unique to humans and song-learning birds. These data suggest that mice possess basic primitive neural substrates for the faculty of "human-like" vocal communication. In addition, as mentioned in the Introduction, the analysis of rodent USVs has been applied extensively and successfully to the study of genetic models of autism spectrum disorders (for recent examples, see [4,26,30,61,69,72–74]). In the context of DS, the study of USVs may shed light on fundamental questions regarding the nature of speech impairments affecting the domains of voice, speech sounds, and potentially even fluency in persons with DS. The mouse is a more amenable system to assess micro- and macroanatomic features of the vocal apparatus when compared to human beings. Mice also allow for the production of functional data under more controlled and homogenous conditions than would be feasible with human experimental participants. For example, the extent to which early language learning is impaired by problems with hearing and musculoskeletal abnormalities (rather than intellectual disability) in people with DS remains an important, but open question that could potentially be addressed systematically in the mouse. More than two-thirds of toddlers with DS suffer from conductive and/or sensorineural hearing loss [59]. Individuals with DS are born with small ear canals, and specific craniofacial features that complicate the proper articulation of speech — including hypoplasia of the bones that enclose or give form to the midface, jaws, oral cavity, trachea, and paranasal sinuses [54]. It is generally thought that hypotonia of the lips and cheeks compounds the effects of facial hypoplasia on breathing and voice production [50].

Given the developmental nature of the speech impairments associated with DS, it will be important to characterize USVs produced by Ts65Dn mice at various ages in future studies. It is known that mice begin vocalizing shortly after birth, with a peak in vocalization rates occurring around postnatal day 8. They continue vocalizing, albeit at reduced rates, throughout adulthood [63]. Reminiscent of human infants, mouse pups are known to produce utterances that have evolved to attract maternal care. Ts65Dn pups show significant delays in the emission of such USVs [27]. The investigation of USVs generated by murine models of DS at different developmental stages is likely to enrich our understanding of the neurophysiological mechanisms controlling speech in individuals with DS. Such studies might also provide us with new surrogate endpoints for the emerging field of translational research of potential pharmacotherapies designed to enhance the cognitive and adaptive skills of persons with DS [14,19].

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