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Altered postnatal developmental patterns of ultrasonic vocalizations in *Dock4* knockout mice

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A R T I C L E I N F O

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A B S T R A C T

Ultrasonic vocalization (USV) characterization is useful for evaluating communication in mouse models of autism spectrum disorder (ASD). Here, by categorizing USVs into 12 types using a comprehensive classification method, we obtained the qualitative and quantitative characteristics of USV repertoire emitted by ASD-related *Dock4* knockout (KO) mice and their wild-type (WT) littermates during social isolation over early postnatal development. Notably, USVs emitted by WT pups exhibited a developmental switch from a pattern with more multiple-note calls, which have more complex acoustic structure, lower pitch and larger volume, into one with more single-note calls, which have simpler acoustic structure, higher pitch and smaller volume. Comparing with WT pups, USVs emitted by *Dock4* KO pups had larger volume and consisted of more multiple-note calls with higher pitch in later developmental stage. These findings collectively reveal a developmental pattern of USV in normal mice and identified a set of alterations in *Dock4* KO pups.

1. Introduction

Ultrasonic –>vocalization (USV) is an essential part of social communication in mice [1], as they emit ultrasonic whistle-like sounds at different ages during a variety of social contexts. At the neonatal stage, when mouse pups are separated from their dam, littermates, or nest, they normally emit a large number of isolation-induced USVs, which is a pre-lingual communicative behavior similar to human infant crying [2]. These USVs are believed to be crucial for building the relationship between the neonatal pups and the dam by eliciting the seeking, retrieval, and maternal care from the dam [3–7]. At adult, mice vocalize a diverse repertoire of USVs during courtship or social interaction with partners or strangers [8]. For example, male mice emit vocalizations in the presence of a female or female pheromones in the urine [9,10]. During social dominance, acoustic communication in male mice is associated with a novel intruding conspecific of same sex [11,12]. It has been revealed that mice are not imitative vocal learners [13,14], and their vocalization behaviors may be affected by genetic factors [15]. Therefore, investigation on USV is crucial for the understanding of not only the molecular neurobiology basis of language development, but also the etiology of language deficits presented in many neurological disorders.

A number of neuropsychiatric or neurodevelopmental disorders are accompanied with communication deficits of varying severity that are evident in the early life. Among them, autism spectrum disorder (ASD) is a complex neurodevelopmental disorder characterized by verbal and non-verbal communication failure as a core symptom. Although the causative pathophysiological mechanism of ASD remains largely unknown, it is clear that genetic factor contributes greatly to the etiology of ASD. Therefore, evaluating early-life social communication levels in mouse models engineered with ASD-related genetic changes will provide substantial evidence on the understanding of social pathophysiology of ASD. Due to relatively simple procedures and high reproducibility, the isolation-induced USV in mouse pups has been so far the most adopted model for evaluating early-life social communication alterations in mice. Similar to all languages, mouse USVs convey communicative information depending on the combinatory use of different syllables (call types), each presenting distinct acoustic parameters including frequency, amplitude, and duration, and these features vary drastically in different social contexts [12,16]. However, analyses were usually performed in a pooled fashion from all call types in the previous studies in mouse pups [17–19], and the information on

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the developmental changes of different call types is missing. It is thus of great importance to dissect the developmental profile of individual call types with more detailed characterizations on their acoustic features.

DOCK4, a gene encoding the RAC1 guanine nucleotide exchange factor DOCK4, has been identified as a risk ASD gene [20,21]. Previously, we generated and characterized a line of *Dock4* KO mice, which displayed ASD-like social recognition deficits, elevated anxiety, and perturbed object and spatial learning [22]. In particular, *Dock4* KO mice emit significantly larger number of ultrasonic calls with longer total duration than wild-type (WT) pups during early postnatal stages [22]. In the current study, by performing comprehensive quantitative and qualitative analyses of USV patterns and acoustic features, more characteristic details of USVs including call types, duration, peak frequency (pF), and peak amplitude (pA) were examined in isolated WT and *Dock4* KO mouse pups during the first two weeks of postnatal development. The results revealed a developmental change of USV patterns in WT pups, which is altered by *Dock4* deficiency.

2. Materials and methods

2.1. Mouse breeding and housing

WT and *Dock4* KO mice have been generated as previously described [22]. All animals are on C57BL/6 J background. Heterozygous males and females were used to breed WT and KO pup littermates. All mice, with *ad libitum* access to food and water, were kept in a room with controlled temperature (24°C) and humidity (55%), in 12/12 h light/-dark cycles with light on from 8:00 AM to 8:00 PM. All experimental procedures involving the use of animals were approved by the Ethics Committee on Animal Experiments at Jinan University, China, and were strictly performed according to the guidelines of the Care and Use of Laboratory Animals.

2.2. USV recording for isolated pups

We recorded the USVs emitted by pups during social isolation at postnatal day (P)3, P6, P9, and P12 as described previously [22]. Each pup was marked on one of the toes after the recording at P3, and the genotypes of pups were blind to the experimenter until four trials were completed. The experiment room temperature was set at 21 ± 1 °C, which is lower than the normal housing temperature and is known to increase isolation-induced USVs in neonatal mice [12,23]. On test days, we transferred the home cage including both the dam and pups to the experiment room, and immediately started to test the pups one by one in random order. Each pup was put in a glass container (diameter: 9 cm; height: 12 cm) with fresh bedding, and the container was put in a soundproof chamber for USV recording for 5 min. An ultrasound-sensitive microphone, placed 10 cm above the isolated pup, was connected to a computer *via* a sound card (Avisoft Bioacoustics, Germany), and USV signals during the isolation periods were displayed in real time with Avisoft SASLab Pro Recorder (16-bit format, 300 kHz sampling frequency to capture sound amplitude up to 150 kHz with a high quality) in the computer screen, meanwhile stored as sonogram files [12]. Both the recording hardware (Avisoft UltraSound Gate 116 Hm with a high-quality condenser microphone) and software (Avisoft SASLab Pro Recorder, version 4.2) were from Avisoft Bioacoustics.

2.3. USV analysis

USVs were analyzed using Avisoft SASLab Pro software, the measurement parameters and analysis procedures were shown below:

1) Call type classification: As described in the classification criteria listed in Table 1, 12 call types were determined by their acoustic features including duration, frequency range, and note number. To separate the calls by types, each call was first selected, and the call type name was typed in the pop-up dialog panel by clicking the option "Tools > Labels > Insert section label from marker". After each call was labeled, by clicking the option "save labeled sections of same named class into single wav files", the calls labeled with the same name were collectively stored as a single sound file.
2) Analyses of pF, pA, and duration: Each file that contains all the calls of same type was analyzed by Avisoft SASLab Pro. By selecting the option "Analyze > Spectrogram Parameters", we set "FFT length" as "1024", "Frame size" as "75%", "Window" as "Hamming", and "overlap" as "75%" to create a "spectrogram" window. In this "spectrogram" window, we set the frequency range to be analyzed as 30kHz – 150 kHz using the option "Display > Cut-Off Frequencies". Subsequently, in the option "Tools > Automatic parameter measurements > Automatic parameter measurements setup", we set the "Element separation" mode as "automatic (whistle tracking)". The "min duration" and "Hold time" were set to 2 ms and 10 ms, respectively, for all call types except the "Short" type, for which the "min duration" and "Hold time" were set to 1 ms and 5 ms, respectively. We then selected the "Duration of element", "Peak frequency" and "Peak amplitude" options to automatically obtain the pF, pA, and duration of each call type.

2.4. Statistical analysis

All statistical analyses were performed using GraphPad Prism software (Version 7.00) or SPSS (Version 21) and $p < 0.05$ was considered significant. The n number of each call type was provided in Table S1. Normal distribution of each comparison group of data was checked using the Kolmogorov-Smirnov test before any parametric analyses, and the normality of each data set is provided in Table S2. In WT pups, proportions of single-note, multiple-note, and Unstructured calls between different days were analyzed using Linear Mixed Models Select Subjects/Repeated Variables. Proportions of pooled single-note, multiple-note, and Unstructured calls of WT and *Dock4* KO pups were analyzed using Chi-square test with z-test. For multiple group comparisons of pF and pA of single-note and multiple-note calls or pA of different call types among different days in WT pups, Kruskal Wallis test with Dunn's multiple comparisons was used if the assumptions for parametric statistics were not met, otherwise, One-way ANOVA followed by Holm-Sidak's multiple comparisons was used. To compare the difference between WT and *Dock4* KO pups or between single-note calls and multiple-note calls in WT pups, Mann Whitney test was used if the assumptions for parametric statistics were not met, otherwise, unpaired *t* test was used. Data are presented as mean ± standard error of the mean (SEM) for each group.

3. Result

3.1. Categorization of USVs in mouse pups

In the previous study, we reported the number and characteristics of total calls between WT and *Dock4* KO pups [22]. We further observed that KO pups tended to emit calls sooner after isolation than their WT littermates, indicated by the shorter latency of the first call in KO pups (Mann Whitney test at P12, $p = 0.0048$; Fig. S1). However, whether there are more alterations in the repertoire of USVs in KO pups have not been investigated. In fact, mouse USVs consist of a complex range of call types that show different sonogram features. Due to different recording conditions and criteria, there is no unifying standard to categorize USV types, and a variety of categorizations have been described in the literature. In the current study, we propose a comprehensive classification method based on the existing criteria from several studies [3,11,19,24,25]. We examined more than 12,000 calls from 13 WT pups and more than 15,000 calls from 13 *Dock4* KO pups during 5-min isolations at P3, P6, P9, and P12. We categorized all USVs into 12 types according to their unique sonogram features (Short, Flat, Downward, Upward, Chevron, Re-chevron, Complex, Composite, Two-components, Frequency-steps, Harmonics, and Unstructured; Fig. 1 and Table 1). In these types, “note” is the most basic acoustic element which shows an intact and clear structure in the sonogram, and one or more notes can be combined to form a “call”, also known as a “syllable” [26,27]. A “call” is a unit of sound separated by a time gap from other sound units [27–29]. According to the note number in different calls, seven types (Short, Flat, Downward, Upward, Chevron, Re-chevron, and Complex) belong to “single-note” call types, as each contains only one note (Fig. 1A); four types (Composite, Two-components, Frequency-steps, Harmonics) belong to “multiple-note” call types, as each contains two or more notes (Fig. 1B). In addition, a noise-like call type without identifiable notes was categorized as “Unstructured” (Fig. 1C and Table 1).

3.2. Proportions of different USV categories are altered in *Dock4* KO pups

To describe the overall changes of USV profiles over development and between WT and *Dock4* KO littermates, we first examined the use of each call type by these pups at different developmental stages. Based on our classification criteria, each call was manually labeled in the audio file and then counted, and proportions of single-note, multiple-note and Unstructured calls, and those of each of the 12 call types were calculated (Figs. 2,3). Intriguingly, the proportion of single-note calls emitted by WT pups was increased from P3 to P12 (37.54%–74.18%; Linear Mixed Models Select Subjects/Repeated Variables, $p < 0.05$; Fig. 2), whereas the proportion of multiple-note calls was decreased (55.27%–24.03%, Linear Mixed Models Select Subjects/Repeated Variables, $p < 0.05$; Fig. 2). By examining the pooled numbers of each call types, we found that the top increased single-note call types from P3 to P12 include Short (11.2%–20.6%), Upward (1.9%–11.6%), Chevron (2.6%–9.2%), and Complex (0.2%–13.3%), and the top decreased multiple-note call types include Composite (20.3%–2.1%) and Frequency-steps (25.7%–8.7%; Fig. 3). Hence, the overall pattern of mouse USVs shows a multiple to single-note conversion during early postnatal development.

Interestingly, the proportion of pooled multiple-note calls emitted by *Dock4* KO pups was larger when compared with WT littermates at each developmental day (Chi-square test with z-test, single-note calls at P3, P6, and P12: $p < 0.05$, at P9: $p > 0.05$; multiple-note calls: P3, P6, P9, and P12: $p < 0.05$; Fig. S2). At P12, KO pups emitted more multiple-note calls of the category Composite (5.5% in KO v.s. 2.1% in WT group) and Frequency-steps (14.4% in KO v.s. 8.7% in WT group; Fig. 3). Conversely, less single-note calls of the category Short (16.2% in KO v.s. 20.6% in WT group), Upward (6.6% in KO v.s. 11.6% in WT group), and Complex (5.5% in KO v.s. 13.3% in WT group) were recorded in KO pups (Fig. 3). These findings collectively reveal a developmental change of the use of different call types in pup communication during isolation, and this pattern is altered in *Dock4* KO pups.

3.3. Acoustic characteristics of single-note and multiple-note calls in WT and *Dock4* KO pups

We next investigated the pF and pA, basic acoustic characteristics of

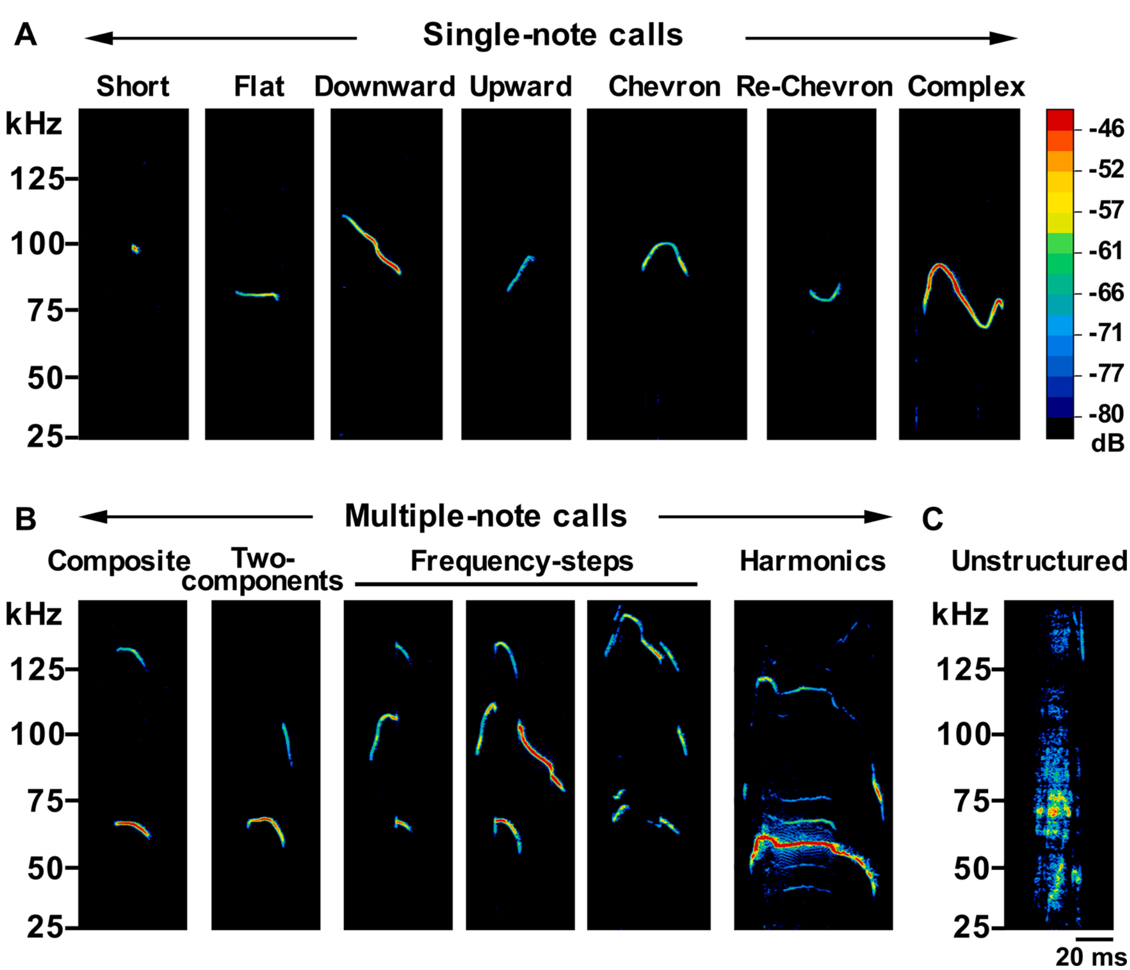

Fig. 1. The representative acoustic structures of different call types recorded in WT pups during social isolation.
(A) The example sonograms of single-note call types, which include Short, Flat, Downward, Upward, chevron, Re-chevron, and Complex. (B) The example sonograms of multiple-note call types, which include Composite, Two-components, Frequency-steps, and Harmonics. Three example calls of Frequency-steps that contain different numbers of notes are shown. (C) The example sonogram of Unstructured calls. Refer to Table 1 for detailed classification criteria.

Table 1
The classification criteria of ultrasonic vocalizations in mouse pups.

Call types	Description
Single-note calls	
Short	The call duration is shorter than 5 ms with the frequency range ≤6.25 kHz.
Flat	The call duration is longer than 5 ms with the frequency range ≤6.25 kHz.
Downward	A downward-shaped sonogram, with a frequency drop range >6.25 kHz.
Upward	An upward-shaped sonogram, with a frequency rise range >6.25 kHz.
Chevron	"Inverted-U" shape; the highest frequency is at least 6 kHz greater than both the starting and ending frequencies.
Re-chevron	"U" shape; the lowest frequency is at least 6 kHz smaller than both the starting and ending frequencies.
Complex	This call type contains at least one frequency drop and one frequency rise (a wave-like shape), with frequency range ≥6.25 kHz.
Multiple-note calls	
Composite	This call type consists of two parallel notes with different frequencies, which are emitted simultaneously.
Two-components	This call type consists of a sequence of two notes and includes one jump in frequency without a time gap.
Frequency-steps	This call type consists of three or more notes, each has a jump in frequency from the others without time gaps.
Harmonics	This call type consists of multiple notes, and the main one with strongest amplitude and longest duration is surrounded by higher or lower noise-like frequencies.
Unstructured	This call type has a wide spectrum of noise-like frequencies without identifiable notes.

sound pitch and volume, respectively, of both single-note and multiple-note calls in WT and KO pups at different stages. As Unstructured calls lack distinct notes, we did not include this call type in the examination. We found that along the development of WT pups, the pF of single-note calls was slightly increased (Kruskal Wallis test with Dunn's multiple comparisons post hoc test, P3 v.s. P6, $p = 0.2368$; P3 v.s. P9, $p = 0.0406$; P3 v.s. P12, $p = 0.0003$), whereas the pF of multiple-note calls was decreased from P3 to P9 but slightly increased at P12 (Kruskal Wallis test with Dunn's multiple comparisons post hoc test, P3 v.s. P6, $p < 0.0001$; P3 v.s. P9, $p < 0.0001$; P9 v.s. P12, $p < 0.0001$; Fig. 4A). Regarding the amplitude, we found that the pA of both single-note and multiple-note calls in WT pups reached to peak at P6, and was then decreased gradually at P9 and P12 (Kruskal Wallis test with Dunn's multiple comparisons post hoc test; single-note calls: P3 v.s. P6, $p = 0.0005$; P6 v.s. P9, $p < 0.0001$; P9 v.s. P12, $p < 0.0001$; multiple-note calls: P3 v.s. P6, $p < 0.0001$; P6 v.s. P9, $p < 0.0001$; P9 v.s. P12, $p < 0.0001$; Fig. 4B). Interestingly, the pF of single-note calls (85.1–87.6 KHz) was always higher than that of multiple-note calls (67.9–80.6 KHz), and the pA of single-note calls (-63.3–-57.8 dB) was smaller than that of multiple-note calls (-56.8–-47.7 dB) at each day (Mann Whitney test, $p < 0.0001$; Fig. 4A,B). These data reveal a notable developmental pattern of USVs of mouse pups during isolation, that is, mouse pups tend to switch the vocalization mode dominated by lower pitch but louder multiple-note calls into the mode dominated by higher pitch but lighter single-note calls.

By analyzing the pF and pA of different categories of USVs in *Dock4* KO pups, we found several alterations. First, the pF of both single- and multiple-note calls was lower in KO pups than in WT littermates at P3 (Mann Whitney test; single-note calls: $p = 0.0230$; multiple-note calls: $p < 0.0001$; Fig. 4C), but the pF of multiple-note calls became significantly higher in KO pups than in WT littermates at later developmental days (Mann Whitney test; P6, P9, and P12: $p < 0.0001$; Fig. 4C). Second, the pA of both single-note and multiple-note calls emitted by KO pups did not show obvious reduction trend as observed in WT littermates, and the pA of both call types was larger in KO pups than in WT littermates at P12 (single-note calls: Mann Whitney test, $p < 0.0001$; multiple-note calls: unpaired *t* test, $p < 0.0001$; Fig. 4D). Therefore, the USVs emitted by *Dock4* KO pups are generally louder than those of WT littermates at later developmental stages. Moreover, the multiple-note calls are not only more frequently used, but also have significantly higher pitch in KO pups than WT littermates at later developmental stages.

3.4. Acoustic characteristics of individual call types in WT and Dock4 KO pups

To further gain detailed picture of USV alterations during development and between WT and *Dock4* KO pups, we examined the pF, pA, and duration of individual call types. Re-chevron was omitted as its proportion was less than 1% in either genotype at any developmental day (Fig. 3). By measuring the pF of each call types in WT and KO at different developmental days, we observed two common changes in KO pups. First, the pF of two single-note calls (Upward, Chevron) and Two-components was lower at early developmental days (P3 and/or P6) in KO pups (statistical information in Table S3; Fig. 5D, E, H). Second, four multiple-note calls (Composite, Two-components, Frequency-steps, and Harmonics) have commonly higher pF at later developmental days (P9 and/or P12) in KO pups (statistical information in Table S3; Fig. 5G–J). These data are consistent with the findings that single-note calls have generally lower pitch at early developmental stages, whereas multiple-note calls have generally higher pitch at later developmental stages in KO pups than WT pups (Fig. 4A).

For the call amplitude, we observed a common reduction trend of pA in all call types along development in WT pups (P3 v.s. P12, Kruskal Wallis test with Dunn's multiple comparisons post hoc test for Short (p =

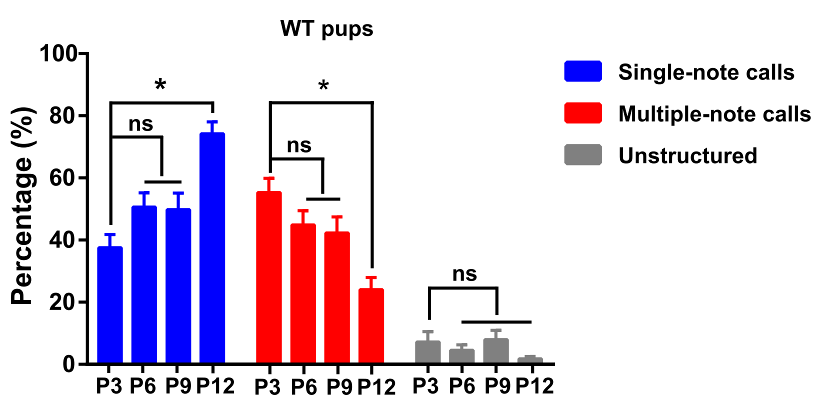

Fig. 2. Percentages of single-note, multiple-note, and Unstructured calls of WT during developmental stages.
Percentage of single-note, multiple-note, and Unstructured calls of WT pups were counted at P3, P6, P9, and P12. All USVs were collected from 13 pups at P3, P6, and P9, and from 10 pups at P12. The number of calls in each category is shown in Table S1. *$p < 0.05$; Linear Mixed Models Select Subjects/Repeated Variables.

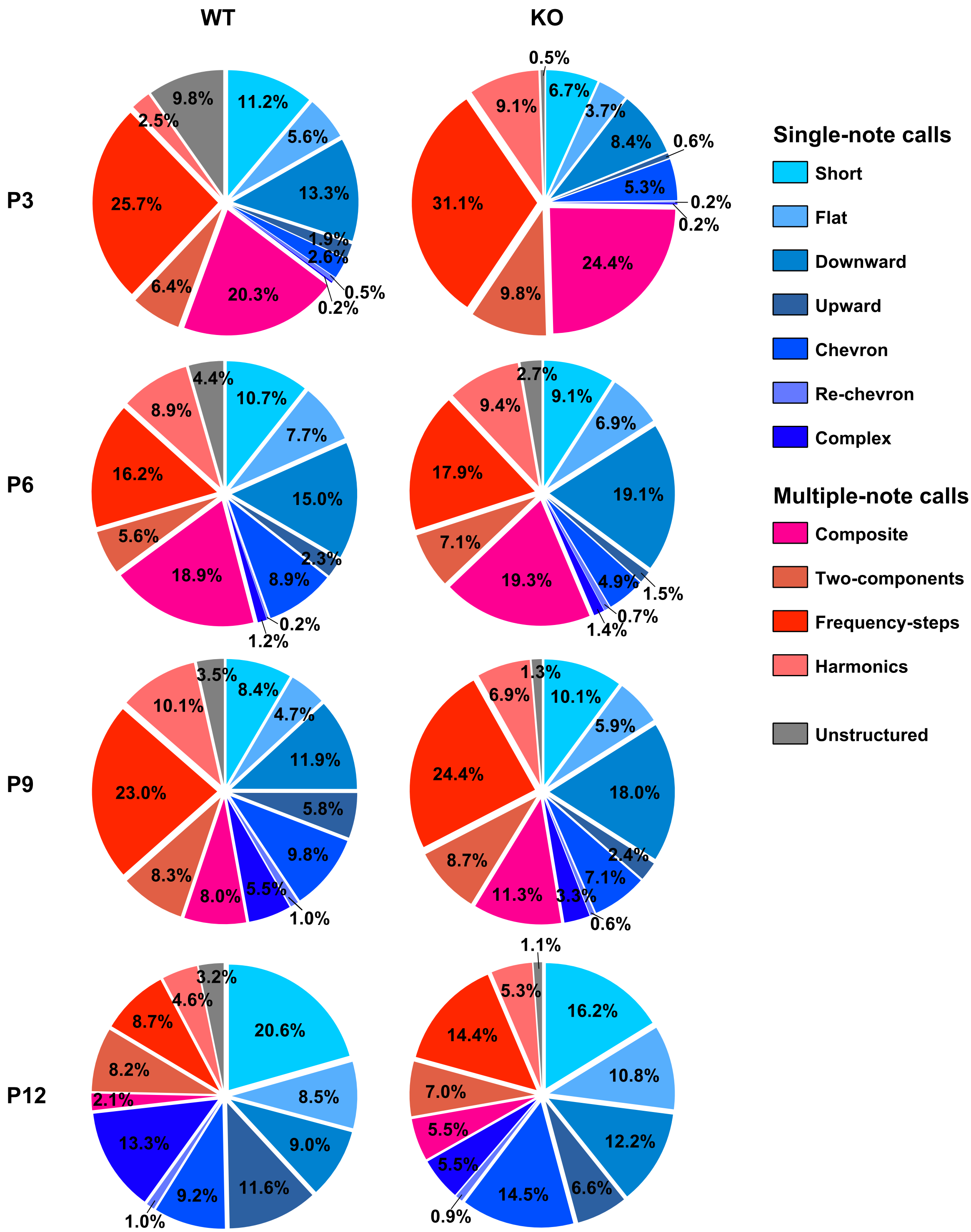

Fig. 3. Proportions of individual USV categories from WT and *Dock4* KO pups during different developmental stages.
Percentage of each call type in WT and *Dock4* KO pups at P3, P6, P9, and P12 are shown in Pie charts. The data were obtained from the total USVs pooled from 10 to 13 pups each genotype at each age. The total USV numbers recorded from WT pups are 1433 (P3, 13 pups), 5434 (P6, 13 pups), 4220 (P9, 13 pups), 1729 (P12, 10 pups); and from KO are: 2409 (P3, 13 pups), 5951 (P6, 13 pups), 4608 (P9, 13 pups), 2571 (P12, 11 pups). The number of calls in each type is shown in Table S1.

0.0011), Flat ($p = 0.2336$), Down ($p = 0.0100$), Up ($p < 0.0001$), Complex ($p = 0.0507$), Composite ($p = 0.5458$), and Frequency steps ($p < 0.0001$); One-way ANOVA with Holm-Sidak's multiple comparisons test for Chevron ($p < 0.0001$), Two-component ($p < 0.0001$), and Harmonics ($p < 0.0001$); Fig. 6), consistent with the finding that USVs emitted by WT pups are gradually lighter along development (Fig. 4B). Moreover, highly consistent alterations were observed in all call types except Composite in KO pups, that is, the pA of each call type was larger in KO pups than their WT littermates since P6 or P9, and became more evident at P12 (statistical information in Table S3; Fig. 6A–J). This result is consistent with the findings that both single- and multiple-note calls emitted by KO pups are louder than WT pups at later developmental stages (Fig. 4B).

We finally examined the duration of individual call types

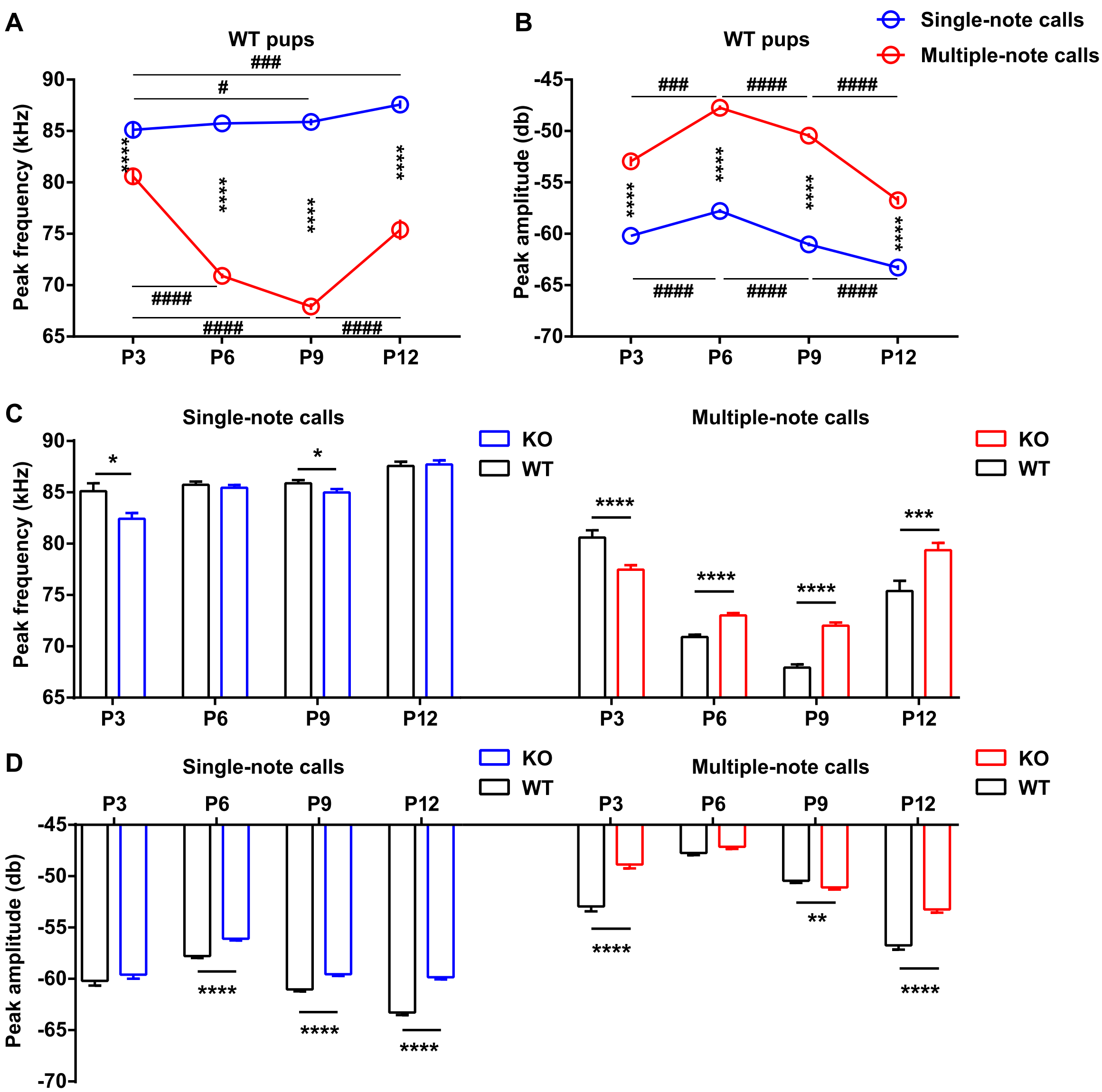

Fig. 4. Developmental patterns of acoustic properties of single-note and multiple-note calls in WT pups and the alterations in *Dock4* KO pups. (A–B) The developmental patterns of peak frequency (A) and peak amplitude (B) were analyzed in WT pups. #p < 0.05, ###p < 0.001, ####p < 0.0001, Kruskal Wallis test with Dunn's multiple comparisons test was used for comparison among developmental days; ****p < 0.0001, Mann Whitney test was used for comparisons between each pair of single-note calls and multiple-note calls at each day. (C–D) The peak frequency (C) and peak amplitude (D) of single-note calls and multiple-note calls were analyzed in WT and *Dock4* KO pups. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, Mann Whitney test for all comparisons except peak amplitude of multiple-note calls at P12, which was compared using unpaired *t* test. The numbers of single-note calls and multiple-note calls, respectively, in WT pups are 506 and 787 (P3, 13 pups), 2497 and 2696 (P6, 13 pups), 1991 and 2083 (P9, 13 pups), 1266 and 408 (P12, 10 pups); the numbers of single-note calls and multiple-note calls, respectively, in KO pups are 607 and 1790 (P3, 13 pups), 2595 and 3196 (P6, 13 pups), 2184 and 2364 (P9, 13 pups), 1717 and 826 (P12, 11 pups) for KO pups. Data are presented as mean ± standard error of the mean (SEM).

(Fig. S3A–J). However, we found that the changes of the duration were rather heterogeneous in different call types in KO pups. For instance, the durations of Short, Flat, Upward, Chevron, Composite, Frequency-steps, and Harmonics were longer in KO pups than in WT littermates, whereas those of Downward were shorter, but the alterations occurred at different developmental days without a common pattern (statistical information in Table S3; Fig. S3). To sum up, the examinations of basic acoustic features of individual call types reveal detailed alterations of each call types at each developmental day in *Dock4* KO pups, and notable common changes occur at the later developmental stages, when all call types emitted by KO pups have a generally louder characteristic, and most of the multiple-note call types by KO pups have higher pitch when comparing to WT pups.

4. Discussion

In the present study, we used a set of refined classification criteria to categorize the isolation-induced USVs of mouse pups into two main types, single-note calls and multiple-note calls, and calls in each group were further subcategorized into seven and four types, respectively. By examining the proportions and acoustic characteristics of each call type emitted by WT and *Dock4* KO mice in the first two weeks of postnatal development, we obtained some findings on the developmental pattern of USVs, and revealed the alterations of USVs in *Dock4* KO pups. First, the intrinsic acoustic features of single- and multiple-note calls are distinct, that is, single-note calls have higher pitch and lower volume than multiple-note calls. Second, multi-note calls are more used in the early developmental stages by WT mouse pups, whereas single-note calls

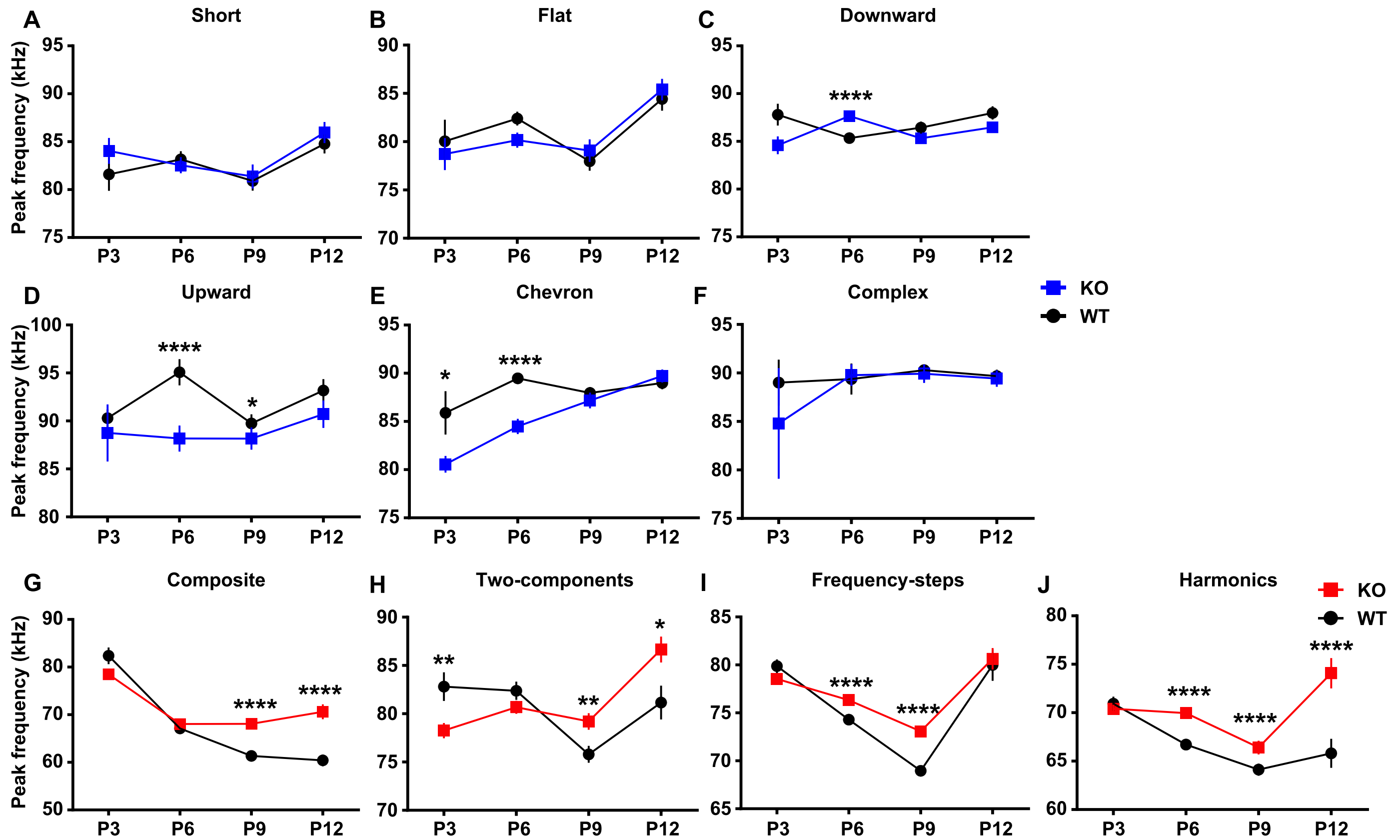

Fig. 5. Peak frequency of different call types between WT and *Dock4* KO pups.
(A–J) Peak frequency of Short (A), Flat (B), Downward (C), Upward (D), Chevron (E), Complex (F), Composite (G), Two-components (H), Frequency-steps (I), and Harmonics (J) between WT and *Dock4* KO pups was analyzed at P3, P6, P9, and P12. The number of calls in each type is shown in Table S1. Data are presented as mean ± standard error of the mean (SEM), *p < 0.05, **p < 0.01, ****p < 0.0001; Mann Whitney test for all comparisons except peak frequency of Complex at P3, which was compared using unpaired *t* test.

are more used in later development stages, and the volume of all calls are gradually lowered along development (Table 2). Third, comparing with WT pups, USVs emitted by *Dock4* KO pups have larger volume and consist of more multiple-note calls with higher pitch at later developmental stages (Table 2).

The classification of single- and multiple-note calls is based on the number of notes in the call. In some literature, single-note calls are also known as simple calls or simple syllables, and multiple-note calls are also known as complex calls or complex syllables [30]. Our findings provide evidence on the validity of this classification by showing the distinct characteristics of each category in addition to the mere structural difference in the sonogram. In terms of sound features, the single-note calls have generally higher pF and smaller pA than the multiple-note calls in each recording day, meaning that single-note calls have higher pitch and lower volume. By examining the change of proportions and acoustic parameters in different developmental stages, we identified that multiple-note calls are mostly used in early development communications, whereas single-note calls dominate at later development. Moreover, the pitch of single-note calls remains relatively the same along development, whereas multiple-note calls have lowered pitch at later development; the volume of both categories is gradually decreased along development. These findings together reveal that each category exhibits uniqueness on intrinsic acoustic properties as well as developmental profile on proportions of usage in communication and sound characteristics.

The switch of vocalization mode from multiple- to single-note calls in mouse pups along development is of great interest for studying the language development in mouse. Similar observation was reported in another study using C57BL/6 N mouse pups [31]. More evidence on call type proportion analysis has been provided using adult mice, in which studies report dominance of single-note calls during different social contexts [8,17,27,32,33]. These observations point to an interesting possibility that usage of single-note calls for communication may indicate the maturation of language development. In particular, the current study showed that the top increased single-note call types during development include Short, Upward, Chevron, and Complex, which took more than half of the USVs at P12. These four types of single-note calls are also highly used at adult [31,32,34]. Therefore, conveying effective communication may depend on the use of these particular types of single-note calls. Our study also adds other parameters to the measurement of the developmental changes of USVs. For instance, the volume of all calls is generally decreased along development. These characteristics may be together used as indicators for mouse language development.

USV is a classical behavior for the evaluation of communication ability in mouse models with mutation or deletion of ASD risk genes. However, most of these studies simply measure the parameters of the total calls, lacking detailed description of the alterations in the call repertoire over development. For example, it has been reported that the isolation-induced USVs in pups either increased in ASD mouse models such as BTBR T+tf/J mice [8], *Nf1*$^{+/-}$ mice [18], *Mecp2* KO mice [35], *Chd8*$^{+/N2373K}$ mice [36], Kirrel3 KO mice [37] *POGZ*$^{WT/Q1038R}$ mice [38], *patDp/+* mice [39], or decreased in ASD mouse models such as *Shank2* female KO mice [24,40] *Shank1* KO mice [9], 16p11.2 KO mice [41], and NEXMIF/KIDLIA KO mice [30]. Similarly, we previously reported that the overall USV number emitted by *Dock4* KO pups is larger than WT pups, and the overall peak frequency is similar between the two genotypes [22]. By comparing USVs emitted by *Dock4* KO pups with

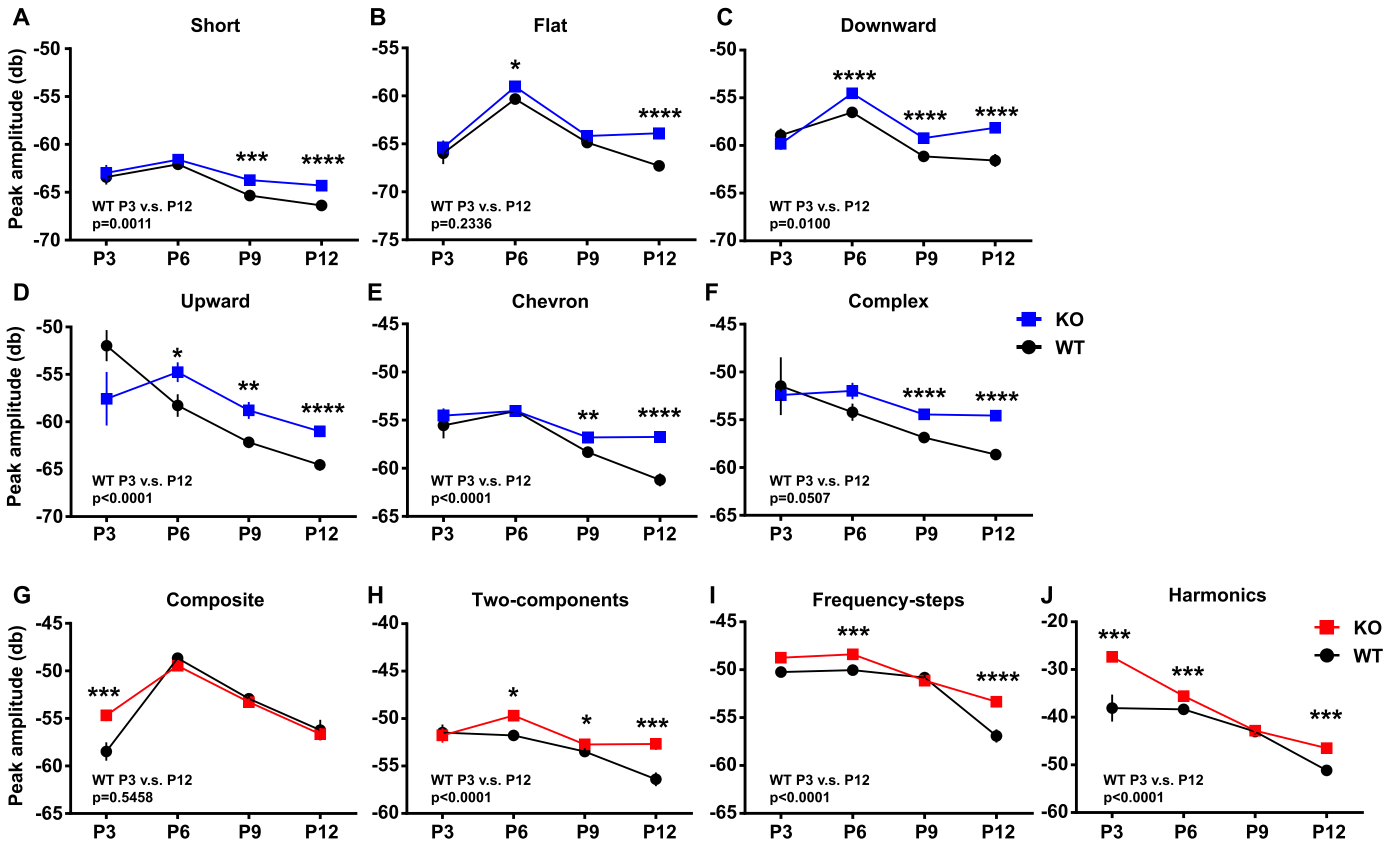

Fig. 6. Peak amplitude of different call types between WT and *Dock4* KO pups.
(A–J) Peak amplitude of Short (A), Flat (B), Downward (C), Upward (D), Chevron (E), Complex (F), Composite (G), Two-components (H), Frequency-steps (I), and Harmonics (J) between WT and *Dock4* KO pups was analyzed at P3, P6, P9, and P12. The number of calls in each type is shown in Table S1. Data are presented as mean ± standard error of the mean (SEM), *p < 0.05, **p < 0.01, ***p < 0.001,****p < 0.0001; Mann Whitney test for peak amplitude of Short, Flat at P12, Downward at P6, Upward and Complex at P9, Composite at P3, Two-components at P9, Frequency steps at P3, P6 and P9, and Harmonics between WT and *Dock4* KO pups; unpaired *t* test for all the other comparisons between WT and *Dock4* KO pups. Kruskal Wallis test with Dunn's multiple comparisons test for peak amplitude of Short, Flat, Down, Up, Complex, Composite, and Frequency steps between P3 and P12 in WT pups; One-way ANOVA with Holm-Sidak's multiple comparisons test for Chevron, Two-component, and Harmonics between P3 and P12 in WT pups. The P value of each comparison was indicated in the bottom left of the corresponding panel.

Table 2
Summary of USV characteristics in WT and KO mice.

Genotype		WT	*Dock 4* KO v.s. WT
Developmental stages		P3 → P12	P12
	Proportion	Increased	Lower
Single-note calls	Peak Frequency	Slightly increased	Unchanged
	Peak Amplitude	Decreased	Larger
	Proportion	Decreased	Higher
Multiple-note calls	Peak Frequency	Decreased	Higher
	Peak Amplitude	Decreased	Larger

those by WT pups using the comprehensive analyzing system presented in this study, we identified a number of notable alterations in the KO pups. First, more multiple-note calls are used by KO pups than WT pups in both early and late development. Second, the volume of all calls is larger, and the pitch of the multiple-note calls is higher in KO pups than in WT pups in the later developmental stages. Moreover, additional alterations at acoustic features were also observed by looking into details of individual call types. Some of these alterations, such as the more proportions of multi-note calls, are also observed in other ASD mouse models [8,42]. Therefore, we uncover a set of quantitative and qualitative deficits of language development in *Dock4* KO pups, and support that *Dock4* KO mice can be studied as an ASD model for social communication. This study also provides an example showing that whereas simple examination of whole calls is not sufficient, comprehensive analyses on different types of the calls are required to provide thorough information on language deficits in mouse models of ASD and other language-related disorders.

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CRediT authorship contribution statement

Xiaoman Yang: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing, Visualization. **Daji Guo:** Formal analysis, Investigation, Resources, Writing - original draft, Writing - review & editing, Visualization, Funding acquisition. **Keshen Li:** Formal analysis, Writing - review & editing. **Lei Shi:** Conceptualization, Resources, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

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Appendix A. Supplementary data

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