Deficiency of the paternally inherited gene *Magel2* alters the development of separation-induced vocalization in mice

Authors:

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Abstract

Offspring behavior results from the combined expression of maternal and paternal genes. Genomic imprinting, however, silences some genes in a parent-of-origin specific manner, a process that, among all animals, occurs only in mammals. How genomic imprinting affects the behavior of mammalian offspring remains poorly understood. Here we studied the effects of the loss of the paternally inherited gene *Magel2* on the emission of separation-induced ultrasonic vocalization (USV) by mouse pups. Using quantitative analysis of more than one hundred thousand USVs, we characterized the rate of vocalizations as well as their spectral features from postnatal days 6 to 12 (P6-P12), a critical phase during mouse development when pups fully depend on the mother for survival. Our analyses show that *Magel2* deficient offspring emit separation-induced vocalizations at lower rates and with altered spectral features. Using methods for a holistic analysis of the entire vocal repertoire of pups, we found that *Magel2* deficient mice at postnatal day 8 (P8) emit USVs that resemble the vocal repertoire of wildtype mice at older ages (P10-12). These results suggest that the deficiency of this paternally inherited gene impairs the expression of separation-induced vocalization in pups, a behavior that supports the pups' growth and development.

Keywords

Genomic imprinting; offspring-parent conflict; Prader Willi Syndrome; Autism spectrum disorders; behavior development.

Introduction

For the normal development of mammals, offspring need copies from both maternal and paternal genomes. Some genes, however, are expressed in a parent-of-origin specific manner. In other words, some genes are always expressed when inherited from the mother and some genes are always expressed when inherited from the father [1–3]. The process that regulates the expression of genes in a parent-of-origin specific manner is called genomic imprinting [2,3].

Genomic imprinting depends on epigenetic modifications of the genome. These modifications do not alter the sequence of the DNA but the chemical structure of the DNA, thereby leading to altered gene expression [4]. An imprinted gene can be silenced in the maternal genome and, therefore, only the paternal allele will be expressed in the offspring. Consider, for example, a series of imprinted genes in human chromosome 15, which illustrates the importance of paternally inherited genes for normal mammalian development [5–9]. The deletion of the paternally inherited genes in chromosome 15 leads to neurodevelopmental disorders, such as Prader-Willi syndrome (PWS). PWS presents with hypotonia and poor feeding early in life, followed by hyperphagia, alteration in social behavior, and cognitive deficits. It should be noted, however, that because PWS involves several genes, it masks the relative contribution of single imprinted genes on the phenotype of the offspring.

Among the PWS-related genes, MAGEL2 is a candidate gene for some of the clinical features of PWS. Humans with loss-of-function mutations in MAGEL2 present clinical aspects of PWS and autism spectrum disorders [10–12], suggesting that this single paternally inherited gene supports at least some of the developmental alterations found in PWS. In agreement with the clinical features of MAGEL2 deficiency in humans, *Magel2* deficient mice show impairments in growth and adult social behaviors which are, however, of small-effect size [10,13,14].

In this study, we investigated whether the loss of paternally inherited *Magel2* in mice alters behaviors that are ecologically relevant for the development of the offspring. We chose to study the vocal behavior of mouse pups when separated from their dams [15–23], as separation-induced vocalizations signal the needs of the pups to the dams [19,24–29]. In contrast to human babies, mouse pups vocalize in the ultrasonic frequency range (45 - 120 kHz) [20,21,23,30], which humans cannot hear. In order to survey the vocal behavior of mice, we recorded the emission of ultrasonic vocalizations (USVs) when pups were separated from the home nest. We performed these studies at postnatal days 6, 8, 10, and 12, since it is during this phase of mouse development that the peak expression of separation-induced vocalizations typically occurs [15,31,32]. We then used VocalMat [33], a software developed by our group, to perform quantitative analysis of mouse vocal behavior. Our analysis shows that the deficiency of *Magel2* in mice impairs the expression of separation-induced vocalizations, a behavior that supports pups' growth and development.

Results

Early waning of vocal behavior in *Magel2*$^{m+/p-}$ deficient pups

To investigate the effects of paternally inherited *Magel2* on the vocal behavior of infant mice, we crossed heterozygote males for *Magel2* deficiency with wildtype females. From this cross, we generated *Magel2* deficient offspring (*Magel2*$^{m+/p-}$) that carry the null allele from the father (p−) and the imprinted allele from the mother (m+). This cross also generates wildtype littermates (*Magel2*$^{m+/p+}$), used as experimental controls. As previously reported[10], *Magel2*$^{m+/p-}$pups display lower body weight compared to controls (genotype: $F_{1, 182} = 19.91$, $P < 10^{-4}$; age: $F_{3, 182} = 70.09$, $P < 10^{-9}$; genotype x age: $F_{3, 182} = 0.02$, $P = 0.996$; two-way ANOVA).

We recorded the emission of USVs during 20 minutes of separation from the home nest at different postnatal ages (P6, P8, P10 and P12; **Figure 1A**). First, we analyzed the total number of USVs emitted during the period of separation using two-way ANOVA. We found a significant effect of genotype, age, and interaction between genotype and age (genotype: $F_{1, 181} = 20.61$, $P < 10^{-4}$; age: $F_{3, 181} = 11.80$, $P < 10^{-6}$; genotype x age: $F_{3, 181} = 3.90$, $P = 0.009$; **Figure 1B**). Post-hoc analysis (Sidak's multiple comparisons test; **Figure 1B**) shows that the total number of USVs is similar among groups at P6 (control: 991 ± 139 USVs, n = 16; *Magel2*$^{m+/p-}$: 787 ± 130 USVs, n = 20; $P = 0.75$), P10 (control: 503 ± 45 USVs, n = 23; *Magel2*$^{m+/p-}$: 466 ± 70 USVs, n = 20; $P = 0.98$), and P12 (control: 594 ± 71 USVs, n = 30; *Magel2*$^{m+/p-}$: 345 ± 46 USVs, n = 28; $P = 0.06$). Compared to controls, however, *Magel2*$^{m+/p-}$ pups show a ≈53% reduction in the emission of USVs at P8 (control: 1045 ± 100 USVs, n = 24; *Magel2*$^{m+/p-}$: 495 ± 65 USVs, n = 28; $P < 10^{-4}$). It is worth noting that P8 *Magel2*$^{m+/p-}$ pups emit a similar number of USVs compared to P10 and P12 control pups, suggesting an early decline in vocal behavior. We also performed these analyses in females and males separately and found similar effects of genotype and age (**Figure 1C-D** and **Supplementary Table 1**). These results show age-specific reductions in the

106 emission of USVs in *Magel2*$^{m+/p-}$ mice, suggesting a non-sex specific effect for paternally
107 inherited *Magel2* on the vocal behavior of the offspring.
108
109 The emission of USVs occurs when the breathing musculature contracts, expelling air from the
110 lungs and propelling it through the larynx [29,34]. Since previous reports found that *Magel2*$^{m+/p-}$
111 mice display hypotonia [35], we considered the hypothesis that the low rate of USV emission in
112 *Magel2* deficient pups is due to a lower capacity to expel air from the lungs. To rule out this
113 hypothesis, we measured the intensity (or volume, in decibels) of the USVs. Since the intensity
114 of the USVs relates to the pressure by which the air is expelled through the larynx [29,34], a lower
115 intensity is expected in cases of hypotonia. This analysis shows that the intensity of the emitted
116 USVs between *Magel2*$^{m+/p-}$ mice and controls is similar in all ages tested (genotype: $F_{1, 175}$ =
117 0.82, P = 0.63; age: $F_{3, 175}$ = 2.64, P = 0.05; genotype x age: $F_{3, 175}$ = 0.68, P = 0.56; two-way
118 ANOVA; **Figure 1E**). Thus, hypotonia does not seem to be a factor of primary significance for
119 the lower rate of USV emission in *Magel2*$^{m+/p-}$ mice [34].
120
121 ***Magel2*$^{m+/p-}$ mice emit vocalizations with distinct spectral features**
122 In addition to the rate of separation-induced vocalizations, the spectral features of the USVs
123 also correlate with altered maternal care [36]. To test the extent to which *Magel2* deficiency affects
124 the spectral features of USVs across ages, we used two-way ANOVA to analyze the frequency
125 characteristics (pitch) and duration (**Figure 2A**) of USVs [33]. We found significant effects of
126 genotype and age for maximal frequency (genotype: $F_{1, 182}$ = 20.38, $P < 10^{-4}$; age: $F_{3, 182}$ = 2.79,
127 P = 0.04; genotype x age: $F_{3, 182}$ = 6.45, P = 0.0004; **Figure 2B**) and bandwidth (genotype: $F_{1, 182}$
128 = 9.79 P = 0.002; age: $F_{3, 182}$ = 6.94, P = 0.0002; genotype x age: $F_{3, 182}$ = 5.88, P = 0.0007;
129 **Figure 2E**). When comparing *Magel2*$^{m+/p-}$ to control mice, post-hoc analysis (Sidak's multiple
130 comparisons test) shows that at P8—but not at P6, P10, or P12—there is a 3% reduction in
131 maximal frequency (control: 88.4 ± 1.5 kHz, n = 24; *Magel2*$^{m+/p-}$: 85.6 ± 1.1 kHz, n = 28; $P < 10^{-}$

^{4}; **Figure 2B**) and a 40% reduction in bandwidth (control: 17.3 ± 1.2 kHz, n = 24; *Magel2*$^{m+/p-}$: 10.4 ± 0.7 kHz, n = 28; $P < 10^{-5}$; **Figure 2E**). Moreover, we found a significant effect of genotype for mean frequency (genotype: $F_{1, 181} = 4.86$, $P = 0.02$; age: $F_{3, 181} = 0.24$, $P = 0.87$; genotype x age: $F_{3, 181} = 0.41$, $P = 0.74$; **Figure 2D**), but not for minimal frequency (genotype: $F_{1, 182} = 0.54$, $P = 0.46$; age: $F_{3, 182} = 1.97$, $P = 0.12$; genotype x age: $F_{3, 182} = 0.47$, $P = 0.83$ **Figure 2C**) or duration (genotype: $F_{1, 175} = 3.54$, $P = 0.06$; age: $F_{3, 175} = 5.78$, $P = 0.0009$; genotype x age: $F_{3, 175} = 1.98$, $P = 0.11$; **Figure 2F**). In addition to the main frequency component, USVs can contain harmonics (**Figure 2G**). We calculated the percentage of USVs with harmonics and found a significantly lower number in *Magel2*$^{m+/p-}$ mice compared to controls at P8 (control: 9.1 ± 1.6 % , n = 24; *Magel2*$^{m+/p-}$: 3.4 ± 0.8 %, n = 28; $U = 146$, $P_{2\text{-tailed}} = 0.0003$, Mann-Whitney test; **Figure 2H**) but not at P6 (control: 2.0 ± 0.2 % , n = 16; *Magel2*$^{m+/p-}$: 2.1 ± 0.2 %, n = 20; $U = 138$, $P_{2\text{-tailed}} = 0.49$, Mann-Whitney test; **Figure 2H**), P10 (control: 5.7 ± 0.8 % , n = 23; *Magel2*$^{m+/p-}$: 6.1 ± 1.2 %, n = 20; $U = 240$, $P_{2\text{-tailed}} = 0.98$, Mann-Whitney test; **Figure 2H**), or P12 (control: 3.6 ± 0.6 % , n = 30; *Magel2*$^{m+/p-}$: 4.9 ± 0.1 %, n = 28; $U = 408.5$, $P_{2\text{-tailed}} = 0.86$, Mann-Whitney test; **Figure 2H**). In sum, these results suggest that the loss of paternally inherited *Magel2* in mice causes discrete changes in the features of separation-induced vocalizations that are most evident at postnatal day eight.

Discrete changes in the use of syllable types by *Magel2*$^{m+/p-}$ mice

Mouse pups emit USVs of distinct classes—i.e., syllable types. Thus, the emission of different syllable types could explain the discrete changes in the spectro-temporal features of USVs in *Magel2*$^{m+/p-}$ (**Figure 3A**) [33,37]. We used machine learning to automatically categorize each USV into one of eleven syllable types based on the morphology of the main component of the vocalization. The output of the method was the probability for each USV to be of a certain syllable type. The highest probability (P_1) defined the syllable type for a given USV (**Figure 3B**). Using this approach, we did not find any significant differences in the distribution of syllable

types emitted by control and *Magel2*$^{m+/p-}$ mice at P6, P10, and P12 (**Figure 3C, 3E, 3F**). At P8, however, *Magel2*$^{m+/p-}$ pups emit 54% less USVs of the type *chevron* (control: 8.7 ± 1.6%; n = 24; *Magel2*$^{m+/p-}$: 4.0 ± 0.9 %; n = 28; $P = 0.008$), 71% less USVs of the type *step-down* (control: 0.7 ± 0.1 %; n = 24; *Magel2*$^{m+/p-}$: 0.2 ± 0.05 %; n = 28; $P < 10^{-4}$), 44% less USVs of the type *step-up* (control: 14.5 ± 1.4 %; n = 24; *Magel2*$^{m+/p-}$: 8.1 ± 1.3 %; n = 28; $P = 0.001$), and 87% less USVs of the type *two-steps* (control: 3.6 ± 1.1 %; n = 24; *Magel2*$^{m+/p-}$: 0.5 ± 0.2 %; n = 28; $P = 0.005$) compared to controls (**Figure 3D**). Conversely, *Magel2*$^{m+/p-}$ pups emit 36% more USVs of the type *flat* (control: 9.7 ± 0.9 %; n = 24; *Magel2*$^{m+/p-}$: 15.2 ± 1.2 %; n = 28; $P = 0.007$) and 33% more USVs of the type *short* compared to controls (control: 23.8 ± 1.8 %; n = 24; *Magel2*$^{m+/p-}$: 35.5 ± 2.0 %; n = 28; $P < 10^{-4}$; **Figure 3D)**. (**Supplementary Table 2** provides a detailed analysis of the spectro-temporal features of each syllable type across all ages tested in controls and *Magel2*$^{m+/p-}$ pups.) In summary, we found that *Magel2*$^{m+/p-}$ mice at P8 use simpler vocalizations that fall under the 'flat' and 'short' classifications. These findings are in line with our previous results **(Figure 1-2)** demonstrating that the largest differences in vocal behavior occur in eight-day-old *Magel2*$^{m+/p-}$ pups.

Altered vocal repertoire of *Magel2*$^{m+/p-}$ mice

As stated above, the vocal analysis pipeline outputs the probability for each USV to be classified as each of the eleven syllable types (P_1, P_2, P_3, … $P_{11;}$ **Figure 3A-B**). This distribution of probabilities allows the qualitative and quantitative comparison of the vocal classification among groups [33]. By considering the distribution of probabilities to classify each USV, it is possible to estimate how similar the vocal repertoire of one group of mice is to another group. To compare the vocal repertoire of mice across all ages studied, we used diffusion maps—a dimensionality reduction technique that decreases the number of dimensions of the probability distribution from eleven classes to three dimensions in a Euclidean space (**Figure 4A**) [33]. Using pairwise comparisons (**Figure 4B**), we estimated the similarity between the vocal repertoire of mice of

different ages and genotypes. Using this method, we found that control pups at P6 and P8 (Cohen's coefficient: **κ** = 0.99) and control pups at P10 and P12 (Cohen's coefficient: **κ** = 0.95) display vocal repertoires that are similar to each other (**Figure 4**). These two age groups (P6-P8 and P10-P12), however, present lower pairwise similarities when compared to each other with **κ** ranging from 0.67 to 0.77 (**Figure 4**). These results suggest that the vocal repertoire of control pups undergoes significant changes between P8 and P10.

Next, we analyzed the same transitions in the vocal repertoire of *Magel2*$^{m+/p-}$ pups. The comparison between the vocal repertoire of *Magel2*$^{m+/p-}$ pups at P6 and P8 show lower pairwise similarity (**κ** = 0.80) compared to control pups (**Figure 4**). In contrast to littermate controls, *Magel2*$^{m+/p-}$ pups at P8 show a higher pairwise similarity with P10 (**κ** = 0.84) and P12 (**κ** = 0.99) pups. These findings suggest that *Magel2*$^{m+/p-}$ pups switch their vocal repertoire at a younger age (P8) to a vocal repertoire that control pups emit at an older age (P10-P12).

Finally, we directly compared the vocal repertoire of *Magel2*$^{m+/p-}$ and control pups. *Magel2*$^{m+/p-}$ pups, at P6, show high pairwise similarity when compared to controls at P6 (**κ** = 1.00) and P8 (**κ** = 1.00), but not at P10 (**κ** = 0.80) and P12 (**κ** = 0.72). At P8, *Magel2*$^{m+/p-}$ pups show relatively low pairwise similarities with control pups at P6 (**κ** = 0.76) and P8 (**κ** = 0.78) but show high similarities with control pups at P10 (**κ** = 1.00) and P12 (**κ** = 0.95). At P10, *Magel2*$^{m+/p-}$ pups show relatively lower pairwise similarities with control pups at P6 (**κ** = 0.67) and P8 (**κ** = 0.70) than at P10 (**κ** = 0.82) and P12 (**κ** = 0.81). This pattern is more evident in P12 Magel2$^{m+/p-}$ pups, which show lower pairwise similarities with control pups at P6 (**κ** = 0.74) and P8 (**κ** = 0.75) than at P10 (**κ** = 0.99) and P12 (**κ** = 0.97). Altogether, these analyses suggest that the vocal repertoire of *Magel2*$^{m+/p-}$ pups transforms at a faster pace than control pups—with *Magel2*$^{m+/p-}$ pups at P8 resembling control pups at P10-P12. Thus, the critical phase of development

209 between P8 and P10 seems to mark an important period for the effect of the maternally

210 imprinted gene, *Magel2*, on the vocal behavior of the offspring.

Discussion

In this study, we recorded and analyzed vocalizations from *Magel2* deficient pups and their wildtype littermates during a 20-minute period at postnatal days 6, 8, 10 and 12. Using custom-built software to automatically analyze vocalizations [33], we counted the number of vocalizations and measured the spectro-temporal features of each vocalization, including its intensity, duration, bandwidth, mean frequency, maximum frequency, minimum frequency, and use of harmonic components. We also assigned a syllable type for each vocalization based on its morphological features in the time-frequency plane. We further used quantitative methods to analyze the vocal repertoire of mice across groups and ages. These methods shed light on discrete changes in the development of separation-induced vocalizations in *Magel2* deficient mice.

Using our software allowed us to expand the period of analysis to 20 minutes. By comparison, previous studies quantified vocal behavior during a much shorter period—typically between 1 to 5 minutes. The longer period of analysis enabled us to characterize more than a hundred thousand vocalizations, which provided an in-depth view of the vocal repertoire of pups. With regards to the vocal behavior of wildtype mice, our results demonstrated that the emission of separation-induced USVs gradually decreases from P6-P8 to P10-P12. This result agrees with previous studies, which show an inverse-U shape profile for separation-induced vocalizations in mice during the first two weeks of life [15,29,31,32,38]. Based on the large number of vocalizations we analyzed, we also found that wildtype mice use simpler vocalizations at older ages (P10-P12) compared to younger ages (P6-P8). These findings in wildtype mice provide the basis for comparisons with *Magel2* deficient pups.

Intriguingly, *Magel2* deficient pups show different dynamics for separation-induced vocalizations. At P6, these pups vocalize comparably to wildtype littermates, but at P8, their vocal number and features resemble wildtype littermates that are older (P10-P12). An explanation for these results is that *Magel2* deficient pups are less responsive to certain social cues. In socially isolated pups, therefore, the deprivation of these cues would not induce the behavior to the same degree as in wildtype pups. An alternative explanation for these results is that for *Magel2* deficient pups' vocal behavior does not have the same fitness value compared to wildtype pups. In the latter case, the behavior begins to change at younger ages due to the lack of reinforcement. While it is difficult to test these interpretations experimentally, the fact that *Magel2* deficient pups have lower body weight during early development suggests a decrease in the fitness of these animals and would support the idea that the development of vocal behavior in these mice is impaired. Whether the change in vocal behavior and the decrease in body weight are causally related warrants further investigation.

The discrepancies in expression of vocal behavior between wildtype and *Magel2* deficient pups support a more general role for imprinted genes on offspring behavior. Consider, for example, previous studies of other imprinted genes. Deletion of the paternally inherited imprinted gene *Peg3* lowers vocal rate in mouse pups [39]. Conversely, deletion of the maternally inherited gene *Gabrb3* increases vocal rate [40]. Thus, imprinted genes inherited from the father seem to increase vocal rate while imprinted genes inherited from the mother seem to decrease vocal rate. In evolutionary terms, these findings support the theory that genomic imprinting evolved to balance the cost of the phenotype for the offspring and for the mother, as well as to balance the best interests of mothers and fathers in altering offspring's phenotype. In the case of paternally inherited genes, therefore, the expression of these genes favors the use of maternal resources, which is in the best interests of the father, by increasing vocal behavior to increase maternal care. Conversely, the loss of these paternally inherited genes favors the conservation of

maternal resources, which is in the best interests of the mother, by decreasing vocal behavior and, consequently, the demand for maternal care [1,41]. Again, the lower body weight of *Magel2* pups supports this argument [10]. Future studies should test this theoretical view more directly by systematically investigating the effect of imprinted genes on the behavior of offspring and on the behavior of mothers towards their offspring.

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Author contributions

M.O.D and G.M.B.O. conceived the hypothesis, designed the study and wrote the manuscript. G.M.B.O. performed experiments. G.M.B.O. and G.M.S. analyzed and plotted the data. All authors read and edited the manuscript.

Methods

Experimental models and subject details

All preweaning mice used in the experiments were 6 to 12 days old from both sexes (see table below for total number of recording used under each age specifying sex and genotype). Litters were provided from 4 separate breeding pairs. Dams used were 2 to 6 months old. To generate experimental pups, we used the following cross: *Magel2*$^{m+/p-}$ (Jax #009062) dams bred with C57BL/6J (Jax #000664) males. Offspring from this cross were either *Magel2*$^{m+/p-}$ or wildtype (*Magel2*$^{m+/p+}$). All mice were kept in temperature- and humidity-controlled rooms, in a 12/12 hr. light/dark cycle, with lights on from 7:00 AM–7:00 PM. Studies took place during the light cycle. Food (Teklad 2018S, Envigo) and water were provided ad libitum. All procedures were approved by IACUC (Yale University).

AGE	***Control*** (F)	***Control*** (M)	***Magel2***$^{m+/p-}$ (F)	***Magel2***$^{m+/p-}$ (M)
P06	9	7	10	10
P08	15	9	15	13
P10	10	13	11	10
P12	15	15	16	12

Behavior test

Pups from the same litter were placed individually in a soundproof chamber containing fresh bedding material [36]. An UltraSoundGate Condenser Microphone CM 16 (Avisoft Bioacoustics, Berlin, Germany) was placed 10 cm above the recording chamber and connected to the UltraSoundGate 416 USGH device to record ultrasonic vocalizations. The recording sessions lasted 20 minutes. Four to eight chambers were recorded simultaneously. After testing, mice were placed back in their home cage with the dam. Pups were tested at postnatal days 6, 8, 10, and 12. Because pups that were naïve for the test—only tested at one specific age—show similar results as pups tested at multiple ages, we pooled all mice together for our analysis.

311 **Vocalization analysis**

312 Ultrasonic vocalizations were automatically extracted from audio recordings using a custom-built
313 tool [33]. In brief, audio recordings were converted from the time-domain to the frequency-domain
314 using a 1024-point Fast Fourier Transform (FFT) through a 512-width hamming window with
315 50% overlap. Spectrograms were computed from the FFT and processed as images. Each pixel
316 in the spectrogram corresponded to the intensity of each time-frequency component. Next, we
317 applied a series of image-processing techniques (e.g., contrast enhancement, binarization,
318 median filter, and morphological operations) to obtain segmentation of candidate vocalizations.
319 A single spectrogram was generated for each candidate vocalization detected. Candidate
320 vocalizations were classified as noise or real vocalization using a local median noise filter. The
321 remaining vocalization candidates are further labeled under one of eleven call type
322 classifications [37] using a Convolutional Neural Network (CNN), or as noise. The CNN was
323 trained using a curated vocalization dataset, containing over 20,000 noise samples and 40,000
324 vocalization samples. Finally, the tool produces one spectrogram centralized on each
325 vocalization for visual inspection, and a table (*xlsx* format) containing spectro-temporal features
326 for each USV, such as time, duration, bandwidth, frequency, and intensity (minimum, mean, and
327 maximum) values.
328
329 **Quantification and statistical analysis**
330 Prism 8.0 or above was used to analyze data and plot figures. Shapiro-Wilk normality test was
331 used to assess normal distribution of the data. Then, data were analyzed using two-way
332 ANOVA or mixed-effects analysis. Sidak's multiple comparisons test was used to find post hoc
333 differences among groups and to calculate the 95% confidence intervals to report effect size. To
334 analyze differences in the use of harmonics, we used the non-parametric Mann-Whitney Test
335 with Bonferroni correction to find statistically different effects. Statistical data are provided in text
336 and in the figures. In the text, values are provided as mean ± SEM. $P < 0.05$ was considered

337 statistically significant and, when necessary and as described above, was corrected using
338 Bonferroni's method.

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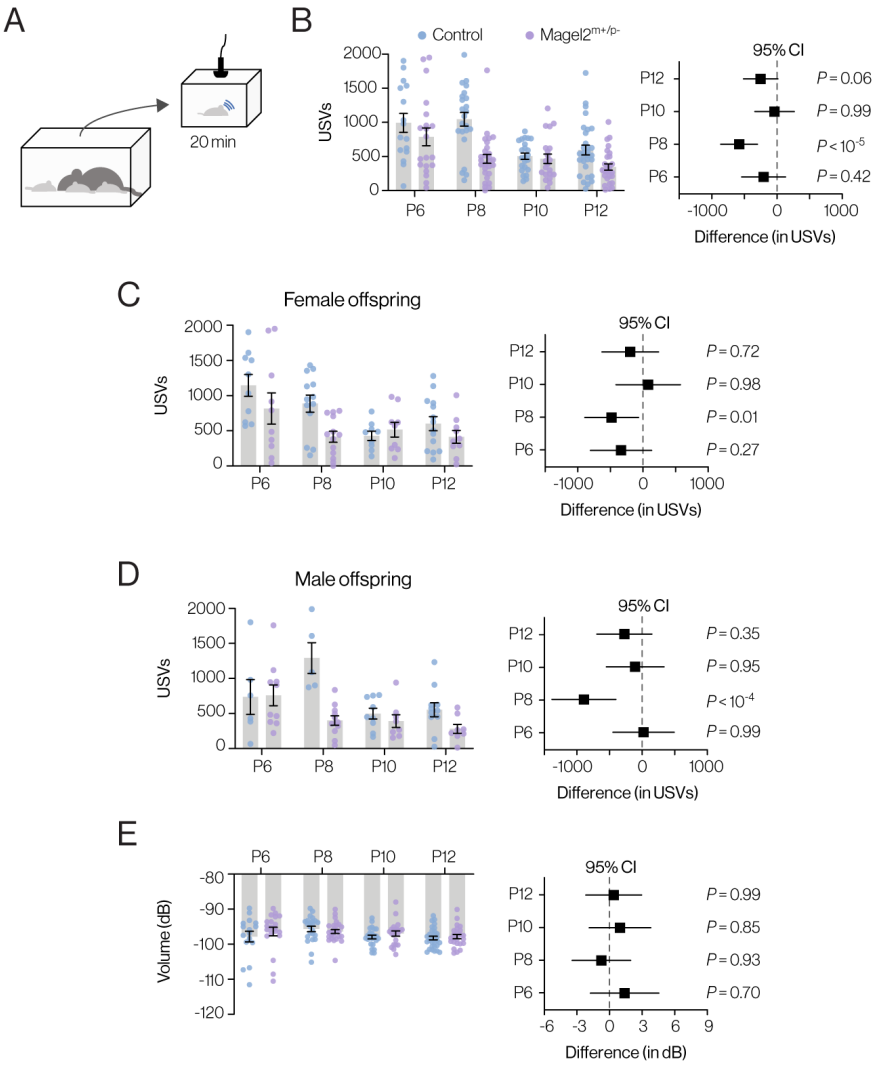

Figure 1. *Magel2* deficiency affects the emission of ultrasonic vocalizations in mice. (**A**) Schematic of the protocol used to record separation-induced USVs in mice (from P6 to P12); pups are separated from the home nest in a new chamber equipped with an ultrasonic microphone and recorded for 20 minutes. (**B**) Total number of USVs emitted by control (blue) and *Magel2* deficient (purple) littermates at P6, P8, P10, and P12; right panel denotes the 95% confidence intervals as a measure of effect size. (**C**) Similar to (B), but only considering female pups. (**D**) Similar to (B), but only considering male pups. (**E**) Average intensity of the USVs measured in decibels; right panel denotes the 95% confidence intervals as a measure of effect size. Bars represent mean value with error bars representing SEM and round symbols representing individual values. When plotting the effect sizes, squared symbols and black lines represent 95% confidence intervals calculated as the different between *Magel2* deficient and control pups. *P* values are provided in the figures as calculated using Sidak's multiple comparison test. The sample sizes for control and *Magel2* deficient pups are: P6, n = 16 and 20; P8, n = 24 and 28; P10, n = 23 and 20; and P12, n = 30 and 28, respectively.

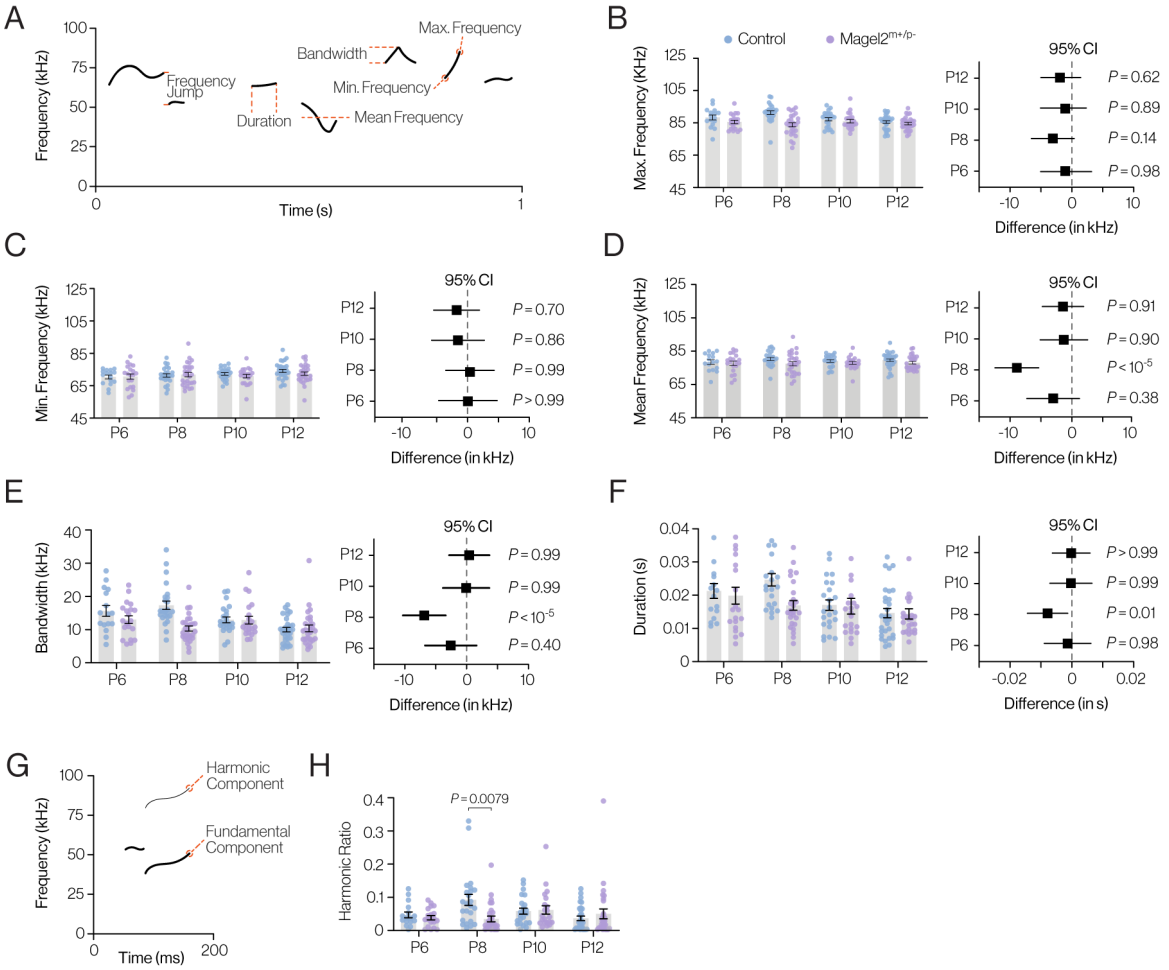

Figure 2. *Magel2* deficient pups emit ultrasonic vocalizations of distinct spectro-temporal features. (**A**) Illustration of a spectrogram with the spectro-temporal features measured for each USV. (**B**) Maximum frequency of the USVs emitted by control and *Magel2* deficient littermates at P6, P8, P10, and P12; right panel denotes the 95% confidence intervals as a measure of effect size. (**C**) Similar to (B) but plotting the minimum frequency of the USVs. (**D**) Similar to (B) but plotting the mean frequency of the USVs. (**E**) Similar to (B) but plotting the bandwidth of the USVs. (**F**) Similar to (B) but plotting the duration of the USVs. (**G**) Illustration of the spectrogram of a single USV with a harmonic component. (**H**) Ratio of harmonic across all USVs emitted by control and *Magel2* deficient littermates. Bars represent mean value with error bars representing SEM and round symbols representing individual values. When plotting the effect sizes, squared symbols and black lines represent 95% confidence intervals calculated as the different between *Magel2* deficient and control pups. In B-F, *P* values are provided in the figures as calculated using Sidak's multiple comparison test post hoc analysis from two-way ANOVA test. In H, *P* values are provided as calculated using Mann-Whitney test. The sample sizes for control and *Magel2* deficient pups are: P6, n = 16 and 20; P8, n = 24 and 28; P10, n = 23 and 20; and P12, n = 30 and 28, respectively.

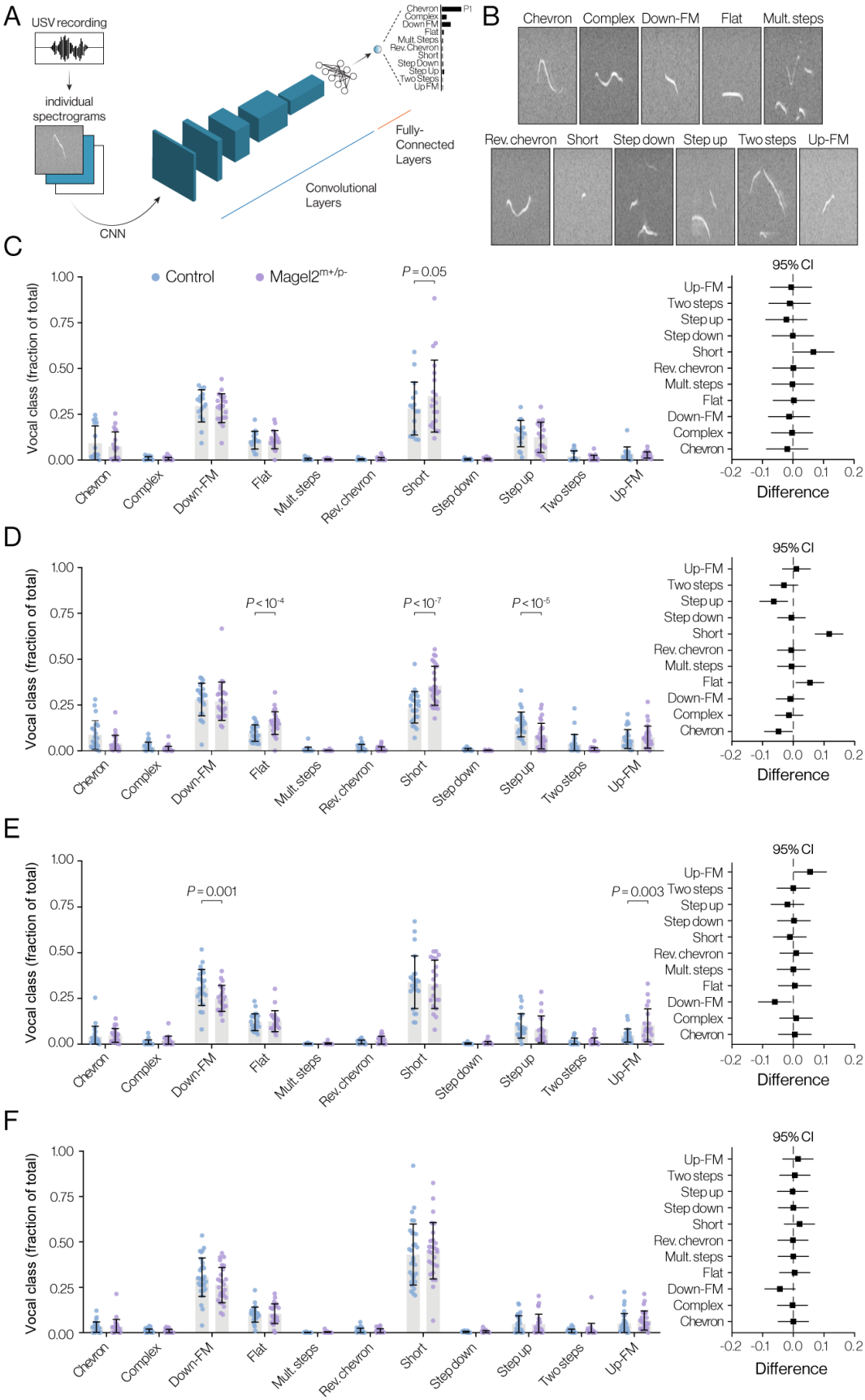

Figure 3. *Magel2* deficient pups emit ultrasonic vocalizations with discrete changes in the distribution of syllable types. (**A**) Illustration of the convolutional neural network used to classify each USV into one of eleven syllable types based on their morphology in spectrograms. (**B**) Spectrograms representing each of the eleven syllable types. (**C**) Distribution of syllable types in P6 pups—control in blue and *Magel2* deficient in purple. Data are showed as fraction of the total number of USVs; right panel denotes the 95% confidence intervals as a measure of effect size. (**D**) Similar to (C), but for P8 pups. (**E**) Similar to (C), but for P10 pups. (**F**) Similar to (C), but for P12 pups. Bars represent mean value with error bars representing SEM and round symbols representing individual values. When plotting the effect sizes, squared symbols and black lines represent 95% confidence intervals calculated as the different between *Magel2* deficient and control pups. *P* values are provided in the figures as calculated using Sidak's multiple comparison test as a post hoc analysis after two-way ANOVA. The sample sizes for control and *Magel2* deficient pups are: P6, n = 16 and 20; P8, n = 24 and 28; P10, n = 23 and 20; and P12, n = 30 and 28, respectively.

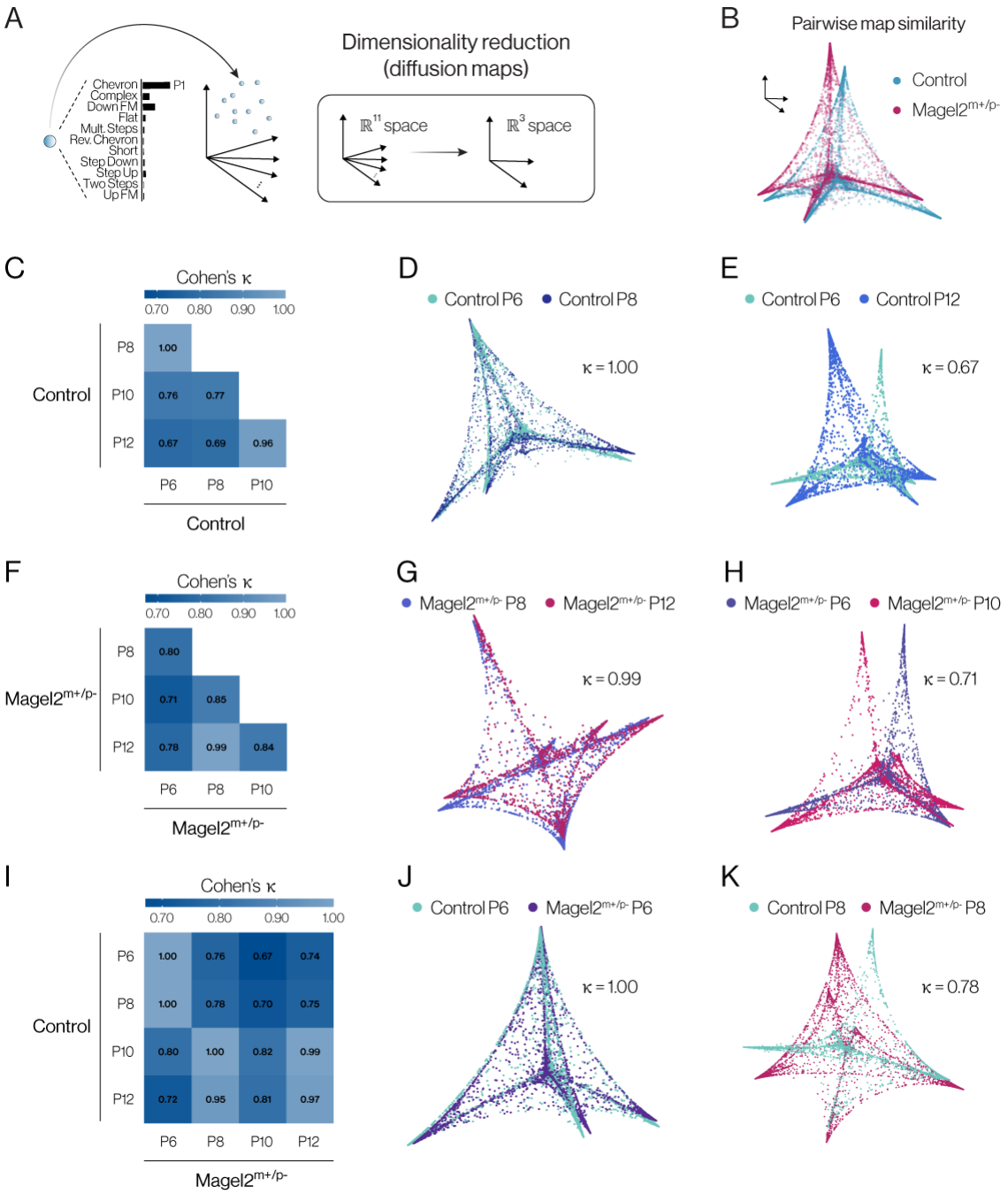

Figure 4. Analysis of the vocal repertoire of pups across ages. (**A**) Illustration of the output of the convolutional neural network, with a distribution of eleven probabilities for vocal classification (one probability for each of the eleven syllable types, with the highest probability defining the syllable type). Using diffusion maps, a dimensionality reduction technique, these eleven dimensions are reduced to three dimensions in the Euclidian space. (**B**) Illustration of a pairwise comparison of the vocal repertoire of pups using diffusion maps and 3D alignment of the manifolds (see methods for more details). (**D**) Comparison of the pairwise distance matrix between control pups at different ages using Cohen's Kappa coefficient. (**D-E**) Examples of two pairwise comparisons with high and low alignment. (**F-H**) Similar to (**D-E**), but for *Magel2* deficient pups. (**I-K**) Similar to (C-H), but comparing control and *Magel2* deficient pups across

ages. The sample sizes for control and *Magel2* deficient pups are: P6, n = 16 and 20; P8, n = 24 and 28; P10, n = 23 and 20; and P12, n = 30 and 28, respectively.