Article

Testosterone increases the emission of ultrasonic vocalizations with different acoustic characteristics in mice

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Abstract: Testosterone masculinizes male sexual behavior through an organizational effect during the perinatal period. We previously reported that the emission of ultrasonic vocalizations (USVs) in male mice was dependent on the organizational effects of testosterone; females treated with testosterone in the perinatal period had increased USV emissions compared to males. Recently, it was revealed that male USVs have various acoustic characteristics and these variations were related to behavioral interactions with other mice. In this regard, the detailed acoustic character changes induced by testosterone have not been fully elucidated. Here, we revealed that testosterone administered to female mice during the perinatal period modulated the acoustic characteristics of USVs. There was no clear difference in acoustic characters between males and females. Call frequencies were higher in TP-treated males and females compared to control males and females. When the calls were classified into nine types, there was also no distinctive difference between males and females, but TP increased the number of calls with a high frequency, and decreased the number of calls with a low frequency and short duration. The transition analysis by call type revealed that even though there was no statistically significant difference, TP-treated males and females had a similar pattern of transition to control males and females, respectively. Collectively, these results suggest that testosterone treatment can enhance the emission of USVs in females, but the acoustic characteristics are not the same as those of intact males.

Keywords: ultrasonic vocalization, mice, masculine behavior, testosterone.

1. Introduction

The mammalian brain initially develops in a sexually undifferentiated manner, and steroid hormones can make the brain sexually differentiated. Early exposure to testosterone masculinizes not only the reproductive function in the brain, but also cognitive, emotional, and neurophysiological functions [1]. We have previously shown that treating neonatal female mice with testosterone propionate induces male sexual behavior [2], indicating that hormonal regulation is more crucial than genetic regulation in mammalian brain sexual differentiation.

Ultrasonic vocalizations are unique behavioral phenotypes in rodents. Upon encountering females or female urinary pheromones, male mice emit ultrasonic vocalizations (USVs), which have common features to the songs of songbirds [3]. Adult males emit more USVs than adult females [4], and male USVs also stimulate female sexual function [5,6]. In addition, characteristics of USVs differ among strains; for example, C57/BL6 (B6) males show a higher peak frequency of calls, shorter intervals between

calls, and more "jump" calls, whereas BALB/cA males present more "harmonic" calls [7]. These differences in song characteristics act as social cues that determine the preferences of female mice. In particular, female mice prefer the USVs from different strains of male mice, which implies that USVs are used to avoid inbreeding [8].

Recent studies have revealed that various types of calls are observed in a social context-dependent manner. Sangiamo et al. examined USV emissions in group-living mice. They analyzed mice social behavior together with USV emissions in a spatio-temporal monitoring system and used deep learning methods to classify the calls into 22 types. Calls with decreased frequency occurred during aggressive behavior, while calls with increased frequency occurred during escaping behavior [9]. Ey et al. revealed that mice used call types in a context-specific manner in same-sex pairs. Interestingly, female pairs emitted more USVs than males when encountering the partner female [10]. Collectively, female mice have the potential to emit USVs, and the emission of USVs also reflects a high level of excitement in social interactions across different contexts [10,11]. In the context of male and female sexual interactions in standard experimental settings, male mice are more excited and emit more USVs than females [12,13].

Sex differences in USV emission during sexual interaction depend on the sexual differences in arousal and motivation elicited by sexual cues [14]. In this regard, female sexual cues stimulate male sexual arousal and motivation, and the neural circuits regulating USV emissions by female cues have also been described. In females, pheromonal information inhibits USV emission, and functional neuronal circuits for USV emission exist in the female brain [15]. We also reported that while male sexual behavior, such as mounting, is mediated via the vomeronasal neural circuits, USV emissions induced by chemosignals are mediated predominantly by the main olfactory system [16], suggesting that these male sexual behaviors are regulated by distinct neural circuits. Recently, we reported that perinatal testosterone treatment in female mice induces comparable numbers of USVs to males, but they are controlled by a different timeline to the testosterone-induced male mounting behavior [17]. Therefore, the neural circuits that regulate male mounting behavior and USVs are both masculinized by perinatal testosterone.

In a previous study, the organizational effect of testosterone was assessed by the number of emissions, and call type analysis was not performed. As mentioned above, male mice emitted various types of calls depending on the social context [9,10]. In addition, although it is still a topic of debate, it has been argued that female USVs produced during male/female interactions have different acoustic characteristics to the male USVs [11,20]. A question arises as to whether treatment with testosterone can also masculinize call types induced by encounters with females. If the neonatal testosterone masculinizes the sexual motivation, testosterone treated female showed equivalent acoustic character and call types to male mouse in a standard experimental setting. In this study, we aimed to address this issue by examining the acoustic characteristics of USVs induced by the neonatal treatment of testosterone.

2. Results

2.1. Acoustic characteristics

Twelve acoustic parameters were compared among the 4 groups. Some group differences were found (Table 1). Sex differences between control males and females were not observed. Similarly, there was no difference between testosterone propionate (TP)-treated males and TP-treated females. TP-treated females showed higher frequency dynamics, delta frequency, mean frequency, and peak frequency as compared to control male and females ($p<0.05$, Kruskal-Wallis test followed by Steel-Dwass test). TP-treated males

showed higher mean frequency compared to control males and females (p<0.05, Kruskal-Wallis test followed by Steel-Dwass test).

2.2. *Clusters of acoustic characteristics*

Acoustic parameters were grouped into three clusters based on the correlations identified (Figure 1). Cluster 1 was composed of "mean frequency total variation (TV)," "frequency dynamic," "linearity index," "frequency TV," and "number (nb) of jumps," and these parameters were related to frequency modulation in calls. Cluster 2 was composed of "mean frequency," "peak frequency," "max frequency", and "min frequency", and these parameters were related to the frequency of the calls. Cluster 3 was composed of "nb modulation," "duration," "vocal (voc) number," and "delta Frequency, " and these parameters were related to the duration and slope of the calls. The loading factors of each parameter are shown in Table S1. There was a group difference in the Cluster 2 score (Figure 2, p<0.05, χ^2=19.9, Kruskal-Wallis test), and scores of TP in Cluster 2 were higher than those of control male and control females (z=3,81, p<0.01; z=2.64, p<.0.05, respectively, Steel-Dwass test). TP-treated males showed higher scores in Cluster 2 than the control males (z=2.89, p<0.05, Steel-Dwass test).
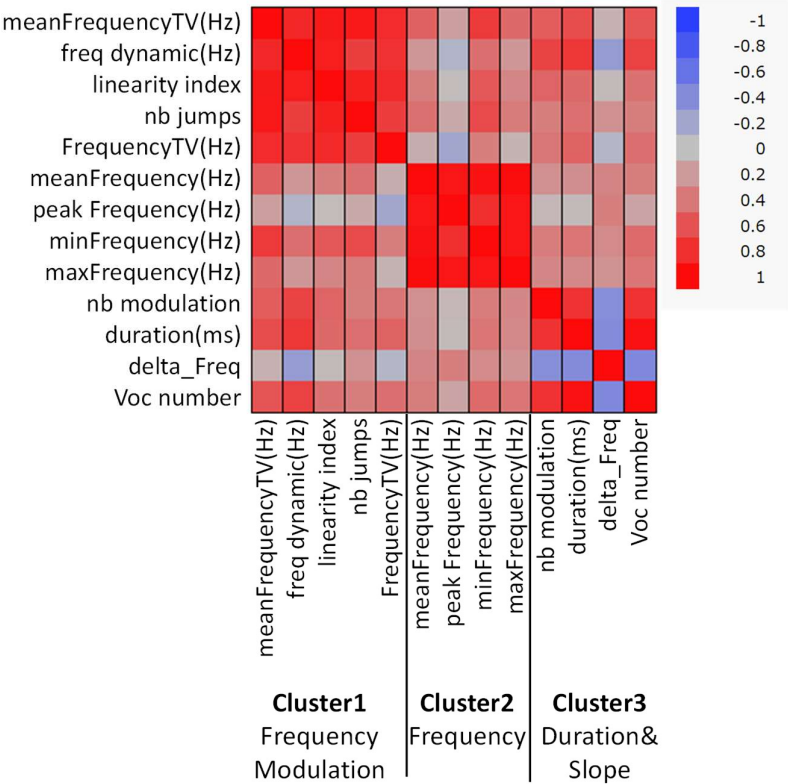

Figure 1. Correlation heatmap of 13 acoustic parameters and clustering with the VARCLUS procedure using orthoblique rotation (JMP, version 14.0, SAS Institute, Cary, NC, USA). Three representative clusters were obtained, as follows: Cluster 1, Frequency modulation; Cluster 2, Frequency; and Cluster 3, Duration and Slope.
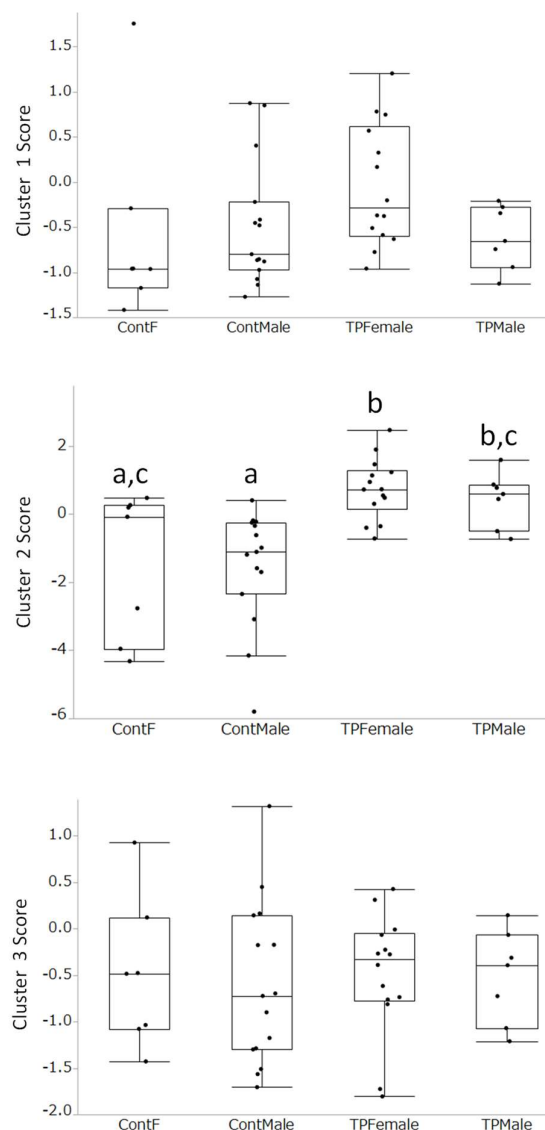
Figure 2. Comparisons of the 3 cluster scores among the 4 groups. There was no sex difference between control male and control female. In Cluster 2, TP-treated females had higher scores than control males and control females. TP-treated males had higher scores than control males. Different alphabet letters (e.g., a and b) indicate statistically significant differences obtained using the Kruskal-Wallis test followed by the Steel-Dwass test.

2.3. Call classification and group comparison.

Calls were classified into nine clusters according to 12 acoustic parameters (Figure 3). The acoustic parameters were averaged for each call type (Table S2) and represented in standardization (z-score, Supplementary Figure S1). The following were the characteristics of the call types: Type 1, jump, high nb of jumps; Type 2, high frequency with short duration; Type 3, short segment with a flat segment; Type 4, long duration with a small slope; Type 5, slope with high frequency; Type 6, high linearly index and frequency of TV; Type 7, upward with frequency modulation; Type 8, high frequency with a short slope; and Type 9, long duration with modulations.
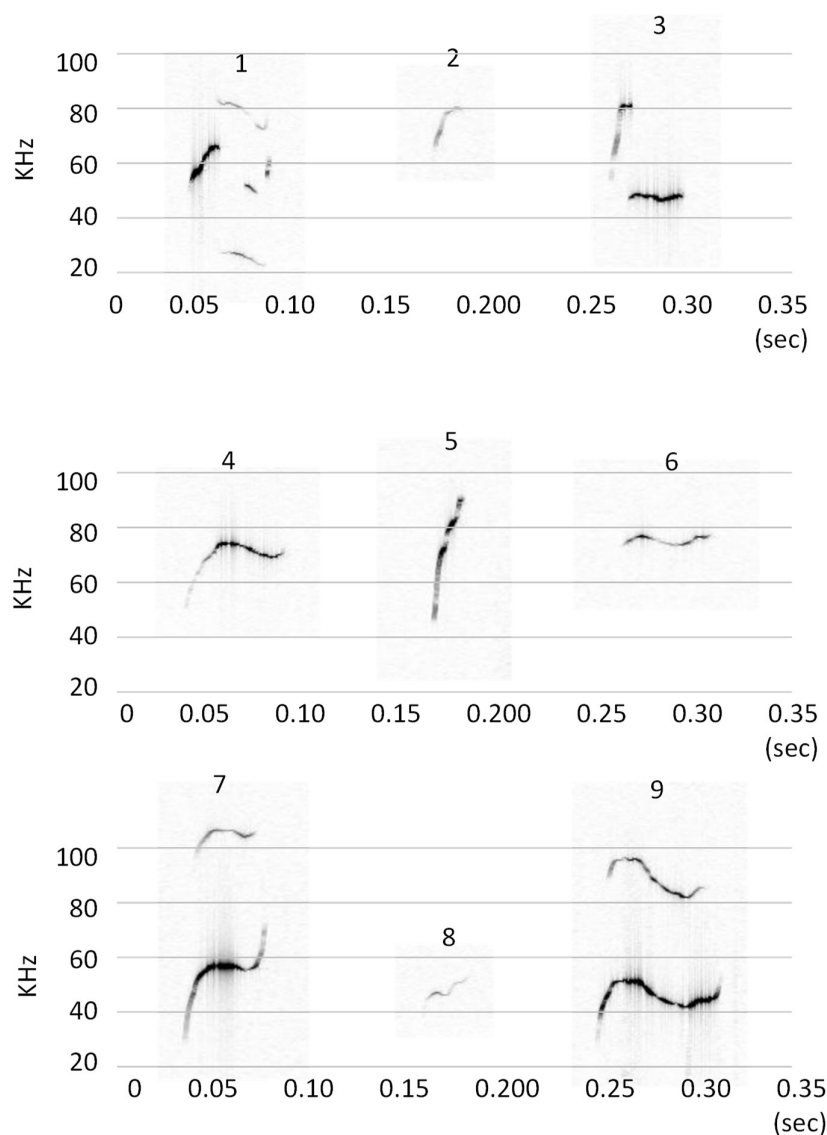
Figure 3. Representative figures of 9 call types. Calls were classified using 12 acoustic parameters by the k-mean clustering method.

The occurrences of each call type were calculated for each animal, and group comparisons were conducted using a multivariate analysis of variance (MANOVA). There were significant group differences (Fig. 4; Roy's largest root =2.07, $F_{(10,31)}$=6.62, $p<0.00001$). In the post-hoc test, TP-treated females were associated with a higher occurrence of Type 2 calls than control females and control males ($p<0.05$). TP-treated males were associated with a higher occurrence of Type 2 calls than control males ($p<0.05$), and TP-treated females were associated with a higher occurrence of Type 7 calls than the other three groups ($p<0.05$). Control males were associated with a higher occurrence of Type 8 calls compared to TP-treated females and TP-treated males ($p<0.05$).
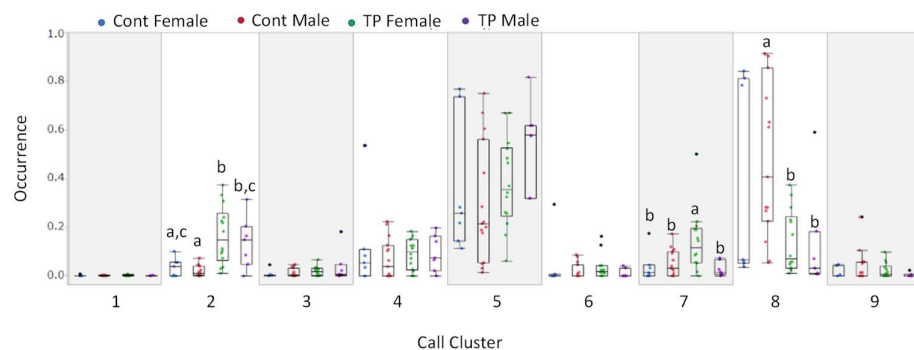
Figure 4. The probability of occurrence of 9 call types. The Y axis represents the probability of the occurrence of each call. Call types 2, 7, and 8 showed group differences.

2.4. Transition probability

Finally, the call type transition was calculated for each mouse; the average transition probability is illustrated in Figure 5. Although there were no statistical differences found, there was a tendency for sex differences; transition from Type 5 to 2 was highest in the control females, and the control males were associated with complex transitions and had a higher transition from Type 2 to 9. In TP-treated females, the transition pattern became complex, but a main transition from Type 5 to Type 2 was maintained in the control females. TP-treated males showed a similar pattern of transition to the control males; a high transition from Type 2 to 9.
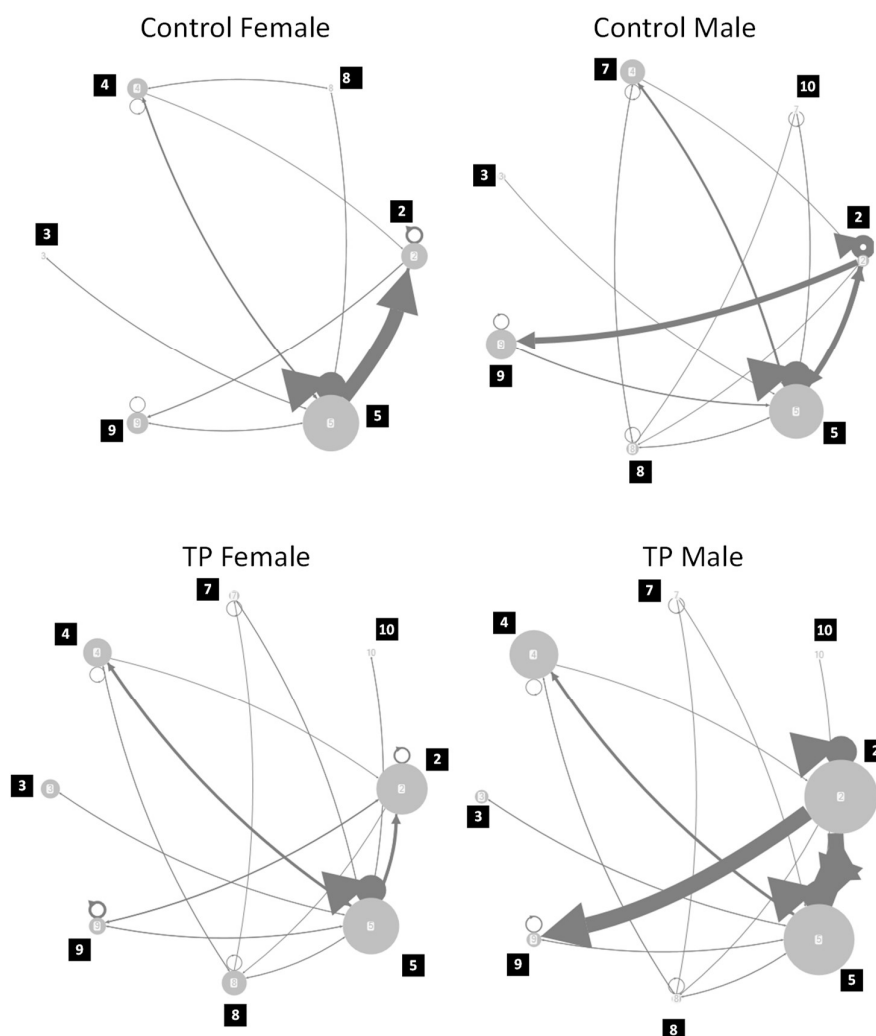
Figure 5. Call type transition probability. Numbers of nodes described the call types, and the width of the edge with arrows represented the probability of transition from one call type to another. For example, transition from type 2 calls to type 9 calls were obvious in control and TP-treated males, as compared to control and TP-treated females. There were self-transitions observed and it was represented with circles and triangles in each node (example, call type 2 and 5).

3. Discussion

In this study, we compared the acoustic characteristics of USVs between males and females, including TP-treated males and females. Perinatal and peripubertal testosterone treatment in females increased USV emissions compared to intact males, while the acoustic characteristics were not similar to those of intact males. Interestingly, there was no clear difference between control males and females, suggesting that male and female emit USVs with similar acoustic characteristics, even though there was a difference in the number of calls. Previous reports described similar results, in which there were only minor differences in male and female USVs when encountering receptive females [11,20]. When both male and female mice were perinatally treated with TP, they showed

similar changes such as an increase in the frequency of calls. The complexity of the calls, such as the number of jumps, modulation, and duration of calls, were not different between males and females, including in the TP-treated males and females. TP can be aromatized into estradiol in the brain, suggesting that the frequency of calls was increased by exogenous androgenic and/or estrogenic stimulations in perinatal period.

When the acoustic parameters were clustered, three clusters were obtained. The first cluster included frequency TV and frequency dynamics, indicating frequency modulation. The second cluster was composed of mean frequency and max frequency, and this was related to the call frequency. The third cluster comprised the duration of calls and the number of modulations, suggesting that this was related to call duration and slope. Interestingly, the number of calls in the test session was positively correlated with other parameters such as duration. This means that the more a mouse emitted USVs, the longer the duration of calls. The scores of the three clusters were compared and there was no obvious sex difference. Cluster 2 scores showed group differences; TP-treated females and males had higher scores, indicating that TP treatment in the perinatal period increased the frequency of calls.

There are several ways to classify call types; we reported one in which the shapes, duration, and frequency of calls were visually determined and the calls were classified into 10 types [7]. Using this method, we revealed strain differences in call types, which were mainly generated by the genetic background of mice [7]. Several reports have presented the acoustic clustering of mouse USVs. For example, Hammerschmidt et al. demonstrated a two-step clustering method and identified three distinctive call types [20]. In this study, we used acoustic parameters to classify call types. We initially set the number of clusters to 10, as we have previously described [19]. As a result, one cluster was identified as noise, and then nine call types were classified. The occurrence of type 2 calls with high frequency and short duration was higher in TP-treated females and male, contrary, type 8 calls were similar in shape as type 2 calls but with lower frequency and short duration, and the occurrence of type 8 calls was high in the control male and female. These were probably the reasons explain why TP-treated mice showed an acoustic characteristic of a higher frequency of calls. TP treatment did not modify the complex type of calls, such as types 1 and 9. These complex calls were frequently observed in mice during the approaching and moving away from counterparts in a large environment [9,10], and these behaviors were difficult to observe in this experiment due to use of a standard cage. Therefore, if the testing area were larger, we could have observed the differences between males and female, and effect of perinatal TP treatment in the occurrence of the call types. Recently, several statistical classifications were developed, revealing various call types [9,10]. An important point in these reports was that the occurrences of call types were social context dependent; a mouse, either male or female, can emit specific types of calls related to specific social behavior. For example, long and modulated calls were observed when the mouse showed close contact with another mouse [10]. When a male mouse escapes from an aggressive male it emits calls with increased frequency, while males emit calls with decreased frequency during aggressive episodes [9]. These results suggest that the occurrence of various call types reflects the emotional status of the subject mouse [10]. The testing area in this study comprised of a standard cage, and future studies are needed to clarify the effect of perinatal TP treatment on the occurrence of the call types.

If the call types reflect the emotional status of the subject, the next question is whether the specific call types can convey the emitter's emotion to the recipient. In a male–female interaction in a large area, males and females synchronously emit USVs when males chasing females, and the presence or absence of female calls could modulate the male-female interaction; female speed was significantly slower during chases with vocal interactions than without vocal interactions [19,20]. This suggests that calls from

females can transmit female emotional status to males, and these USV interactions can change the interactions. In four male mice groups, dominant calls from the chaser stimulated the speed of locomotion in the recipient males, indicating that the USV can convey emotional information from the emitter to modulate social behavior in the recipient [11,20]. We reported playback experiments in which male USVs were regenerated by the ultrasound emitter and observed the behavioral changes in females [18]. These reports revealed that females can recognize some specific characteristics of male USVs and show approaching behavior in response to the USVs. In the future, context-dependent and/or social behavior-dependent playback experiments are needed to clarify the social function of each call type in a complex social environment.

The pattern of transition of call types tended to be different between males and females. We reported that the transition patterns of male USVs were different among strains [18,19]. In addition, mouse pups' USVs also contained the specific call transition pattern, and the pattern was different between wildtype B6 and the genetic model of autism of TBX1 pups. If the call transition sequence was artificially randomized in B6 pup calls, the mother mouse did not show typical approach behavior to the randomized call sequences, even if the numbers of calls and types of calls were identical [11,25]. These results suggest that the call type transition patterns are important for acoustic communication in mice. TP treatment in the perinatal period was not effective in modulating the tendency of the difference between males and females, implying that the call type transition patterns are relatively dependent on genetic sex differences. Some reports showed that mouse *Sry* genes can be expressed in the central nervous system and modulate behavior in male mice [12,13]. Thus, even though there was no evidence of this in our study, it would be worth exploring this subject to reveal the function of sex-related genes in the central nervous system in relation to the pattern of transition of call types.

Sex differences in the acoustic characteristics of USVs are still controversial. In this study, we could not find any obvious sex differences in USV acoustic characteristics, which supports previous findings [12,13]. However, Warren et al. revealed the following sex differences: the bandwidth and slope of vocal signals emitted by male and female mice were consistently different; female calls were narrower in bandwidth and females had more rapid changes in pitch than males [18]. However, differences in acoustic characteristics between male and female USVs may be due to the complexity of behaviors that the animals engage in as they vocalize, because the call characteristics were largely dependent on social contexts [16,27]. Our study was conducted in a standard small cage and the behavioral variation was limited; therefore, it may have not been possible to detect sex differences in acoustic characteristics because of the behavioral testing context in the present study.

Several reports have demonstrated the emission of USVs by females in male-female interactions [17,27]. In these reports, a pair of mice were introduced into a relatively large cage and they displayed a repertoire of complex behavior. To contrast, in the present study a pair of mice were in a small cage and USV emission by females might have been limited, as previously reported [16]. However, we could not eliminate the possibility that the recorded USVs contained USVs emitted by the female mice. Indeed, the control females were encountered with sexually primed female opponents; thus, half of the USVs recorded in the control females could be from the counterpart subjects. The acoustic features of control females were composed of subject females and counterpart females, indicating that these calls were from two intact females. In other groups, the subject mice (control males, TP-treated males, and TP-treated females) showed intensive sniffing and approached the counterpart females, suggesting that these calls were mainly made by the subject mice as previously reported [14]. Therefore, there was a little data contamination, but the overall results were not influenced by contamination.

Another concern was the dose of TP injections. The acoustic features of TP-treated females and males were different to those of control males and females, suggesting that TP treatment did not mimic the physiological level of intact male pups. If the testosterone and estrogen levels associated with TP treatment were equivalent to those of the control male pups, the acoustic features of TP-treated females would be similar to those of control males; however, the results were different. Therefore, the TP dose in this study was not adequate and in fact was probably excessive when compared to the physiological levels in intact male pups. We previously reported the detailed time-dependent secretion pattern of testosterone in intact male and female pup brains [15], but we do not yet know whether TP treatment can replicate the differences or not. Further analysis of testosterone and estrogen in the mouse brain after TP injections is needed to clarify this issue. In contrast, adult testosterone treatment has been shown to mimic testosterone secretion in intact males [17]. These perinatal and adult treatments induced USVs and mounting behavior in females at a similar level to that seen in intact males, indicating that testosterone was sufficient to induce male sexual behavior, as previously reported [8,17].

The neural mechanisms underlying the emission of USVs with complex call types, different frequency calls, and the pattern of transition of call types are yet to be uncovered. We previously reported that the neural circuits underscoring USV emissions and mounting behavior differ; the emission of USVs depends on the main olfactory circuit [2,17]. Mice lacking the dorsal region of the main olfactory bulb demonstrated a decrease in USV emissions. We also previously reported clear sex differences in olfactory information processing in the medial amygdala, especially in the posterior regions [28]. Genetic deletion of the vomeronasal neurons in females resulted in mounting behavior [15]. These results suggest that USV emissions depend on the main olfactory bulb-anterior olfactory nucleus-medial amygdala-hypothalamus. Further studies are needed to elucidate the temporal and spatial details of the androgenic/estrogenic modification of acoustic characteristics of USVs in the control of neural circuits.

4. Materials and Methods

All the vocalization data were acquired in our previous study [17], and we analyzed the acoustic characteristics of the calls using a state-of-the-art machine learning method [10] which was not available at the time of the initial publication. We have described the methods briefly below.

4.1. Animals

C57BL/6J male mice were originally obtained from CLEA Japan, Inc. (Shizuoka, Japan). All experimental procedures were approved by the Ethics Committee of Azabu University (# 160303–6).

4.2. Measurement of USVs

The procedures for USV measurement was in line with our previous studies [8,17]. In brief, sexually naive males were singly housed (172 mm × 240 mm × 129 mm) 1 week before the USV recording. The cage that contained the male mouse was placed in a sound-proof chamber and an unfamiliar virgin female (8–12 weeks old) was introduced. USVs were recorded for 5 min using a microphone (CM16/CMPA, Avisoft Bioacoustics, Brandenburg, Germany) and an A/D converter (Avisoft-UltraSoundGate116H, Avisoft Bioacoustics). The recorded calls were as follows: control females (n=7, median 332 calls), control males (n=15, median 522 calls), TP-treated females (n=14, median 614 calls), TP-treated males (n=7, median 1,249 calls); therefore, there were in total 43 mice and 38,957 calls.

4.3.   Testosterone treatments

Testosterone treatments were performed as reported in our previous study [2,17]. In brief, female mice were treated with TP (Wako Pure Chemicals, Osaka, Japan) during the neonatal period and in adults. For perinatal treatment, pregnant dams were injected with TP on embryonic days (EDs) 15, 16, and 19. Two injections were administered to pups on post-natal (PD) 0 and 2. Control injections were performed with corn oil, and the TP was also dissolved in corn oil. Administration was performed as follows: TP injection in pups, 1.25 μg/0.02 mL, s.c; TP injection in dams, 1.25 μg/0.02 mL, s.c.

On PD21, steroid capsules were surgically implanted, which comprised cholesterol powder (25%, Wako Pure Chemicals, Osaka, Japan) with TP (75%) packed in a 7-mm silicone tube (external diameter=2 mm; internal diameter=1 mm), with silicone at both ends. Four groups were used in the following analysis: control females (n=7), control males (n=15), TP-treated females (n=14), and TP-treated males that received excessive testosterone as compared to control males (n=7).

4.4. Acoustic characteristics analysis

The calls were automatically detected using online software developed by Ey et al. (USV detector: https://usv.pasteur.cloud) [10]. In brief, background noise was removed by filtering the spectrum data, and then signals were extracted using machine learning. The following acoustic characteristics were computed for each USV: duration (ms), frequency dynamic (Hz), mean frequency (Hz), frequency TV (Hz), delta frequency (Hz), mean frequency TV (Hz), linearity index, nb of modulations, nb of jumps, minimal frequency (Hz), maximal frequency (Hz), and peak frequency (Hz). These parameters and the number of calls (Voc number) underwent variable clustering with the VARCLUS procedure using orthoblique rotation (JMP, version 14.0, SAS Institute, Cary, NC, USA), in which correlation among parameters was evaluated. In this method, a large set of variables can often be replaced by a set of cluster components automatically with little loss of information. The first principal component of each cluster was calculated, was the most representative variable, and was assigned a cluster score. The 12 acoustic parameters and the cluster cores were compared among the 4 groups using the non-parametric Kruskal-Wallis test, followed by the post-hoc Steel-Dwass test.

Call classification was conducted by k-means clustering using the abovementioned parameters, with the cluster number set as 9. The frequency of occurrence of these nine call types was compared among the four groups by MANOVA, followed by the Tukey-Kramer HSD post-hoc test.

Finally, the syllable transition was calculated for each mouse. Inter-call intervals of more than 2 s were assigned as gaps, and the frequency of the occurrences of the transition between the call was calculated. The average of the occurrences of the transition (more than 5%) in each experimental group was visualized in transition figures.

Author Contributions: Conceptualization, T.K. and K.M.; methodology, T.K., and K.N.; software, E.E., F.C, and T.B.; data collection, M.S., Y.Y. and T.K.; formal analysis, T.K. and M.N.; writing—original draft preparation, T.K.; writing—review and editing, K.M., K.N., E.E., F.C. and T.B.; funding acquisition, T.K. All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement: All experimental procedures were approved by the Ethics Committee of Azabu University (# 160303–6).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

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