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Research report

Vocal and physical phenotypes of calsyntenin2 knockout mouse pups model early-life symptoms of the autism spectrum disorder

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ABSTRACT

This study discovered a novel acoustic phenotype in Calsyntenin2 deficient knockout (Clstn2-KO) pups in the neurodevelopment period of 5–9 postnatal days (PND 5–9). The narrowband ultrasonic calls (nUSVs) were less complex (mostly one-note, shorter in duration and higher in peak frequency) in Clsnt2-KO than in wild-type (WT) C57BL/6 J pups. The wideband ultrasonic calls (wUSVs) were produced substantially more often by Clstn2-KO than WT pups. The clicks were longer in duration and higher in peak frequency and power quartiles in Clstn2-KO pups. The elevated discomfort due to additional two-minute maternal separation coupled with experimenter's touch, resulted in significantly higher call rates of both nUSVs and clicks in pups of both genotypes and sexes compared to the previous two-minute maternal separation, whereas the call rate of wUSVs was not affected. In Clstn2-KO pups, the prevalence of emission of wUSVs retained at both sex and both degrees of discomfort, thus providing a reliable quantitative acoustic indicator for this genetic line. Besides the acoustic differences, we also detected the increased head-to-body ratio in Clstn2-KO pups. Altogether, this study demonstrated that lack of such synaptic adhesion protein as calsyntenin2 affects neurodevelopment of vocalization in a mouse as a model organism.

1. Introduction

Wild domestic mouse (*Mus musculus domesticus*) serves as an ancestor form for creating numerous laboratory strains [1], including various genetic models of human autism spectrum disorder (ASD) [2–5]. Substantial differences in ultrasonic vocalization (USV) are reported between different mice strains with ASD-like phenotypes [6–9]. Genetically modified mice can also display modified USVs compared to wild-type (WT) strains [10–16]. In addition, substantial differences in pup physical development are reported between different mouse models related to ASD, from a severe growth retardation during the first postnatal weeks [17] to accelerated developmental milestones and growth rates [18]. Effects of sex on pup size and physical development in mice models of ASD can also be strain-specific [19].

Neonatal mice pups isolated from the nest emit USVs with fundamental frequencies (f0) over 20 kHz [20], often accompanied with sonic and ultrasonic clicks [11]. These isolation-induced calls elicit a maternal approach [20–24] being therefore similar in function to human infant cry [25,26]. Atypical preverbal vocalizations are prognostic of ASD in humans [27–31]. In human ontogeny, ASD is also responsible for language problems [10,11].

Analysis of atypical neonatal calls in mouse pups of ASD-related genetic lines [1–3,5,15,32,33] may help to understand the exact nature and underlying mechanisms of vocal and developmental disorders in children with autism [25,26,34,35]. For children, early diagnosis of ASD is critical, because early intensive treatment greatly improves its prognosis [26].

Laboratory mice with ASD –like behavior differ by peaks of

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ultrasonic emission, from the earliest at 3 postnatal days (PND) pups in C57BL/6 J strain till 6–8 PND in the BTBR and FVB strains [18,32]. Many previous studies restricted their recordings of mice pup USVs only at one single day (mostly with 7–10 PND pups) [10,12,32,36], because ages from about 3–10 PND in mice pups correspond to preterm and term human infants, respectively [3,37]. So, for comparative studies of USVs between strains, the range of pup's ages of about 5–9 PND seems to be optimal [18,32,33,38].

In laboratory mice, the USVs of genetic strains displaying ASD-like phenotypic traits, may be altered in call rate and the acoustic structure, compared to WT mice [2,8,17–19,39–42]. A few well-established mouse pup of ASD-like genetic strains display a changed call rate and/or acoustic variables associated with a more limited vocal repertoire of USVs and clicks compared to WT pups, as *e.g.* in BTBR pups [18], Cd157-deficient pups [1,33], Tph2-deficient pups [17], Fox2-deficient pups [10,11], Nlgn4-deficient pups [19,43,44], Shank1 knockout pups [39,45,46] and Neurexin 1α knock out pups [42]. In some pup mouse models of ASD, USV deficits are stronger in females, as *e.g.* in Nlgn4-deficient pups [19] and Shank1 knockout pups [45,46], however this sex effect was not detected in another study Shank1 knockout pups [39].

Aside from the genetic background of the mutant mouse line [18,38,47], an important factor, affecting USVs in mouse models, is the degree of discomfort [17,48–50]. In addition, methodological approaches used to analyze USVs data are fast evolving tools [51–53] and significantly affect the final outcomes.

Calsyntenin2 knockout (Clstn2-KO) adult mice express some ASD-like behavior including hyperactivity, stereotypy, deficient spatial long-term memory, and impaired social behavior [54–56]. Notably, that Clstn2-KO males but not females demonstrate lack of social motivation and abolished social recognition [55] in spite of hyperactivity and repetitive behavior detected in both sexes of mutant mice, further supporting potential role of Clstn2 gene in ASD-related pathology since autism is more common in men than in women with a ratio 4:1 [57]. In addition, Clstn2-KO male mice also demonstrate deficient social communication evident in the social transmission of food preference (STFP) task [55].

Calsyntenin-2 (Clstn2) belongs to the superfamily of cadherins, which is important for the synaptic adhesion and our recent study detected a reduction of length of synaptic contacts, post-synaptic density, and amount of inhibitory interneurons, accompanied by the simplified type of synapses in the cortex [58]. Notably, that lack of Clstn2 results in a selective reduction of functional inhibitory GABA synapses with no changes of excitatory functional synaptic response, together with decreased expression of GAD65 [54], supporting further the role of Clsnt2 in ASD given that imbalance between the excitatory to inhibitory (E/I) cortical activity underlies social deficiencies in autistic patients [59]. Moreover, analysis of copy number of variances in clinics revealed a deletion of intron2 in Clstn2 gene in several ASD patients [60]. Given that in humans, the Clstn2 locus is associated with verbal episodic memory at different ages [61–63] and children with ASD have difficulties developing language skills [57], it is becoming highly important to characterize vocalization in Clstn2-KO pup mice.

Most studies suggest the semi-automatic or automatic approaches for detecting and measuring the acoustic variables in different strains of laboratory mice [40,64–66]. However, the fully or partially automatic extraction and measuring of USVs may be unsuccessful in detecting although small but meaningful acoustic differences between knockout and WT mice strains [67,68]. For instance, applying the automatic measurements of dominant frequency and duration with manual control of 10–20 % audio files failed to reveal any difference in USV variables between Celf6 knockout and WT control C57BL/6 J mice [64]. So, in this study, we applied the manual method with full human control for measuring the acoustic variables in the USVs and clicks of the Clstn2-KO and WT mice pups.

In addition to the narrowband USVs, domestic mice produce short wideband clicks in audible through the ultrasonic range of frequencies [10–13,21]. Whereas a production mode for the USVs in rodents is an aerodynamic whistle mechanism resulting from the blowing the air through the narrowings of the vocal tract [69–71], the mechanism for production of the clicks in rodents is unknown [72] and may represent the tongue clicks, as in tenrecks [73], piebald shrews *Diplomesodon pulchellum* [74] and Egyptian fruit bats *Rousettus aegyptiacus* [75] or single pulses of the glottis.

Therefore, the aim of this study was to reveal and to describe the differences in call rate and the acoustic structure of both USVs and of the clicks, produced at the experimental situation of isolation from a mother and littermates, between (Clstn2-KO) mice pups and wild-type (WT) mice pups. In addition, we estimate the effect of sex and degree of discomfort (basic or elevated), by comparison the two successive experimental stages, isolation and touch, on call rate and the acoustics of USVs and clicks. We supposed, following the studies by [76], that subject 5–9 PND pups experienced a higher discomfort during the touch stage than during the isolation stage, because of the cumulative effect of time passed since isolation from the nest and of the touching with a cotton bud.

2. Methods

2.1. Mice

Subject pups of Clstn2 homozygous knockout mice (Clstn2-KO) and of wild-type (WT) C57BL/6 J mice were bred and maintained in the animal facility at the Federal State Budgetary Scientific Institution «Scientific Research Institute of Neurosciences and Medicine». The generation of Clstn2-KO mice has been previously described [54]. To maintain this knockout line, Clstn2-KO mice were mated with C57BL/6NCrl mice. Next, Clstn2 heterozygous offspring were intercrossed to generate Clstn2-KO mice and their WT littermates [55]. Experimental animals were genotyped by PCR using primers: "cls2-ko-F1" (knockout forward: 5′ AAGTTTTGGGCTTGTAGATCCAGC TCT GTC) and "neo-ko-R1" (knockout reverse: 5′ AAATTGCATCG-CATTGTCTGAGTA GGTGTC) and "cls20ko-R2" (wild-type reverse: 5′ GATGTCTTATTGAGCACCACAGCCTCAAAG). WT and Clstn2-KO amplicons were ~162 base pairs and ~364 base pairs, respectively [55].

2.2. Subjects and housing

The 5–9 PND subject pups were kept together with their mothers and littermates under inverted light regime (light from 18.00 p.m. to 6 a.m.) and temperature was maintained about 23 °C. Subject pups were 36 (18 male, 18 female) pups of a Clstn2-KO strain originated from 6 litters and 44 (21 male, 23 female) pups of a WT strain originated from 7 litters. The 13 study litters, with 6 pups per litter on average, were obtained from the 13 different parental pairs.

By age, both Clstn2-KO and WT pups during test trials and morphometric assessments belonged to two age groups: 5–6 PND and 8–9 PND pups; the 7 PND pups lacked in pup sample. Within Clstn2-KO strain, there were 25 5–6 PND and 11 8–9 PND pups. Within WT strain, there were 27 5–6 PND and 17 8–9 PND pups.

Before the predicted day of birth, pregnant females were isolated singly or together with another pregnant female in a cage 34 × 29 × 15 cm (OptiMice Biotech A.S.) and then checked two times per day for the appearance of a litter. Mice had *ad libitum* access to rodent food pellets (Ssniff, Germany) and water in home-cages. Day of birth was considered day zero of pup life. Study pups were sexed before experimental test trials, based on the distance between the anus and the urethra. Pups were not tattooed of otherwise marked, as each pup was only tested once.

2.3. Experimental setup

Pup USVs and clicks were recorded during experimental isolation and touch test trials. The test trials were conducted at the Scientific Research Institute of Physiology and Basic Medicine, Novosibirsk, Russia, from 24 April to 12 August 2017. All test trials were conducted in a separate room where no other animals were present, at room temperature 22−25 °C during the daytime, at the same level of background noise. Each pup participated only once in one experimental trial. Each individual was tested singly. Immediately before an experimental trial, the focal pup was taken from the home cage and transferred in a small clean plastic hutch to the experimental room within the same floor of the building. Time from the removal of the focal pup from the cage to the start of an experimental trial did not exceed 60 s. During the trial, the animal just was isolated in an experimental setup, the clean plastic hutch (190 × 130 × 70 mm) placed on even plastic table surface. The plastic hutch was open from above, *i.e.* from the side where the microphone was placed.

2.4. Experimental procedure

Each test trial (one per individual pup) had two stages: the isolation stage (120 s) and the touch stage (90 s). For the duration of the isolation stage (Stage1), the focal pup was alone in the experimental setup. For the duration of the touch stage (Stage2), the experimenter (IAV) gently touched the focal pup with a cotton bud, approximately two times per second. Aside isolation, the focal pups experienced also a cooling, due to the imperfect thermoregulation. We considered that pup discomfort increases from Stage1 to Stage2 because of cumulative effects of time of pup isolation from the nest and of touching [72,76]. The morphometric assessments (body measurements and weighting) were conducted after the test trials. After the morphometric assessments, the focal pup was placed to a heating hutch with a bedding of a cotton fabric in the neighboring room. Test trials with all littermates were done consequently in the same manner. Then all the litter in total was returned to their home cage to their mother; the time of pup stay out of the nest did not exceed 40 min. Although pups were not individually identified, the sequential test trials with littermates allowed controlling that each pup participated in the experiment only once. The experimental setup was rubbed with napkin wetted with alcohol after each test trial, to avoid effect of the smell on vocalization of the next focal animal in the next test trial [77–79].

We considered the touch stage as more discomfort than the isolation stage [76]. Elevated discomfort of a focal pup during the experimental touch stage was primarily promoted by additional two-minute maternal separation compared to the isolation stage, whereas the simultaneous pup touch by the experimenter was applied for stimulating the focal pup to elicit more calls and, in case if applied alone without prolonged isolation, could potentially have any emotional valence, negative (perceived as discomfort), positive (perceived as *e.g.* maternal approach for withdrawal a pup to the nest) or bivalent.

2.5. Acoustic recording

For acoustic recordings during the test trials (sampling rate 384 kHz, 16-bit resolution), we used a Pettersson D1000X recorder with built-in microphone (Pettersson Electronik AB, Uppsala, Sweden). The microphone was established stationary at distance 25 cm above the animal. The obtained recordings had a high signal/noise ratio, the reverberation practically lacked. Recording of each trial was stored as a wav-file, one file per individual pup. In total, we recorded 80 audio files with USVs and clicks from 5 to 9 PND pups, 36 files from Clstn2-KO and 44 files from WT pups.

2.6. Morphometric assessments

After a test trial, the experimenter measured body length, head length, foot length and tail length of a focal pup with an electronic caliper (Kraf Tool Co., Lenexa, Kansas, US) with an accuracy of 0.01 mm, continuing keeping it in hands. We measured body length from the tip of the snout to the anus, and head length from the tip of the snout to the occiput. We measured foot length from the heel to the tip of the middle toe, and tail length from anus to the tip to the tail [79]. These measurements were repeated three times and the mean value was taken for analysis. Then the focal pup was weighed on the electronic scales G&G TS-100 (G&G GmbH, Neuss, Germany) with an accuracy of 0.01 g, in the same plastic hutch which was used for transferring the animal to the experimental setup.

2.7. Call samples

Based on visual inspection of spectrograms, we classified pup calls, blindly to subject genotype, to three categories: the narrowband ultrasonic calls (nUSVs), the wideband ultrasonic calls (wUSVs), and the clicks (with a variable frequency range, from the audible to ultrasonic; see a detailed description of these call categories in Results). Some pups also produced the audible calls in the experimental setup; these calls were not analysed in this study.

For calculating call rates, we counted the number of calls of each of the three call types separately for the experimental Stage1 and Stage2. For nUSVs and wUSVs, we counted only calls longer 5 ms. Calls separated with intervals 20 ms or more were counted as separate calls; if the interval was less than 20 ms, the call parts were considered as belonging to the same call. Simultaneously, we scored the number of test trials (= individual pups), in which calls of each category were presented at the experimental Stage1 and Stage2.

For further detailed analysis of acoustic variables, we selected calls of good quality, appropriate for measurements of all acoustic variables. For each pup, the first 10 high-quality calls of each call type were included in analysis of acoustic variables for Stage 1 and for Stage 2, 30 calls from Stage 1 and 30 calls from Stage 2, 60 calls per test trial. In cases if less than 10 calls of each category were recorded, all available measurable calls have been analyzed. In total, we included in analysis 3222 calls, 1532 calls from the 36 Clstn2-KO pups (653 nUSVs, 155 wUSVs and 724 clicks) and 1690 calls from the 44 WT pups (756 nUSVs, 63 wUSVs and 871 clicks).

2.8. Call analysis

Measurements of acoustic variables of the calls have been conducted with Avisoft SASLab Pro software (Avisoft Bioacoustics, Berlin, Germany) and exported to Microsoft Excel (Microsoft Corp., Redmond, WA, USA). For analysis, we used sampling rate 386 kHz, FFT-length 1024, Frame 50 %, Overlap 87.5 % for the nUSVs and wUSVs and Overlap 93.75 % for the clicks. We applied 0.5 kHz high-pass filtering to remove the background noise in the audio files.

For each call irrespectively of call type, we measured, by using the semi-automatic option of Avisoft, the duration (duration), the peak frequency (fpeak) of the entire call spectrum, the lower (q25), medium (q50) and upper (q75) power quartiles of the entire call (covering respectively 25 %, 50 % and 75 % of call energy), the bandwidth of peak frequency at minus 10 dB from the maximum (bndw) and the entropy (entropy) as a measure of the harmonic energy in call spectra. Additionally, for nUSVs and wUSVs, we measured the minimum and the maximum values of fundamental frequency (f0min and f0max) with a reticule cursor in the spectrogram window of Avisoft (Fig. 1).

For USVs, we noted the presence of nonlinear vocal phenomena: frequency jumps, biphonations and deterministic chaos [79–82]. Frequency jump (Fig. 2a) was denoted when f0 suddenly changed for > 10 kHz up or down [18,79,82–85]. Biphonation (Fig. 2b) was denoted

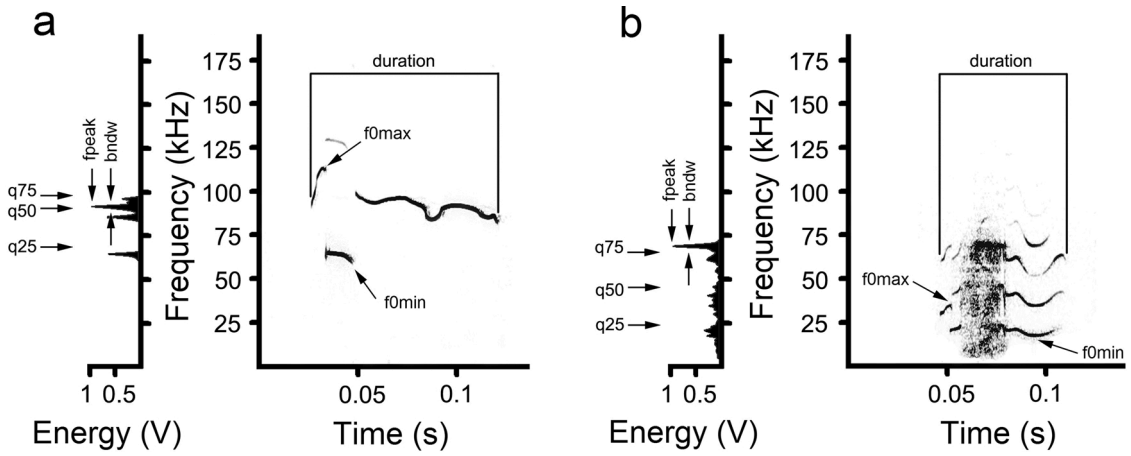

Fig. 1. Spectrogram (right) and power spectrum (left) illustrating the measured acoustic variables in the narrowband (nUSV) ultrasonic call (a) and the wideband (wUSV) ultrasonic call (b) of 5-9 PND pup mice. Designations: duration – call duration; f0max – the maximum fundamental frequency; f0min – the minimum fundamental frequency; fpeak – the frequency of maximum amplitude; q25, q50, q75 – lower, medium and upper quartiles; bndw – the bandwidth of the fpeak at the distance of 10 dB from the maximum. The spectrograms were created with Hamming window; 386 kHz sampling rate; FFT 1024 points; frame 50 %; and overlap 87.5 %.

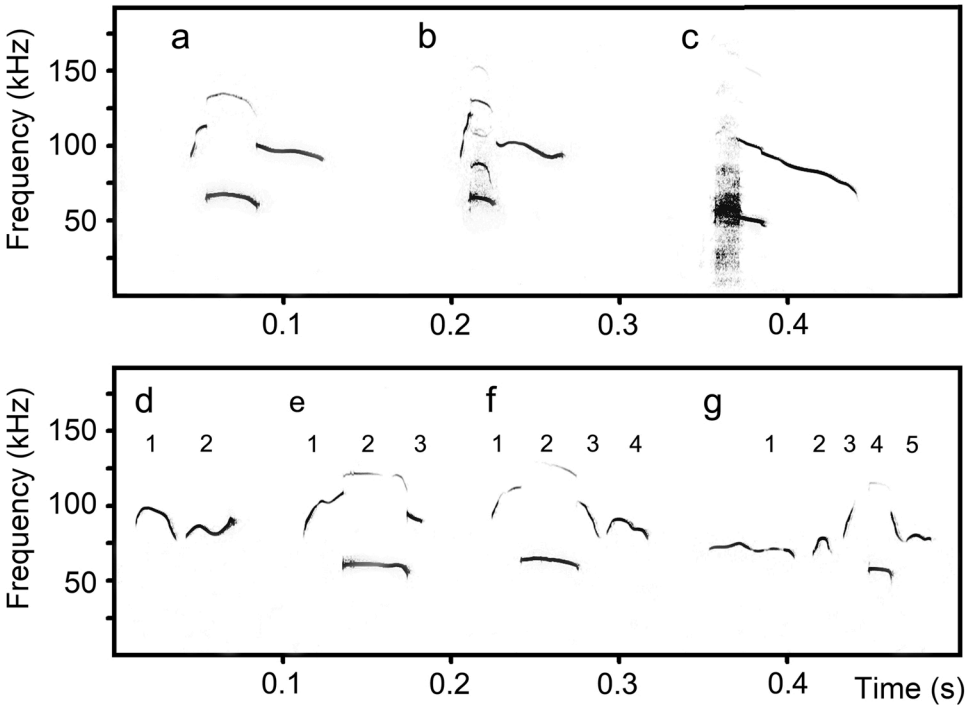

Fig. 2. Spectrogram illustrating the complexity of the narrowband ultrasonic calls (nUSVs) of 5-9 PND pup mice. Three types of nonlinear vocal phenomena: frequency jump (a), biphonation (b) and deterministic chaos (c), and four different note compositions: two-note (d), three-note (e), four-note (f) and five-note (g). The spectrogram was created with Hamming window; 386 kHz sampling rate; FFT 1024 points; frame 50 %; and overlap 87.5 %. The audio file is available at Supplementary file 1.

when two independent fundamental frequencies and their combinatory frequency bands were found in a USV call [18,79,82]. Deterministic chaos (Fig. 2c) was denoted when the chaotic segments were found in call spectra; these chaotic segments were different from the background noise by the existence of residual harmonic structures within the chaotic episodes [80,81].

In each USV call we also scored the number of notes (= continuous fragments) of which USV call was composed. We considered that a call was continuous (*i.e.* consisting of only one single note), when the breaks of call frequency contour did not exceed 10 kHz in frequency and 5 ms in duration [85]. In cases where a frequency jump exceeded 10 kHz and/or where the break of call contour lasted from 5 ms to 20 ms, we considered such call fragments as separate notes within the same call (Fig. 2d–g).

2.9. Statistical analyses

Statistical analyses were made with STATISTICA, v. 8.0 (StatSoft, Tulsa, OK, USA), all means are given as mean ± *SD*. Significance levels were set at 0.05, and two-tailed probability values are reported.

We used a one-way ANOVA to compare pup age and number of pups per litter between genotypes (Clstn2-KO *vs* WT). We used two-way MANOVA to compare the effects of factors genotype, sex and joint effect of sex and genotype on pup body mass and dimensions.

We used a General Linear Model (GLM) with Tukey HSD (honestly significant difference) post hoc test for call type, to compare the acoustic variables of the three call types, with pup sex, genotype, age (5–6 *vs* 8–9 PND) and degree of discomfort (isolation *vs* touch) as covariates, introduced in analysis as fixed factors. We did not introduce pup ID in this analysis, as pup ID involved also genotype, sex and age, so that these

key factors for this study were neglected by the statistical model in case when pup ID was added in the model. We used a Discriminant Function Analysis (DFA) standard procedure based on 7 acoustic variables which were measured in calls of all three types, to calculate the probability of the assignment of calls to the correct call type. We used Wilks' Lambda values to estimate how strongly the acoustic variables contribute to the discrimination of call types. To validate our DFA results, we calculated the random values of correct assignment of calls to call types by applying randomization procedure with macros, created in R [86]. The random values were averaged from DFAs performed on 1000 randomized permutations on the data sets as described by [87].

We used a Repeated Measures ANOVA to compare the effect of degree of discomfort on call rate, separately for each of the three call types, with inclusion in analysis of pup genotype, sex and age as covariates. We used a one-tailed Fisher exact test to estimate the effect of pup genotype on complexity of nUSVs and wUSVs (estimated *via* percentage of one-note *vs* multi-note USVs and percentage of USVs with different nonlinear vocal phenomena) and on the occurrence of different call types during the experimental trials. We used a Nested Design ANOVA with individual nested in pup genotype to estimate the effect of the Clstn2-KO *vs* WT genotype on the acoustic variables, separately for each of the three call types, with genotype as fixed factor and individual as random factor.

3. Results

3.1. Body variables

Six litters of Clstn2-KO mice contained 6.0 ± 3.6 pups per litter, and seven litters of WT mice contained 6.3 ± 2.1 pups per litter ($F_{1,11} = 0.03$, $p = 0.86$). The age of Clstn2-KO pups was 6.2 ± 1.5 days on average; the age of WT pups was 6.6 ± 1.7 days on average. The age did not differ between pup genotypes, neither at comparison of the litters ($F_{1,11} = 0.20$, $p = 0.66$), nor at comparison of the individuals ($F_{1,78} = 2.82$, $p = 0.10$).

Pup genotype affected pup mass and the dimensions: 5–9 PND Clstn2-KO pups had a significantly lower body mass (2.87 ± 0.75 g *vs* 3.58 ± 1.26 g) and the significantly shorter body length (36.81 ± 3.29 mm *vs* 39.47 ± 4.78 mm), foot length (9.37 ± 1.15 mm *vs* 10.13 ± 1.74 mm) and tail length (19.31 ± 1.86 mm *vs* 21.74 ± 4.49 mm), but did not differ from WT pups by head length (14.28 ± 1.43 mm *vs* 14.65 ± 1.86 mm) (Table 1). The head/body length ratio was also significantly higher in Clstn2-KO than in WT pups (0.388 ± 0.015 *vs* 0.372 ± 0.024) (Table 1). Pup sex did not influence pup body mass or body dimensions, either in a total sample of all pups or within strains (Table 1). Within-sex comparison of genotypes showed that only female Clstn2-KO pups had a significantly lower body mass, body length, foot length, tail length and head/body length ratio than female WT pups, whereas the male pups did not show the differences between genotypes, for the exclusion of head/body length ratio (Table 1).

Table 1
Values (mean ± SD) for body mass and body dimensions of 5-9 PND Clstn2-KO and wild-type (WT) pup mice and two-way ANOVA results for their comparison. Significant differences are given in bold.

Genotype	WT		Clstn2-KO		
Sex	Males (n = 21)	Females (n = 23)	Males (n = 18)	Females (n = 18)	MANOVAs
Body mass (g)	3.61 ± 1.48	3.55 ± 1.04	2.96 ± 0.75	2.80 ± 0.76 *	Genotype: $F_{1,76} = 8.64$, $p = 0.004$ Sex: $F_{1,76} = 0.21$, $p = 0.64$ Genotype x Sex: $F_{1,76} = 0.04$, $p = 0.84$ Genotype: $F_{1,76} = 7.83$, $p = 0.007$
Body length (mm)	39.01 ± 5.18	39.89 ± 4.45	37.21 ± 3.28	36.41 ± 3.34 **	Sex: $F_{1,76} = 0.002$, $p = 0.97$ Genotype x Sex: $F_{1,76} = 0.79$, $p = 0.38$ Genotype: $F_{1,76} = 0.88$, $p = 0.35$
Head length (mm)	14.54 ± 1.99	14.75 ± 1.77	14.43 ± 1.42	14.13 ± 1.46	Sex: $F_{1,76} = 0.02$, $p = 0.90$ Genotype x Sex: $F_{1,76} = 0.44$, $p = 0.51$ Genotype: $F_{1,76} = 4.88$, $p = 0.03$
Foot length (mm)	10.04 ± 1.87	10.21 ± 1.64	9.52 ± 1.17	9.22 ± 1.14 *	Sex: $F_{1,76} = 0.04$, $p = 0.85$ Genotype x Sex: $F_{1,76} = 0.47$, $p = 0.49$ Genotype: $F_{1,76} = 9.02$, $p = 0.004$
Tail length (mm)	21.73 ± 5.08	21.75 ± 3.98	19.53 ± 1.75	19.09 ± 1.99 *	Sex: $F_{1,76} = 0.07$, $p = 0.79$ Genotype x Sex: $F_{1,76} = 0.08$, $p = 0.78$ Genotype: $F_{1,76} = 12.30$, $p<0.001$
Head/Body length ratio	0.373 ± 0.021	0.370 ± 0.027	0.388 ± 0.017 *	0.388 ± 0.014 *	Sex: $F_{1,76} = 0.09$, $p = 0.76$ Genotype x Sex: $F_{1,76} = 0.10$, $p = 0.75$

* $p < 0.05$.
** $p < 0.01$ for comparison of Clstn2-KO with WT pups of the same sex.

3.2. Call types

3.2.1. Narrowband ultrasonic calls (nUSVs) (Fig. 3)

These calls always display a well-visible narrow ultrasonic band (thereafter "tonal component") with the maximum fundamental frequency ranging from 46.5–152.2 kHz and the minimum fundamental frequency ranging from 37.8–104.6 kHz between calls (Table 2). Some calls contain a noisy component; in these cases, the fundamental frequency band is still accented quite well and can be traced along the entire call duration. The peak frequency and all the three quartiles of the power spectrum of nUSVs exceed substantially and significantly the values of these variables in the wideband ultrasonic calls (wUSVs) and in the clicks (Table 2). The values of entropy and bandwidth of peak frequency are the lowest compared to the wUSVs and the clicks, what indicates a high degree of the harmonic energy and the low degree of the noisy energy in call spectra.

3.2.2. Wideband ultrasonic calls (wUSVs) (Fig. 3)

These calls are longer in duration than nUSVs (Table 2). The wUSVs obligatory contain the wideband noisy component and optionally also the tonal component, occurring before/after the wideband one or simultaneously with it. Where presented, the tonal component cannot be tracked along the entire call duration, being strongly masked with the wideband component. The fundamental frequency of the tonal component is substantially lower in wUSVs than in nUSVs (Table 2), so the harmonics of the fundamental frequency band are often visible on the spectrogram. The values of the peak frequency and the three power quartiles are intermediate between those of nUSVs and of the clicks. The values of entropy are the highest and the values of bandwidth of peak

Table 2
Values (mean ± *SD*) of the acoustic variables of three call types: nUSVs, wUSVs, and clicks in Clstn2-KO and WT pups at 5-9 PND and GLM results for the effects of call type, sex, genotype, age and degree of discomfort on the acoustic variables. The same superscripts indicate that the values did not differ significantly between call types (Tukey HSD test). Significant differences are given in bold. Designations: Duration – call duration; fpeak – the peak (maximum amplitude) frequency; bndw – call bandwidth at minus 10 dB from the maximum; q25, q50, q75 – the lower, the medium and the upper quartile of call power spectrum (covering respectively 25 %, 50 % and 75 % of call energy); entropy – call entropy; f0min – the minimum fundamental frequency; f0max – the maximum fundamental frequency.

Acoustic variable	Call type			GLMs				
	nUSV (n = 1409)	wUSV (n = 218)	Click (n = 1595)	Call type	Sex	Genotype	Age	Degree of discomfort
Duration (ms)	33.7 ± 23.9 [a]	46.9 ± 26.6 [b]	1.46 ± 0.26 [c]	$F_{2,3215} = 1668.1$, $p<0.001$	$F_{1,3215} = 6.11$, $p = 0.01$	$F_{1,3215} = 50.7$, $p<0.001$	$F_{1,3215} = 0.90$, $p = 0.34$	$F_{1,3215} = 1.83$, $p = 0.18$
fpeak (kHz)	74.77 ± 15.91 [a]	43.94 ± 20.33 [b]	10.06 ± 8.04 [c]	$F_{2,3215} = 9364.1$, $p<0.001$	$F_{1,3215} = 1.95$, $p = 0.16$	$F_{1,3215} = 73.1$, $p<0.001$	$F_{1,3215} = 0.18$, $p = 0.67$	$F_{1,3215} = 0.0$, $p = 1.0$
bndw (kHz)	4.26 ± 2.72 [a]	8.58 ± 7.20 [b]	8.06 ± 3.57 [b]	$F_{2,3215} = 463.7$, $p<0.001$	$F_{1,3215} = 15.5$, $p<0.001$	$F_{1,3215} = 1.89$, $p = 0.17$	$F_{1,3215} = 11.4$, $p<0.001$	$F_{1,3215} = 1.84$, $p = 0.17$
q25 (kHz)	70.99 ± 16.44 [a]	27.69 ± 8.42 [b]	9.19 ± 4.70 [c]	$F_{2,3215} = 10858$, $p<0.001$	$F_{1,3215} = 0.34$, $p = 0.56$	$F_{1,3215} = 34.1$, $p<0.001$	$F_{1,3215} = 1.56$, $p = 0.21$	$F_{1,3215} = 0.01$, $p = 0.93$
q50 (kHz)	76.04 ± 15.39 [a]	43.66 ± 11.20 [b]	14.59 ± 8.14 [c]	$F_{2,3215} = 9933.6$, $p<0.001$	$F_{1,3215} = 0.20$, $p = 0.66$	$F_{1,3215} = 69.2$, $p<0.001$	$F_{1,3215} = 0.0$, $p = 1.0$	$F_{1,3215} = 0.30$, $p = 0.59$
q75 (kHz)	82.54 ± 17.13 [a]	59.92 ± 11.76 [b]	28.19 ± 11.27 [c]	$F_{2,3215} = 5680.5$, $p<0.001$	$F_{1,3215} = 0.76$, $p = 0.38$	$F_{1,3215} = 42.8$, $p<0.001$	$F_{1,3215} = 42.7$, $p = 0.55$	$F_{1,3215} = 0.31$, $p = 0.59$
entropy	0.24 ± 0.09 [a]	0.49 ± 0.13 [b]	0.37 ± 0.08 [c]	$F_{2,3215} = 1252.3$, $p<0.001$	$F_{1,3215} = 5.58$, $p = 0.02$	$F_{1,3215} = 31.4$, $p<0.001$	$F_{1,3215} = 21.5$, $p<0.001$	$F_{1,3215} = 0.85$, $p = 0.36$
f0min (kHz) *	65.13 ± 15.97	20.09 ± 6.84		$F_{1,1559} = 1344.5$, $p<0.001$	$F_{1,1559} = 0.14$, $p = 0.70$	$F_{1,1559} = 84.4$, $p<0.001$	$F_{1,1559} = 0.01$, $p = 0.94$	$F_{1,1559} = 1.44$, $p = 0.23$
f0max (kHz) *	87.72 ± 15.90	31.10 ± 6.37		$F_{1,1559} = 1958.2$, $p<0.001$	$F_{1,1559} = 0.07$, $p = 0.79$	$F_{1,1559} = 14.9$, $p<0.001$	$F_{1,1559} = 22.1$, $p<0.001$	$F_{1,1559} = 0.0$, $p = 1.0$

* n = 1564 for the wUSV.

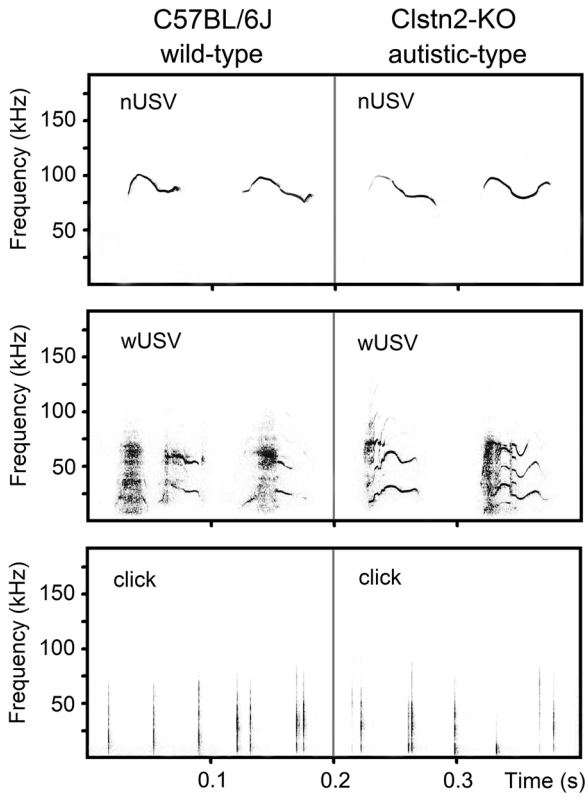

Fig. 3. Spectrogram illustrating three call types: the narrowband ultrasonic calls (nUSVs, upper panel), the wideband ultrasonic call (wUSVs, central panel) and the clicks (lower panel) of 5-9 PND wild-type and CLStn2-KO pup mice. Spectrogram was created with Hamming window; 386 kHz sampling rate; FFT 1024 points; frame 50 %; and overlap 87.5 %. The audio files are available at Supplementary file 2 and Supplementary file 3.

frequency are twice higher than in nUSVs, what indicates a low proportion of the harmonic energy and the high proportion of the noisy energy in call spectra.

3.2.3. Clicks (Fig. 3)

Very short wideband calls, often produced in series. The tonal component is lacking. On the spectrogram, the clicks are looking as vertical pillars, with intensity steadily decreasing from the lower to the higher frequencies. The values of the peak frequency and power quartiles are the lowest compared to USVs, with most click energy presented in the human-audible range of frequencies (below 20 kHz) (Table 2). The values of entropy are intermediate compared to USVs, the values of bandwidth do not differ from those of the wUSVs. On human' ear, the clicks sound as a clatter.

GLM showed that factors call type and genotype had a significant effect on all acoustic variables (Table 2). At the same time, the degree of discomfort did not influence any acoustic variable. Sex affected three acoustic variables: call duration (was shorter in females), bandwidth (was narrower in males) and entropy (was higher in males). Age affected four acoustic variables: bandwidth and the maximum fundamental frequency (were higher in 8–9 PND pups); the upper power quartile and entropy (were higher in 5–6 PND pups) (Table 2).

DFA correctly classified 96.93 % of the total number of 3222 calls to the correct call type (Table 3, Fig. 4). The average value of the correct classifying was significantly higher ($p < 0.001$) than the random value (33.90 ± 0.42 %). The particular values of correct classification to call type varied from 71.56 % for the wUSV to 99.50 % for the clicks. Analysis of misclassifications showed that nUSVs are misclassified with wUSVs, and the clicks are misclassified with wUSVs (Table 3). The plot based on the first two discriminant functions indicates that wUSVs are

Table 3
Classifying calls of 5-9 PND pup mice to correct call type with Discriminant Function Analysis based on seven measured acoustic variables. Designations: nUSV - narrowband ultrasonic calls; wUSV - wideband ultrasonic calls; Click - click.

Call type	Number of calls assigned to the predicted call type			Total calls	Correctly classified calls, %
	nUSV	wUSV	Click		
nUSV	1380	28	1	1409	97.94
wUSV	13	156	49	218	71.56
Click	0	8	1587	1595	99.50
Total calls	1393	192	1637	3222	96.93

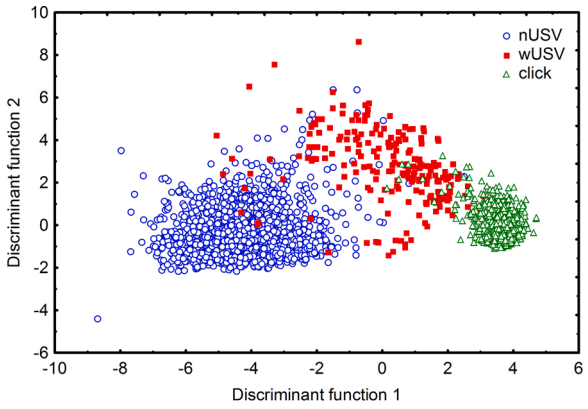

Fig. 4. Scatterplot showing separation produced by the first two discriminant functions of DFA for three call types (nUSV; wUSV, click) of 5-9 PND pup mice. Designations: nUSV – narrowband ultrasonic calls; wUSV – wideband ultrasonic calls; click - clicks.

substantially mixed with nUSVs and clicks, what could be the reason of the lowest value of correct classification for this call type (Fig. 4). Wilks' Lambda values revealed that the variables contributing primarily to discrimination included duration, entropy and q75 (Table 2).

3.3. Effects of discomfort, genotype, sex and age on call rate

Separately for each call type (nUSVs, wUSVs, clicks), we estimated the effects of genotype, sex, age and degree of discomfort (basic *vs* elevated) on call rate (Table 4). The Repeated Measures ANOVA did not reveal any effect of sex on call rate. Genotype affected call rates of clicks, higher in Clstn2-KO than in WT pups (Table 4). Age did not affect on call rate of clicks and wUSVs (Table 4), but affected call rates of nUSVs, with a higher call rate in 8–9 PND pups than in 5–6 PND pups of either genotype at both basic and elevated discomfort (Fig. 5). The only significant difference ($p = 0.01$, Tukey HSD test) was the lower call rate in WT pups at 5–6 PND than at 8–9 PND at elevated discomfort (Fig. 5).

Discomfort affected call rates of nUSVs and clicks, but not wUSVs (Table 4). Call rates of nUSVs and clicks were significantly higher in pups of both genotypes and both sexes under the elevated discomfort, induced by the experimenter' touch compared to the basic level during the isolation stage (nUSVs: 1.08 ± 0.87 *vs* 0.56 ± 0.55 calls/s; clicks: 1.43 ± 1.05 *vs* 1.01 ± 1.25 calls/s), whereas call rate of wUSVs (0.04 ± 0.12 *vs* 0.04 ± 0.12 calls/s) was not affected (Table 4). Although the call rate of wUSVs was three times higher in Clstn2-KO than in WT pups (Table 4), no significant difference was detected. Within-sex comparison of genotypes showed that only male Clstn2-KO pups had a significantly higher call rates of nUSV, wUSVs and clicks than male WT pups under the basic but not under the elevated discomfort, whereas the female pups did not show the differences between genotypes (Table 4).

3.4. Call type usage and the acoustics

We compared the usage of call types (nUSVs, wUSVs, clicks) by calculating the absolute number and percentage of test trials (one per individual pup) in which the given call type was noted. Clicks were detected in all (100 %) trials of both Clstn2-KO and WT pups. The nUSVs were detected in 35 out of 36 (97.2 %) trials with Clstn2-KO pups and in 42 out of 44 (95.5 %) trials with WT pups (one-tailed Fisher exact test, $p = 0.58$). However, wUSVs were used more often by Clstn2-KO pups (in 21 trials out of 36, 58.3 %) than in WT pups (7 trials out of 44, 15.9 %) (one-tailed Fisher exact test, $p = 0.001$).

We have analyzed the complexity of nUSVs produced by Clstn2-KO and WT pups by comparing the percentages of multi-note nUSVs and by comparing the percentages of nUSVs with nonlinear phenomena. The nUSVs were less complex in Clstn2-KO pups, as they more often included only one note (64.0 % *vs* 52.4 %, one-tailed Fisher exact test, $p < 0.001$) and more rarely contained non-linear phenomena than nUSVs of WT pups (33.7 % *vs* 41.4 %, one-tailed Fisher exact test, $p < 0.001$).

Nested ANOVA showed that nUSVs of Clstn2-KO pups were shorter and had a higher peak frequency, power quartiles, entropy, as well as the higher minimum and maximum fundamental frequencies of the tonal component compared to WT pups (Table 5). Only bandwidth values did not differ between nUSVs of Clstn2-KO and WT pups. The wUSVs of Clstn2-KO pups had higher entropy and both the minimum and maximum fundamental frequencies of the tonal component in comparison with WT pups (Table 5). Additionally, clicks of Clstn2-KO pups were longer, higher in the peak frequency and had the higher q25 and q50 power quartiles (Table 5).

4. Discussion

In this study, we characterized acoustic communication in Clstn2-KO pups assessed by the detection of USVs and clicks produced in the context of maternal isolation and described, for the first time, morphometric features of their body. Briefly, Clstn2-KO female pups had lower body weight with shorter bodies, and both sexes had the increased head/body length ratio than their WT counterparts. The analysis of the acoustic communication showed that both male and female Clstn2-KO pups preferred to use the wideband USV calls characterized by higher entropy and emitted more simplified narrowband USV calls with shorter duration and higher peak frequency. Elevated aversive conditions induced by the tactile stimulation similarly increased vocal activity in pups of both genotypes specifically detected by nUSVs and clicks, but not wUSVs.

Table 4
Values (mean ± SD) for call rates (calls/s) in 5-9 PND pup mice at basic (isolation stage) and elevated (touch stage) discomfort, calculated separately for three call types (nUSVs, wUSVs, clicks), and Repeated Measures ANOVA results for the effects of degree of discomfort, genotype, sex and age on call rate. Significant differences are given in bold.

Genotype	WT				Clstn2-KO			
Sex	Males		Females		Males		Females	
Discomfort	Basic	Elevated	Basic	Elevated	Basic	Elevated	Basic	Elevated
nUSVs	0.34 ± 0.48	0.90 ± 0.97	0.67 ± 0.64	1.28 ± 0.82	0.68 ± 0.47 *	1.02 ± 0.66	0.56 ± 0.57	1.10 ± 1.02
Discomfort: $F_{1,72} = 11.03$, $p<0.001$; Genotype: $F_{1,72} = 0.01$, $p = 0.98$; Sex: $F_{1,72} = 1.65$, $p = 0.20$; Age: $F_{1,72} = 11.10$, $p = 0.001$								
wUSVs	0.01 ± 0.01	0.02 ± 0.05	0.03 ± 0.14	0.02 ± 0.07	0.09 ± 0.15 *	0.07 ± 0.14	0.05 ± 0.11	0.06 ± 0.20
Discomfort: $F_{1,72} = 0.01$, $p = 0.98$; Genotype: $F_{1,72} = 1.97$, $p = 0.16$; Sex: $F_{1,72} = 0.12$, $p = 0.73$; Age: $F_{1,72} = 0.0$, $p = 1.0$								
Clicks	0.48 ± 0.47	1.22 ± 0.86	1.08 ± 1.80	1.47 ± 1.34	1.46 ± 0.96 **	1.79 ± 0.81	1.10 ± 1.14	1.30 ± 1.03
Discomfort: $F_{1,72} = 16.96$, $p<0.001$; Genotype: $F_{1,72} = 5.48$, $p = 0.02$; Sex: $F_{1,72} = 0.01$, $p = 0.97$; Age: $F_{1,72} = 0.03$, $p = 0.86$								

* $p < 0.05$.
** $p < 0.01$ for comparison of Clstn2-KO with WT pups of the same sex.

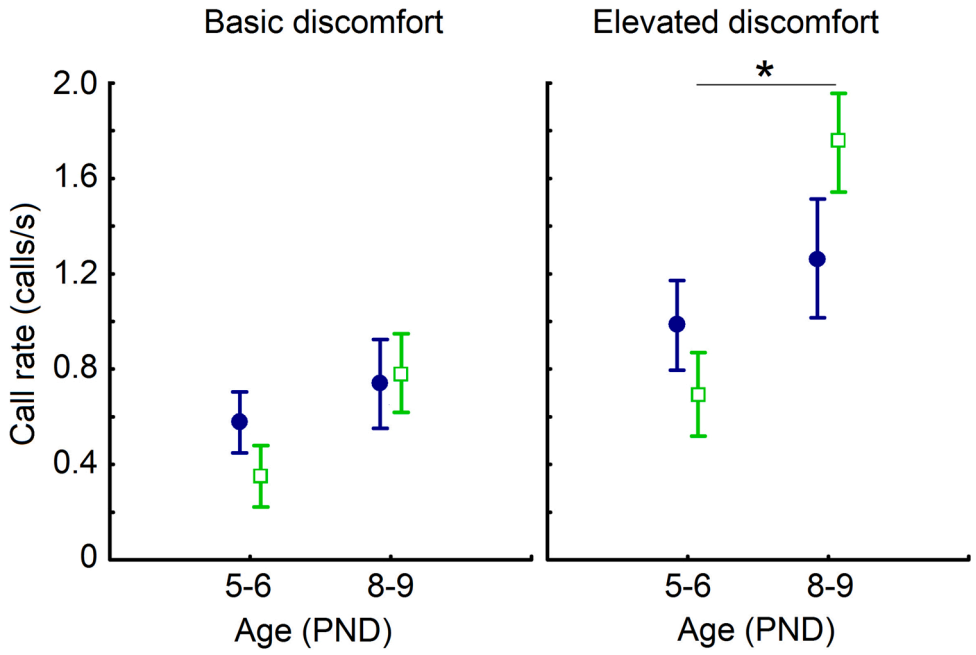

Fig. 5. Call rate of the narrowband ultrasonic calls (nUSVs) in 5-6 PND and 8-9 PND pups of Clstn2-KO (filled circles) and WT (empty squares) mice at basic and elevated discomfort. Central points indicate means, whiskers indicate SE. * - $p = 0.01$, Tukey HSD test.

4.1. Anatomical indexes of body and head

We found a higher ratio between head and body length in Clstn2-KO pups, which was detected due to the smaller body with a comparable head length between the genotypes. A significantly higher growth rate in body length/height and weight was detected in ASD patients [88], so it would be important to track the growth and development of Clstn2-KO mice with expression of ASD-like phenotypes [54–56], in future studies. Although currently little is known about the brain and body development in mouse genetic lines with autism-like behavior, BTBR outbred mice mimicking idiopathic autism-like behavior [89] showed faster acquisition of neurodevelopmental milestones, including the more rapid growth of the body and tail length, faster developed righting reflex, negative geotaxis, the opening of the eyes, or incisor eruption [18]. However, lack of Nlgn4, autism-related synaptic-adhesion protein in mice, did not cause alterations in anatomical and neurophysiological development [19]. Interestingly, the analysis of mouse embryos with the reduced KCTD13 gene expression on ~70 % revealed macrocephaly accompanied by increased neuronal proliferation and no changes in apoptosis [90], resembling the increased head size in autistic children with 16p11.2 deletion [91]. Hence, the detailed characterization of neurodevelopmental phenotypes in the Clstn2-KO genetic mouse line would shed a light on the Clstn2-dependent molecular mechanisms underlying anatomical phenotypes related to autism in early childhood.

4.2. The acoustic features of nUSV, wUSV and clicks in Clstn2-KO pups

Juvenile pups when briefly isolated from their mothers produce frequency-modulated tones in the high ultrasonic range, accompanied by clicks [21]. We characterized vocalization in our experimental pups as nUSV, wUSV calls and clicks, based on the range of acoustic variables, including *e.g.* duration, frequency, and entropy.

Both Clstn2-KO and WT mice emitted nUSVs similarly often (97.2 % *vs* 95.5 %, respectively), but nUSVs have been characterized by their shorter duration, higher peak frequency, power quartiles and entropy together with higher fundamental frequencies of the tonal component in Clstn2-KO pups. Moreover, Clstn2 deficient pups elicited the simplified, 1-note nUSVs rather than multi-note nUSVs, more often than WT pups, directly supporting the role of Clstn2 in language development in healthy populations [61–63] and patients with ASD who expressed delayed language skills [57].

At the same time, Clstn2-KO pups elicited wUSV calls three times more often than the WT pups (58.3 % *vs* 15.9 %), which reflected their unstructured vocalization. However, the acoustic structure of wUSVs displayed only minor differences between Clstn2-KO and WT pups, displaying higher entropy and higher minimum and maximum fundamental frequencies of the tonal component in wUSVs of Clstn2-KO pups. Altogether, nUSVs and wUSVs features of Clstn2-KO pups indicate the impaired vocalization. Notably, one-year old babies who would later be diagnosed with autism expressed a different pattern of cry, characterized by less waveform modulation and more dysphonation compared to healthy children [25].

Clicks were detected in 100 % of trials emitted by pups of both genotypes. However, clicks of Clstn2-KO pups were longer, higher in peak frequency and had higher lower and medium power quartiles. Interestingly, FOXP2-R552H homozygous mice, carrying the point mutation associated with inherited speech and language disorder in humans [92], also expressed more pronounced clicks [11], supporting clicks as a new acoustic phenotype related to the language disorders.

4.3. Discomfort-reactivity and USVs

The additional two-minute maternal separation coupled with stimulation of pups by touching, providing the elevated level of discomfort in our experimental pups [76], increased USVs rate in pups of both genotypes regardless of sex. More precisely, discomfort robustly increased the rate of nUSVs and clicks in all experimental animals but did not affect wUSV emission. Hence, these findings directly support categorical perception mechanisms of social infant-mother interaction [93], suggesting that nUSVs and clicks carry a more significant value of social communication than noise-contained wUSVs. The precise mechanisms of such a phenomenon are currently unknown and it remains to be explored in future experiments. Previously, the higher level of low-frequency ultrasonic calls was found in adult male wild-type C57BL/6 J mice in the context of elevated discomfort [94].

Accumulating studies showed that isolation-induced USV that are emitted by pups when they are separated from their mothers and littermates is a sensitive tool to assess neurodevelopment of social

Table 5
Values (mean ± *SD*) of the acoustic variables for three call types (nUSVs; wUSVs, clicks) in Clstn2-KO and WT pups at 5-9 PND and nested ANOVA results for the effect of genotype (with individual nested in genotype). Significant differences are given in bold. Designations: Duration - call duration; fpeak - the peak (maximum amplitude) frequency; bndw - call bandwidth at minus 10 dB from the maximum; q25, q50, q75 - the lower, the medium and the upper quartile of call power spectrum (covering respectively 25 %, 50 % and 75 % of call energy); entropy - call entropy; f0min - the minimum fundamental frequency; f0max - the maximum fundamental frequency.

Call type	nUSV		wUSV		Click	
Genotype	Clstn2-KO (n = 653)	WT (n = 756)	Clstn2-KO (n = 155)	WT (n = 63)	Clstn2-KO (n = 724)	WT (n = 871)
Duration (ms)	27.8 ± 21.4	38.9 ± 24.8	48.5 ± 26.0	43.0 ± 27.8	1.54 ± 0.31	1.39 ± 0.17
	$F_{1,1331}$ = 93.65, p<0.001		$F_{1,191}$ = 0.63, p = 0.43		$F_{1,1515}$ = 257.5, p<0.001	
fpeak (kHz)	78.43 ± 15.8	71.60 ± 15.4	44.94 ± 19.9	41.47 ± 21.2	10.80 ± 9.0	9.44 ± 7.1
	$F_{1,1331}$ = 74.30, p<0.001		$F_{1,191}$ = 0.01, p = 0.93		$F_{1,1515}$ = 13.93, p<0.001	
bndw (kHz)	4.32 ± 2.8	4.21 ± 2.6	8.93 ± 7.6	7.73 ± 6.2	8.04 ± 3.4	8.08 ± 3.8
	$F_{1,1331}$ = 1.62, p = 0.20		$F_{1,191}$ = 0.42, p = 0.52		$F_{1,1515}$ = 0.11, p = 0.75	
q25 (kHz)	73.42 ± 17.6	68.89 ± 15.0	27.70 ± 8.3	27.68 ± 8.8	9.69 ± 5.3	8.78 ± 4.1
	$F_{1,1331}$ = 31.78, p<0.001		$F_{1,191}$ = 0.09, p = 0.77		$F_{1,1515}$ = 20.18, p<0.001	
q50 (kHz)	79.53 ± 15.1	73.03 ± 15.0	44.55 ± 10.3	41.49 ± 12.9	15.11 ± 9.1	14.15 ± 7.2
	$F_{1,1331}$ = 83.09, p<0.001		$F_{1,191}$ = 0.18, p = 0.68		$F_{1,1515}$ = 7.27, p = 0.007	
q75 (kHz)	87.09 ± 16.2	78.61 ± 16.9	61.39 ± 11.4	56.30 ± 11.9	27.75 ± 12.5	28.56 ± 10.1
	$F_{1,1331}$ = 126.08, p<0.001		$F_{1,191}$ = 2.83, p = 0.09		$F_{1,1515}$ = 3.19, p = 0.07	
entropy	0.26 ± 0.10	0.22 ± 0.08	0.51 ± 0.1	0.43 ± 0.1	0.37 ± 0.1	0.37 ± 0.1
	$F_{1,1331}$ = 79.95, p<0.001		$F_{1,191}$ = 19.01, p<0.001		$F_{1,1515}$ = 0.59, p = 0.44	
f0min (kHz) *	69.08 ± 16.3	61.71 ± 14.9	21.36 ± 6.9	16.97 ± 5.6		
	$F_{1,1331}$ = 89.01, p<0.001		$F_{1,132}$ = 7.82, p = 0.006			
f0max (kHz) *	88.94 ± 15.2	86.66 ± 16.5	32.40 ± 6.5	27.90 ± 4.8		
	$F_{1,1331}$ = 14.27, p<0.001		$F_{1,132}$ = 11.22, p = 0.001			

* n = 111 for the wUSVs of Clstn2-KO pup mice and n = 45 for the wUSVs of WT pup mice.

communication and emotional reactivity at early-life period [83,95–97]. Isolation-induced USVs in mouse pups are very important for mother-infant interactions as pups increased USV call rate in the first week of life, but then decreased in the second postnatal week [18,38]. We found that in both Clstn2-KO and WT pups, call rate was lower at 5–6 PND than at 8–9 PND. At the same time, in most studies the peak of pup USV call rate occurred at 5–6 PND, with decrease of call rate to 8 PND and subsequent days of age [18,40,41,98]. However, some mice strains displayed the peak of pup USV call rate at 8–9 PND [99]. A delayed peak of call rate compared to WT control only was reported for Down syndrome pup mice [100]. In other studies, including our study, the peak of call rate was found coinciding between the control and deficit mice strains [40,41,98,99].

The communication value of pup USVs was demonstrated for the first time in 1956, when mothers receiving isolation-induced USVs, collected vocalizing pups back to the nest [101]. The idea was further confirmed, using tape recordings of mouse pups at PND 5 played back to lactating females as the stimulating signal in the elegant experiment [22]. Currently, multiple evidences further support the communication function of pup USVs, which can induce nest building, pup retrieval or nursing in mothers [24,102,103]. Mothers respond to variety of ultrasonic stimuli within a certain frequency range, indicating categorical perception mechanisms of such infant-mother interaction [93].

Emission of isolation-induced USVs strongly depends from genetics and shows a big variation between inbred strains [18,38,47] and gene-modified lines [17,18,50,104]. The increased USVs rate detected in Clstn2-KO pups may reflect their enhanced emotional state [105]. For instance, anxiolytic-like compound, allopregnanolone, reduced USV rate in rat pups [48], or diazepam and chlordiazepoxide decreased USVs in mouse pups [49]. The reduced emission of USVs was detected in 5-HT genetically modified mice [17,50], including the tryptophan hydroxylase 2 (Tph2) null mutant (Tph2-/-) pups, which also displayed deficits in call clustering and temporal organization in the emission of isolation-induced USV [17]. In opposite, the increased USVs were recorded in *e.g.* mouse model of Angelman's syndrome with increased anxiety [104].

The body thermoregulation also plays an important role in USVs production since it is not mature yet at the early stage of neurodevelopment [76,106,107] and the role of Clstn2 gene remains unknown in this physiological process. However, mice lacking L1 adhesion molecule, expressed the decreased nociceptive heat sensitivity *via* L1 interaction with NMDA receptors [108], which regulate nociception and plasticity. Furthermore, NMDAR antagonists, memantine and neramexane, in low doses enhanced USVs in mouse pups [49]. Hence, given the ability of Clstn2 to reduce the density and functionality of GABAergic inhibitory interneurons [54] and tight regulations between GABA and NMDA systems [109], these studies suggest the potential role of Clstn2 to regulate USVs production through NMDAR-dependent mechanisms.

Some recent studies indicate that in laboratory rat *Rattus norvegicus*, strains selected for vocal behavior (*e.g.*, high rates of USVs in pups) display some changes in vocal morphology in adults [110,111]. In addition, small lesions to the ventral pouch in laboratory rats cause dramatic changes in USV production [71]. From these studies, we can infer a possibility that differences in USV production of the KO mice could reflect abnormal oral morphology or musculature, given the differences seen in general body morphology. This unlikely but possible cause can be investigated in future through morphological oral-nasal evaluation of post mortem samples.

5. Conclusions

Altogether, our current study discovered novel acoustic phenotypes in early-life neurodevelopment period in Clstn2-KO pups, further supporting the idea that this genetic mouse line resembles ASD-like behavior. Isolation-induced USV emission in Clsnt2-KO pups was characterized by production of less complex nUSVs with shorter duration, higher peak frequency and more often usage of one-note calls. Clstn2 deficient pups preferred to communicate *via* wUSVs emission characterized by its high entropy, together with production clicks with longer duration and higher peak frequency and power quartiles. Besides the USVs and clicks features, we also detected the increased head-to-body ratio in Clstn2-KO pups at PND 5–9, which is in agreement with clinical studies [88]. Future studies are essential to precisely explore the role of Clstn2 gene in pathological molecular-cellular and neurobiological mechanisms related to autism during neurodevelopment to improve its early-life diagnostics and preventive therapy.

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Declaration of Competing Interest

The authors have no competing interests to declare.

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Appendix A. Supplementary data

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