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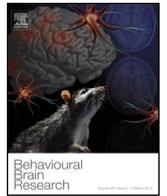
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Neurobiology of Autism Special Issue paper

## The Autism *ProSAP1/Shank2* mouse model displays quantitative and structural abnormalities in ultrasonic vocalisations



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### HIGHLIGHTS

- High level of organisation in sequences of ultrasonic vocalisations.
- Very limited sex-related variations in mouse ultrasonic vocalisations.
- Lower peak frequency in ultrasonic vocalisations of *ProSAP1/Shank2*<sup>-/-</sup> mice.

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### ABSTRACT

Mouse ultrasonic vocalisations have been often used as a paradigm to extrapolate vocal communication defects observed in patients with autism spectrum disorders (ASD). The role of these vocalisations as well as their development, structure and informational content, however, remain largely unknown. In the present study, we characterised in depth the emission of pup and adult ultrasonic vocalisations of wild-type mice and their *ProSAP1/Shank2*<sup>-/-</sup> littermates lacking a synaptic scaffold protein mutated in ASD. We hypothesised that the vocal behaviour of *ProSAP1/Shank2*<sup>-/-</sup> mice not only differs from the vocal behaviour of their wild-type littermates in a quantitative way, but also presents more qualitative abnormalities in temporal organisation and acoustic structure. We first quantified the rate of emission of ultrasonic vocalisations, and analysed the organisation of vocalisations sequences using Markov models. We subsequently measured duration and peak frequency characteristics of each ultrasonic vocalisation, to characterise their acoustic structure. In wild-type mice, we found a high level of organisation in sequences of ultrasonic vocalisations, suggesting a communicative function in this complex system. Very limited significant sex-related variations were detected in their usage and acoustic structure, even in adult mice. In adult *ProSAP1/Shank2*<sup>-/-</sup> mice, we found abnormalities in the call usage and the structure of ultrasonic vocalisations. Both *ProSAP1/Shank2*<sup>-/-</sup> male and female mice uttered less vocalisations with a different call distribution and at lower peak frequency in comparison with wild-type littermates. This study provides a comprehensive framework to characterise abnormalities of ultrasonic vocalisations in mice and confirms that *ProSAP1/Shank2*<sup>-/-</sup> mice represent a relevant model to study communication defects.

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### 1. Introduction

Autism spectrum disorders (ASD) are characterised by impairments in reciprocal social communication, restricted interests and repetitive behaviours [1]. Cognitive and language deficits are often observed, but not necessarily present. The causes of ASD remain largely unknown [2]; however, twin and family studies suggest a strong genetic contribution [3]. Many studies have now demonstrated that mutations in genes coding synaptic proteins are playing an especially important role in ASD

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(reviewed in [4]). For example, an increasing number of studies highlights the association between ASD and mutations in the post-synaptic scaffolding proteins SHANK1, PROSAP1/SHANK2 and PROSAP2/SHANK3 (*PROSAP2/SHANK3* [5–7]; *PROSAP1/SHANK2* [8–10]; *SHANK1* [11]). Mouse models carrying mutations in these genes have been generated to unravel the mechanisms underlying ASD phenotypes. These mice present specific synaptic abnormalities such as a reduction of the number of synapses for *ProSAP1/Shank2*<sup>-/-</sup> and *ProSAP2/Shank3*<sup>-/-</sup> [12–16] or a reduction of large head dendritic spines for *Shank1*<sup>-/-</sup> mice [17–19]. Remarkably, these mice also present abnormalities in social and vocal behaviours, and stereotyped behaviours reminiscent of the clinical features observed in patients with ASD (reviewed in [14]; see also [16,20]). *ProSAP2/Shank3*<sup>-/-</sup> and to a lesser extent *ProSAP1/Shank2*<sup>-/-</sup> mice displayed increased self-grooming in comparison with wild-type littermates. In contrast, all three models, *Shank1*<sup>-/-</sup>, *ProSAP1/Shank2*<sup>-/-</sup> and *ProSAP2/Shank3*<sup>-/-</sup> mice, displayed reduced interest for social interactions either in the three-chambered test or in free social interactions and quantitative abnormalities in the emission of ultrasonic vocalisations [12–19]. These mice are especially useful for the analysis of cellular/synaptic defects caused by their mutations; however, the relevance of these mouse models in the study of abnormalities in social interactions and communication remains to be established.

In patients with ASD, vocal communication deficits range from the complete absence of spoken language to just a delayed development or slower acquisition. When spoken language is present, deficits can occur in syntax (abnormal sentence construction), semantics (use of words with an unusual meaning), and to a larger extent in prosody (unusual or incongruous intonation, unusual tempo), phonology (abnormal systematic organisation of sounds) and/or pragmatics (reduced understanding of the way in which contexts contribute to the meaning, inadequacy of interventions, echolalia [21]; reviewed in [22]). Only a few studies have investigated in detail the abnormalities in the acoustic structure of speech. They have highlighted an abnormal control of pitch and volume, an increased number of syllables with atypical phonation, a larger pitch range (reviewed in [23]), and a larger variability in pitch [23].

Wild-type mice have been known to produce abundant vocal signalling in the ultrasonic range. Pups isolated from dam and littermates during their first two weeks of life emit a large number of ultrasonic vocalisations [24], which serve to trigger the mother's retrieval behaviour (e.g., [24–26]). Ultrasonic vocalisations have been also reported in adult males in the presence of an oestrus female or female pheromones (e.g., reviewed in [27,28]) and when a female mouse encounters an unknown female conspecific (e.g., juveniles [29]; adults: [30]; reviewed in [31]). These vocalisations appear to function as signals for maintaining social proximity [32] or for determining social hierarchy [30] in male–female and in female–female interactions, respectively. More recently, ultrasonic vocalisations have been observed during the encounter of an adult male (socially isolated for several days) with another male (e.g., [33,34]). One of the main aims of the current studies on mouse vocal communication is to determine the type of information encoded in the different call types and in their organisation within sequences, refining the functions of ultrasonic vocalisations in adult mice.

The study of ultrasonic vocalisations in mouse models for ASD would benefit from the analytical methods and the knowledge acquired in these previous studies, and vice versa [28]. On the one hand, the analytical methods used to study vocal behaviour in wild-type mice can provide a thorough characterisation of the vocal communication of mouse models carrying known genetic mutations. On the other hand, mouse models carrying identified genetic mutations could help to determine some of the genetic

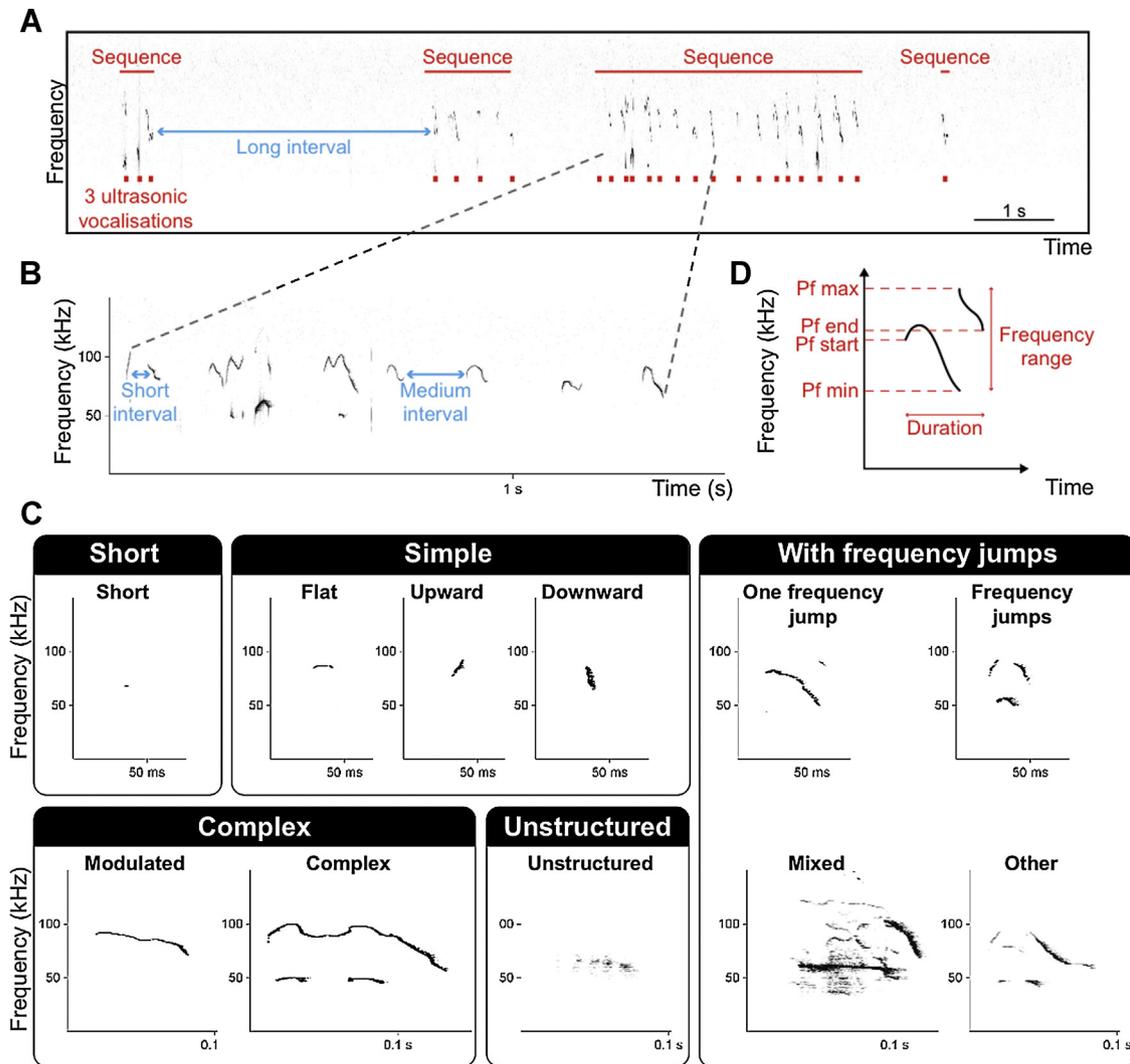
and neural bases of vocal communication abilities. To date, only relatively simple methods have been used to characterise the ultrasonic vocalisations in mouse models of ASD, mostly a quantification of call rate [35] and in a few studies the call distribution (e.g., [14,34,36]; reviewed in [37]). To our knowledge, none of these studies has investigated whether the mutation affects the organisation of sequences of ultrasonic vocalisations.

Here we report an in-depth characterisation of the ultrasonic vocal behaviour of *ProSAP1/Shank2*<sup>-/-</sup> mice and their wild-type littermates. We further analyse data recorded on the first characterisation of this mouse model, using a refined dataset [14]. Indeed, ultrasonic vocalisations were recorded in pups and adults. In pups, *ProSAP1/Shank2*<sup>-/-</sup> females called at a significantly higher rate than wild-type females at P4 and at P10, but there were no significant differences at P6. In adults, a significantly longer latency to emit the first vocalisation was observed in pairs involving a *ProSAP1/Shank2*<sup>-/-</sup> mouse compared with pairs involving wild-type mice. Only pairs involving a *ProSAP1/Shank2*<sup>-/-</sup> adult female emitted fewer ultrasonic vocalisations than pairs involving wild types. Notably, in both female–female and male–female interactions, pairs involving *ProSAP1/Shank2*<sup>-/-</sup> mice used a repertoire different from the one used in pairs involving two wild-type mice ([14]; see also [16]). Therefore, in the present study, we examined usage (call rate, temporal organisation, sequence organisation, repertoire; Fig. 1A–C) and acoustic structure (duration, frequency characteristics; Fig. 1D) of ultrasonic vocalisations of mice during social isolation at P2, P6, P10 and during social interactions at the adult stage (male–oestrus female and female–female; Suppl. Figure 1). We tested for differences between age classes, between sexes and between genotypes in pups and between sexes and genotypes in adults.

## 2. Materials and methods

### 2.1. Mouse breeding and housing

*ProSAP1/Shank2*<sup>-/-</sup> mice were generated at the Institute for Anatomy and Cell Biology at Ulm University (Ulm, Germany). Their generation and first behavioural characterisation were described elsewhere [14]. At the Institut Pasteur, mice were housed in a colony room maintained at 23 ± 1 °C on a 12:12 h light/dark cycle, with lights on at 8:00 AM. All experiments were conducted between 9:30 AM and 6:00 PM. Two cohorts of mice were generated. Cohort 1 (backcrossed for 10 generations on C57BL/6) underwent a developmental study from P2 to P12. A second cohort was generated to test adult behaviour while avoiding the potential behavioural effects of repeated early manipulations. Cohort 2 included 4–6 month old adult mice (backcrossed for 11 generations on C57BL/6). All mice were weaned at 4 weeks of age. Males were housed individually from weaning on, given their high aggressiveness towards each other (no other solution such as excluding the apparently dominant animal or introducing enrichment tubes was efficient). Females were housed in groups of 2–4 mice (mixed genotypes). In both cohorts, we tested the offspring of *ProSAP1/Shank2*<sup>+/-</sup> mice crossings (i.e., wild-type and *ProSAP1/Shank2*<sup>-/-</sup> littermates; data from *ProSAP1/Shank2*<sup>+/-</sup> mice were not included in the present study since no significant difference in their vocal behaviour was found in comparison with wild-type littermates [14]). The experimenters were blinded to the genotype of the tested animals for data collection (pups were only genotyped after the experiment; genotypes of adults were not included in cage labels) and analysis (the experimenters had access to only the name of the animals when analysing audio files). The ethical committee of Ile-de-France approved all behavioural procedures (CEEA Ile-de-France Comité 1).



**Fig. 1.** Usage and structure of ultrasonic vocalisations in mice. A. Spectrogram of four sequences of ultrasonic vocalisations of a P4 pup. Variables related to call usage are measured: call rate (i.e., the number of ultrasonic vocalisations per time unit), and intervals between sequences of ultrasonic vocalisations (long intervals). B. Spectrogram of a part of a sequence, with short and medium intervals between ultrasonic vocalisations within a sequence. C. Presentation of the five call types (“short”, “simple”, “complex”, “unstructured”, and “with frequency jumps”), with spectrograms of examples of vocalisations within each call type. D. Acoustic variables used to define the acoustic structure of each ultrasonic vocalisation in the present study: duration, peak frequency at the beginning (Pf start) and at the end (Pf end) of the call, maximum (Pf max) and minimum (Pf min) peak frequency within the call, and the frequency range (Pf max–Pf min).

## 2.2. Behavioural procedures

For the developmental study (as described in [14]), parent males were separated from pregnant females 1 or 2 days before birth. Births were checked each morning and evening. Pups of both sexes (cohort 1) were individually identified with long-lasting sub-cutaneous tattoos (green tattoo paste, Ketchum Manufacturing Inc., Ottawa, Canada) on the paws on post-natal day 1 (P1). For audio-recordings (sampling frequency: 300 kHz, FFT-length: 1024 points, 16-bit accuracy), each pup was isolated from dam and littermates and placed in a small enclosure with soft plastic surface (cleaned with 10% ethanol between each pup) in a soundproof chamber (temperature  $T = 24 \pm 1$  °C). Audio recordings lasted 5 min and were conducted every 2 days (P2, P4, P6, P8, P10 and P12), in a random order for each litter. Only data from P2, P6 and P10 were analysed in depth for the present study. We selected P2 as the very first recordings of pups, at the beginning of pup development, and P6 as the mid-development point. We chose P10 as being a time point towards the end of the time period in which pups emit isolation calls at a rate high enough to provide robust data. All pups from each litter were recorded. We

subsequently randomly chose 2–6 pups per litter to be included in the present analyses (randomly chosen after genotyping from the data set of [14]). We analysed 10 pups per genotype and per sex (but only eight *ProSAP1/Shank2*<sup>-/-</sup> female pups) that could be individually followed in the three age classes studied. Recording hardware (UltraSoundGate 416-200, Condenser ultrasound microphone Polaroid/COMPA) and software (Avisoft SASLab Pro Recorder) were from Avisoft Bioacoustics (Berlin, Germany).

To examine adult vocal behaviour (as described in [14]), mice of both sexes aged between 4 and 6 months (cohort 2) were recorded in a soundproof chamber first during same-sex social interactions in the resident–intruder test (male–male and female–female) and second during the interaction of a male with an oestrus C57BL/6 female from Charles River Laboratory. Tested females were isolated 3 days before the resident–intruder test to increase their motivation for social interactions. At least 3 weeks elapsed between the two consecutive tests for males. We tested and analysed 16 mice per genotype and per sex (but only 13 *ProSAP1/Shank2*<sup>-/-</sup> females). Between each mouse, the experimental cage was emptied from bedding, cleaned with soap water, dried, and new fresh bedding was used.

### 2.2.1. Resident–intruder test

The tested mouse was left for 30 min for habituation in the experimental cage (Plexiglas, 50 × 25 × 30 cm; 100 lx; clean sawdust bedding [38]). After this time, an unfamiliar C57BL/6 mouse of the same sex was introduced. The two animals were allowed to freely interact for 4 min. Ultrasonic vocalisations were recorded with the equipment and the same settings as described above. However, very few ultrasonic vocalisations were emitted during male–male interactions (see [14]) in comparison with previous studies [33,39]. Resident males were very aggressive towards intruders, probably because they were isolated from weaning on. Therefore, we did not consider data from male–male interactions in this study. In the female–female interaction context, ultrasonic vocalisations are known to be uttered mainly by the tested resident animal habituated to the cage, based on previous experiments with anaesthetised animals (e.g., [40]). We therefore make the assumption that the vocalisations recorded were mainly from the resident female.

### 2.2.2. Male–female interactions

All males had a previous experience of 3 days with a mature female, at least 2 days before the experiment. The tested male was left in the experimental cage (Plexiglas, 50 × 25 × 30 cm; 100 lx; clean sawdust bedding) for 10 min habituation. An unfamiliar C57BL/6 female in oestrus (confirmed through vaginal smears) was then introduced. In this context, the vocal contribution of the female is very limited, based on previous experiments with anaesthetised or devocalised animals (e.g., [40,41]). We therefore make the assumption that the vocalisations recorded were mainly from the male. Ultrasonic vocalisations were recorded for 3 min with the same equipment and settings as cited above.

### 2.3. Analyses of audio recordings

In the pup developmental study, we detected USV automatically (pulse train detection analyses from Avisoft SASLab Pro, Avisoft Bioacoustics, Germany; hold time: 7 ms). Files were additionally verified by a trained experimenter and detection was adjusted if necessary. For vocalisations recorded in adult animals, we manually detected the calls using the software Avisoft SASLab Pro (Avisoft Bioacoustics, Germany; 75% overlap; time resolution: 0.853 ms; frequency resolution: 293 Hz; Hamming window).

For both pups and adults, we labelled all calls using the labelling function of Avisoft SASLab Pro to build the vocal repertoire of each individual. Each call was classified manually in one call type over five. These call types regrouped call subtypes from [14] based on [36] to improve analyses clarity, especially when using Markov models. Call types were defined based on characteristics of duration, frequency modulations (i.e., frequency range covered by the vocalisation) and frequency jumps (Fig. 1C):

- **Short:** duration shorter than 5 ms and frequency range  $\leq 6.25$  kHz.
- **Simple:** duration longer than 5 ms and frequency range  $\leq 6.25$  kHz (flat), or frequency modulation in only one direction (upward or downward) with frequency range  $> 6.25$  kHz.
- **Complex:** frequency modulations in more than one direction and frequency range  $> 6.25$  kHz (modulated), or inclusion of one or more additional frequency components (harmonic or non-linear phenomena, but no saturation) but no constraint on frequency range (complex).
- **Frequency jumps:** inclusion of one jump (one frequency jump) or more jumps (frequency jumps, others) in frequency without time gap between the consecutive frequency components, with (mixed) or without any noisy part within the pure tone call.
- **Unstructured:** no pure tone component identifiable; “noisy” calls.

The repertoire, the temporal organisation of sequences (successive inter-call intervals, types of inter-call intervals) and the sequence structural organisation (successive call types) were evaluated on the complete dataset, including all calls recorded and labelled (see Suppl. Table I).

To describe the acoustic structure, we manually measured the acoustic variables, to be able to collect them even if recordings were perturbed by background noise, especially in adults. Duration was measured on the spectrogram of each call, using the labelling function of Avisoft SASLab Pro. Therefore, duration was measured for all calls on the complete dataset (see Suppl. Table I). Acoustic variables related to peak frequency (i.e., frequency with the highest amplitude) (maximum, minimum, at the beginning of the call, at the end of the call) were measured manually only for short, simple (flat, upward, downward), complex (modulated, complex) and frequency jumps (one frequency jump, frequency jumps; see Fig. 1C and D). Mixed, unstructured, saturated calls and those combining characteristics of two call types were not considered. We also calculated the frequency range representing the frequency domain covered by the vocalisation, i.e., the difference between the maximum and the minimum peak frequency. We measured these variables on the first 3–62 calls of each type to avoid any excessive contribution of one animal to the data set and calculated the average value for each call type and each animal. When an animal emitted less than three vocalisations of a type, we excluded this call type from this animal from the analysis to base our average calculation on a minimal number of vocalisations (here three vocalisations of one call type; see Suppl. Table I).

### 2.4. Sequence analyses

Overall, we tested for differences between the probability of an event in a sequence (for example the probability to observe a given call X,  $P(X)$ ) and the distribution of the same event but in a special temporal situation, for example the first call of the sequence  $P(X_{\text{first}})$ . We then tested the null hypothesis  $H_0$  that  $P(X) = P(X_{\text{first}})$ . If  $H_0$  was rejected, we concluded that there was a specific temporal organisation in the sequence and that for example the probability to observe X was increased when the first event of the sequence was considered. If  $H_0$  was not rejected, it indicated that statistically there was no temporal organisation and that the probability to observe the event X was the same whatever the position of the event within the sequence. Two aspects of the organisation of sequences were analysed.

1/The first aspect consisted of symbolic chains of time intervals between successive ultrasonic vocalisations. Time intervals were coded with three symbols, i.e., short (SI), medium (MI) and long (LI) intervals. Considering these three interval categories, a sequence of calls is defined as ultrasonic vocalisations separated by short or medium intervals. Sequences were thus separated from each other by long intervals. Different variables were thus calculated. The length of a call sequence (i.e., the number of ultrasonic vocalisations in this sequence), the proportion of sequences with more than three calls and the probability of occurrence of a given interval  $X = \text{SI or MI}$ , given by  $P(X) = n(X)/(n(\text{SI}) + n(\text{MI}))$ , where  $n(\text{SI})$  represents the total number of SI intervals (similarly for MI). These probabilities were used as references to compare with:

- $P(X|\text{SI})$ : the probability to observe  $X = \text{SI or MI}$  after a short interval (SI).  $P(X|\text{SI}) = n(X|\text{SI})/(n(\text{SI}|\text{SI}) + n(\text{MI}|\text{SI}))$ , where  $n(X|\text{SI})$  is the number of consecutive SI-X intervals. A difference between  $P(X|\text{SI})$  and  $P(X)$  indicates that X is not independent from the previous interval.
- $P(X_{\text{first}})$  and  $P(X_{\text{last}})$ : the probability to begin and end a sequence by X, respectively. Again a difference among  $P(X_{\text{first}})$  or  $P(X_{\text{last}})$

with  $P(X)$  indicates temporal organisation (i.e., non-random sequence construction).

2/In the second step, sequences of calls (coded by a catalogue of five call types  $X = \{a, b, c, d, e\} = \{\text{short, simple, complex, unstructured, frequency jumps}\}$ ) were analysed. Sequences of ultrasonic vocalisations were thus considered as Markov chains. In this context, information theory was used to quantify the distribution of call categories and the time structure at a two-vocalisation series level. We generalised the analysis performed on a two-symbol sequence of interval length (see above). In a zero-order Markov process (measured by the entropy rate  $H1$ ), the occurrence of a vocalisation ( $n + 1$ ) does not depend on the previous one ( $n$ ), but only on the proportion of the different call categories, that is,  $P(X|a) = P(X|b) = P(X|c) = P(X|d) = P(X|e) = P(X)$ . In contrast, in a first-order process, the transition probability from a vocalisation ( $n$ ) to the next vocalisation ( $n + 1$ ) depends only on the vocalisation ( $n$ ) and not on the previous ones ( $n - 1, n - 2, \dots$ ), that is,  $P(X|a) \neq P(X|b) \neq P(X)$ . The entropy rate of a zero-order Markov process is defined as  $H1 = -\sum p(X) \log_2(p(X))$  with  $X = \{a, b, c, d, e\}$ . The more uniform the distribution among the five call types, the greater the disorder of the system, and therefore the higher  $H1$  is. The entropy rate of a first-order Markov process is defined as  $H2 = -\sum p(X) \sum p(X|Y) \log_2 p(X|Y)$  with  $X = Y = \{a, b, c, d, e\}$ , which measures the organisation at the two-vocalisation series level. We calculated  $H1$  and  $H2$  for sequences of more than three calls. A decrease of the entropy between the two entropic orders  $H1$  and  $H2$  indicates at least a first-order Markov process, and thus a temporal organisation of the sequences of call types.

### 2.5. Statistical analyses

To examine the rate of emission, the length of call sequences, the distribution of the five call types (repertoire), call duration and call frequency characteristics, we used an ANOVA with repeated measures over age classes for pups, with age class as a within-subject factor and genotype and sex as between-subject factors. If sex did not have a significant effect, the ANOVA was re-conducted without this factor. It is used in conjunction with paired Wilcoxon signed rank tests for post hoc testing between age classes within wild-type pups and with non-paired Wilcoxon rank sum tests between genotypes within age classes. For adults, we used non-paired Wilcoxon rank sum tests for these variables (except call repertoire) to examine sex-related differences in wild-type mice, as well as genotype-related differences separately for males and females (given the difference in the context of vocalisation elicitation). We used chi-squared tests to compare the usage of the different call types (repertoire) in adult mice.

We used ANOVA (as above) and paired Student's *t*-tests when paired comparisons were made for the temporal succession of the interval types within sequences, the type of intervals at the beginning and at the end of a sequence, and the position of the call types within sequences. Entropies were compared using Student's *t*-tests. We used a sequential Bonferroni adaptation to counteract the problem of multiple comparisons and control the family-wise error rate. For example, when three null hypotheses are tested with  $\alpha = 0.05$ , unadjusted *p*-values ( $\pi$ ) obtained from the three *t*-tests were ordered by rank. The smallest were compared with  $0.05/3$  and the null hypothesis was rejected if  $\pi < 0.05/3$ . The second *p* value is compared with  $0.05/2$  and the last with  $0.05$ .

In some cases (concerning the organisation of sequences), the probability cannot be estimated because too few calls were recorded for one individual. These individuals were therefore not taken into account in the statistics. The number of individuals considered for each statistic is systematically indicated using the *X/Y* annotation, *X* being the number of estimations and *Y* the number

of mice. For example, in P2 pups,  $12.5 \pm 1.74, n = 17/18$  indicated that the variable has been estimated including 17 over 18 pups at P2. This problem appeared particularly for *ProSAP1/Shank2*<sup>-/-</sup> adult mice that exhibit a reduced number of call sequences.

All analyses were conducted with the computing and statistical software R (R Developmental Core Team 2009).

## 3. Results

### 3.1. Definitions of usage and structure of ultrasonic vocalisations

An ultrasonic vocalisation is a vocal utterance with energy distributed in the ultrasonic range of frequencies, usually concentrated in one main frequency at each instant (pure tone). These vocalisations encompass additional frequency components (harmonic or non-linear) or noisy parts in some cases. The usage of ultrasonic vocalisations is defined as the number of vocalisations uttered within a given time (call rate), the distribution over time (temporal organisation, which allows defining a sequence of ultrasonic vocalisations), the proportion of the different call types emitted (vocal repertoire) and the succession of the different call types within a sequence (sequence composition; Fig. 1A–C). To build the vocal repertoire, we determined five call types according to their duration, frequency modulations and frequency jumps (see Section 2 and Fig. 1C). The structure of ultrasonic vocalisations is defined by their acoustic characteristics, such as duration, peak frequency characteristics and frequency range (Fig. 1D).

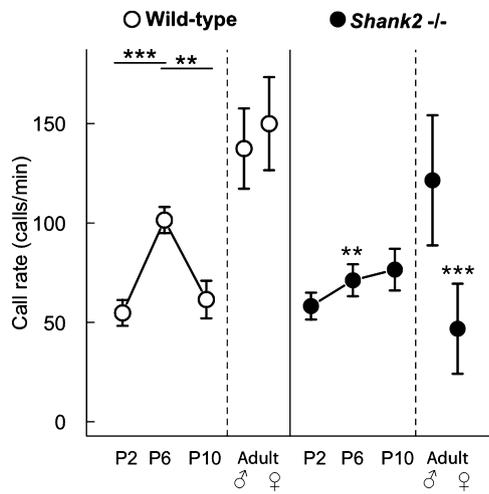
### 3.2. Call usage I: age- and genotype-related variations in call rate

We measured the rate of emission of ultrasonic vocalisations during social isolation for P2, P6 and P10 pups, as well as during the interaction of an adult male with an oestrus female and an adult female with another adult female. In pups, we did not find significant effect of sex on call rate, so we pooled male and female data and conducted an ANOVA with repeated measures on age classes with only genotype as a between-subject factor. We found a significant effect of age class ( $F_{(2,72)} = 9.238, p < 0.001$ ) and of the interaction between age class and genotype ( $F_{(2,72)} = 5.338, p = 0.007$ ). In wild-type mice, P6 pups called at a significantly higher rate in comparison with P2 pups ( $V = 2, p < 0.001$ ) and with P10 pups ( $V = 178, p = 0.007$ ; paired Wilcoxon test; Fig. 2). In adults, wild-type male and female mice emitted ultrasonic vocalisations at a similar rate, despite differences in the context of emission ( $W = 115, p = 0.859$ ; non-paired Wilcoxon test; Fig. 2).

Major differences in call rate between wild-type and *ProSAP1/Shank2*<sup>-/-</sup> mice occurred in P6 pups ( $W = 274, p = 0.006$ ) and in adult females ( $W = 170, p < 0.001$ ), with *ProSAP1/Shank2*<sup>-/-</sup> mice emitting ultrasonic vocalisations at a lower rate in comparison with wild-type littermates (non-paired Wilcoxon test; Fig. 2).

### 3.3. Call usage II: age- and genotype-related variations in the temporal organisation of ultrasonic calls

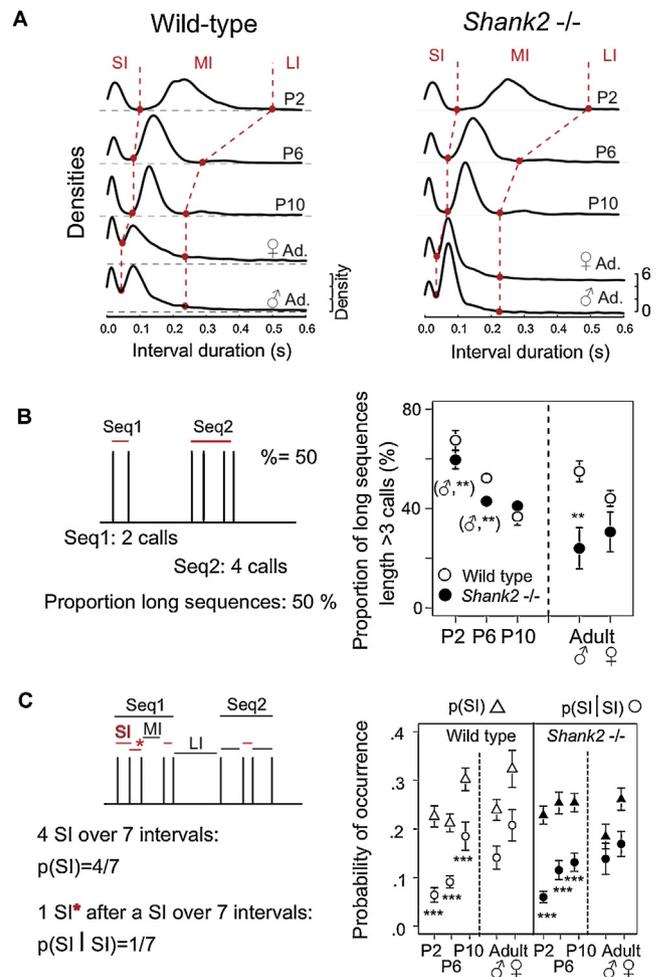
We measured the duration ( $i_n$ ) of the intervals between consecutive ultrasonic vocalisations. We first examined the distribution (density) of the duration of these intervals during pup development and in adulthood (Fig. 3A). There were no significant differences between sexes in any age class, so we pooled the data from males and females. In wild-type mice, independently of the age, ( $i_n$ ) were distributed bimodally with a long tail, then naturally defining three classes of time intervals. Limits between these three classes varied with age (Fig. 3A, left panel, red lines). The first category consisted of very short inter-call intervals of 0–0.10 s in P2 pups, 0–0.08 s in P6 and P10 pups, and 0–0.04 s in adults (maximum limit still larger



**Fig. 2.** Rate of emission of ultrasonic vocalisations in pups and in adult male and female mice. Rate of emission of ultrasonic vocalisations in P2, P6 and P10 pups (males and females pooled) and in adult males and females for wild-type mice (white) and for their *ProSAP1/Shank2*<sup>-/-</sup> littermates (black). Data are presented as means  $\pm$  SEM (\*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ ; significant differences between genotypes are represented by grey stars; significant differences between age classes and sexes are represented by black stars). Wild-type mice:  $N_{P2} = 10$  males + 10 females,  $N_{P10} = 10$  males + 10 females,  $N_{Adult\ males} = 16$ ,  $N_{Adult\ females} = 15$ ; *ProSAP1/Shank2*<sup>-/-</sup> mice:  $N_{P2} = 10$  males + 8 females,  $N_{P10} = 10$  males + 8 females,  $N_{Adult\ males} = 16$ ,  $N_{Adult\ females} = 13$ .

than the classic “hold-time” limit of 0.01–0.007 s taken in previously published papers; e.g., [14,20,36]). These short intervals (SI) separated calls emitted very rapidly one after the other. The second category consisted of medium length intervals (MI), that lasted for 0.10–0.50 s in P2 pups, 0.08–0.27 s in P6 pups, 0.08–0.23 s in P10 pups and 0.04–0.23 s in adults. Finally, the third category regrouped long intervals (LI) of more than 0.50 s in P2 pups, 0.27 s in P6 pups and 0.23 s in P10 pups and adult mice. Considering these three interval categories, we defined a sequence of calls as ultrasonic vocalisations separated by short or medium intervals; sequences were separated from each other by long intervals. For wild-type mice, we observed a dramatic shortening of the duration of the medium intervals during development (Fig. 3A, left panel). This temporal organisation was conserved in *ProSAP1/Shank2*<sup>-/-</sup> mice (Fig. 3A, right panel).

Different variables were then extracted to analyse the complexity of call sequences across ages and genotypes. We first analysed the length of a call sequence (i.e., the total number of successive calls that are separated by short or medium intervals) and the percentage of sequences with more than three calls (two or more intervals). In wild-type pups, the mean bout length decreased from  $13.88 \pm 1.39$  calls ( $n = 20/20$ ) in P2 pups to  $6.05 \pm 0.37$  calls ( $n = 20/20$ ) in P6 pups and  $3.24 \pm 0.26$  calls ( $n = 20/20$ ) in P10 pups ( $F_{(2,67)} = 64.7$ ,  $p < 0.001$ ). Similar results were observed in *ProSAP1/Shank2*<sup>-/-</sup> pups (P2 pups:  $12.5 \pm 1.74$  calls,  $n = 18/18$ ; P6 pups:  $5.21 \pm 0.38$  calls,  $n = 18/18$ ; P10 pups:  $3.78 \pm 0.28$  calls,  $n = 17/18$ ), with no difference with wild-type pups. In contrast, a decreased length of call sequences was observed in *ProSAP1/Shank2*<sup>-/-</sup> adult males ( $3.43 \pm 0.98$  calls,  $n = 13/13$ ) when compared with wild-type animals ( $6.63 \pm 0.75$  calls,  $n = 15/15$ ; t.test,  $df = 26$ ,  $p = 0.015$ ). This difference was not observed in adult females (t.test,  $df = 30$ ,  $p = 0.410$ ). Similarly the percentage of sequences with more than three calls decreased with age in pups ( $F_{(2,67)} = 40.3$ ,  $p < 0.001$ ; Fig. 3B). However, in this case, an interaction with sex ( $F_{(1,33)} = 4.2$ ,  $p = 0.048$ ) and genotype ( $F_{(1,33)} = 4.5$ ,  $p = 0.048$ ) appeared. It indicates a decrease of the percentage of long sequences in *ProSAP1/Shank2*<sup>-/-</sup> at P2 (t.test,  $df = 18$ ,  $p = 0.008$ ) and at P6 (t.test,  $df = 18$ ,  $p = 0.008$ ) in male pups when



**Fig. 3.** Temporal organisation of sequences of ultrasonic vocalisations. A. Distribution of the duration of intervals between ultrasonic vocalisations in P2, P6 and P10 pups (males and females pooled) and in adult males and females in wild-type mice (left panel) and in *ProSAP1/Shank2*<sup>-/-</sup> littermates (right panel). B. Proportion of sequences of ultrasonic vocalisations containing three or more calls (i.e., 2 or more inter-call intervals) in wild-type (white circles) and in *ProSAP1/Shank2*<sup>-/-</sup> littermates (black circles) in P2, P6 and P10 pups (males and females pooled) and in adult males and females. An example is depicted in the left panel. C. Probability of occurrence of one single short interval (triangles) and of two consecutive short intervals (circles) within sequences of three or more ultrasonic vocalisations in wild-type mice (left panel) and in *ProSAP1/Shank2*<sup>-/-</sup> littermates (right panel). An example is depicted in the left panel. Data are presented as means  $\pm$  SEM (\*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ ; significant differences between genotypes are represented by grey stars; significant differences between age classes and sexes are represented by black stars). See exact sample sizes in Suppl. Table 1.

compared to wild-type pups. In adults, and as for the length of a call sequence (see above), a decrease of the percentage was observed in *ProSAP1/Shank2*<sup>-/-</sup> adult males when compared with wild-type littermates (t.test,  $df = 26$ ,  $p = 0.002$ ). This difference was not observed in adult females (t.test,  $df = 30$ ,  $p = 0.120$ ). Taken together, these results indicated a shortening of sequences especially pronounced in *ProSAP1/Shank2*<sup>-/-</sup> adult male mice compared with their wild-type littermates.

We next considered the temporal succession of intervals within a sequence (Fig. 3C). We tested whether, within sequences with more than three vocalisations, successive interval durations (SI or MI) were independent from one another (i.e., whether there was an effect of the preceding interval length on the next interval within a bout). For this purpose, the frequency of occurrence of each possible two-interval series ((SI-SI), (SI-MI), (MI-SI) and (MI-MI)) was compared with the probability of individual events  $P(SI)$  or  $P(MI)$

(see methods). Overall, results are similar whatever the sex of the animal and thus for simplicity we pooled male and female pups for this analysis. The observed sequences were significantly different from the random hypothesis (i.e., the probability of occurrence of an interval duration depends on the previous interval), reflecting a first-order structure within the sequence. For example, the probability ( $P(SI|SI)$ ) of observing a short interval (SI) given that the preceding one was also short (i) increases with age and (ii) was lower in comparison with the marginal probability  $P(SI)$  of observing a short interval in P2, P6 and P10 pups and in adults in wild-type mice (Fig. 3C, left panel;  $p < 0.001$  at P2 ( $n = 20/20$ ), P6 ( $n = 20/20$ ), P10 ( $n = 19/20$ ) and in adult mice ( $n = 15/15$  males and 16/16 females), paired Student's t-tests with sequential Bonferroni correction). The probability of observing a medium interval (MI) following a short interval is then increased (data not shown). Furthermore, there was an increased probability to observe a short interval (SI) after a medium interval (MI) in P6 and P10 pups and in adults ( $p < 0.001$ ) but not in P2 pups in wild-type mice. Overall, a similar organisation was observed in *ProSAP1/Shank2*<sup>-/-</sup> mice (Fig. 3C, right panel, with no genotype effect on  $P(SI)$  and  $P(SI|SI)$ ; P2 pups:  $n = 18/18$ , P6 pups:  $n = 18$ , P10 pups: 17/18, adult males:  $n = 6/13$ , adult females:  $n = 8/16$ ).

We next examined the type of intervals most likely to begin a sequence. The probability to start a sequence with a medium interval  $P(MI_{\text{first}})$  was lower than expected in adult males ( $P(MI_{\text{first}}) < P(MI)$ ;  $p < 0.01$ ), but  $P(MI_{\text{first}})$  was not different from  $P(MI)$  in P2, P6 and P10 pups (P2 pups:  $n = 18/18$ , P6 pups:  $n = 18$ , P10 pups: 17/18, adult males:  $n = 6/13$ , adult females:  $n = 8/16$ , paired Student's t-test with sequential Bonferroni correction). In *ProSAP1/Shank2*<sup>-/-</sup> pups, we observed  $P(MI_{\text{first}}) > P(MI)$  at P2, no change at P6 and  $P(MI_{\text{first}}) < P(MI)$  at P10 ( $p < 0.001$ , paired Student's t-test with sequential Bonferroni correction). In adult mice,  $P(MI_{\text{first}})$  was never significantly different from  $P(MI)$ .

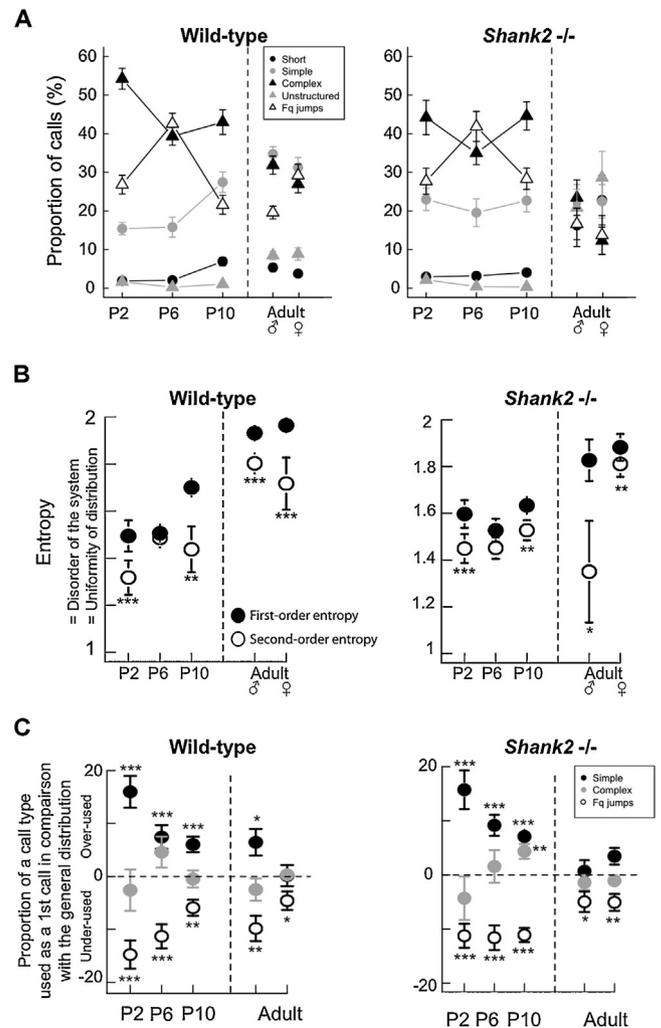
In parallel, we also highlighted that the probability to end a sequence with a medium interval  $P(MI_{\text{last}})$  was higher than expected in wild-type pups ( $P(MI_{\text{last}}) > P(MI)$ ;  $p < 0.001$  at P2, P6 and P10; paired Student's t-test with sequential Bonferroni correction), but  $P(MI_{\text{last}})$  was not significantly different from  $P(MI)$  in adults. Similar results were obtained in *ProSAP1/Shank2*<sup>-/-</sup> pups ( $P(MI_{\text{last}}) > P(MI)$  in P2, P6 and P10 pups).

Finally, we compared  $P(MI)$ ,  $P(MI_{\text{first}})$  and  $P(MI_{\text{last}})$  between wild-type and *ProSAP1/Shank2*<sup>-/-</sup> mice. There were no statistically significant differences at P2 and P10 ( $p > 0.05$ , Student's t-test with sequential Bonferroni correction). However, in adult mice, the probability of ending a sequence with a medium interval  $P(MI_{\text{last}})$  was significantly lower in wild-type males in comparison with *ProSAP1/Shank2*<sup>-/-</sup> mice ( $p < 0.01$ , Student's t-test with sequential Bonferroni correction), despite the fact that there was no genotype-related difference in  $P(MI)$ .

Overall, these results indicated the presence of a temporal structure of inter-call intervals in wild-type and *ProSAP1/Shank2*<sup>-/-</sup> mice. Minimal differences in this temporal organisation between wild-type and *ProSAP1/Shank2*<sup>-/-</sup> mice were observed, but the main structure was conserved.

### 3.4. Call usage III: reduced stereotypy in emission of call categories with age and abnormal call type usage in *ProSAP1/Shank2*<sup>-/-</sup> adult mice

We defined five call types according to characteristics of duration, frequency modulations and presence/absence of frequency jumps (Fig. 1C). Given the absence of a significant effect of sex in pups, we pooled together male and female data and re-conducted the ANOVA with repeated measures over pup developmental data, with age class as a within-subject factor and genotype as a between-subject factor (Fig. 4A). Age class had a significant effect on all call



**Fig. 4.** Vocal repertoire and composition of sequences of ultrasonic vocalisations. A. Distribution of ultrasonic vocalisations among the five different call types in wild-type mice (left panel) and in their *ProSAP1/Shank2*<sup>-/-</sup> littermates (right panel) in P2, P6 and P10 pups (males and females pooled) and in adult males and females. B. First and second order entropy to evaluate the complexity of call sequences (three or more calls) in wild-type mice (left panel) and in their *ProSAP1/Shank2*<sup>-/-</sup> littermates (right panel) in P2, P6 and P10 pups (males and females pooled) and in adult males and females; the higher the entropy is, the higher the complexity of the sequences is. C. Usage of a call type (simple, complex or frequency jump) as the first call of a sequence, in comparison with its total distribution over the sequences. Data are presented as means  $\pm$  SEM ( $\cdot$ :  $p < 0.05$ ,  $\bullet$ :  $p < 0.01$ ,  $\blacktriangle$ :  $p < 0.001$ ). See exact sample sizes in Suppl. Table I.

types ( $F_{(2,71)} < 0.005$  in all cases). In wild-type mice, major differences in call repertoire occurred between pups at P10 and younger pups (P2 and P6). P2 pups emitted a majority of complex calls in comparison with P6 and P10 pups ( $p < 0.01$ , Wilcoxon paired test) and P6 pups uttered a majority of calls with frequency jumps ( $p < 0.001$ , Wilcoxon paired test). P10 pups emitted significantly more short and simple calls in comparison with P2 and P6 pups ( $p < 0.001$ , Wilcoxon paired test). Adult mice of both sexes emitted a majority of simple, complex and frequency jumps calls, with no significant differences between sexes in the proportions of the call types used ( $p > 0.05$ , chi-squared tests).

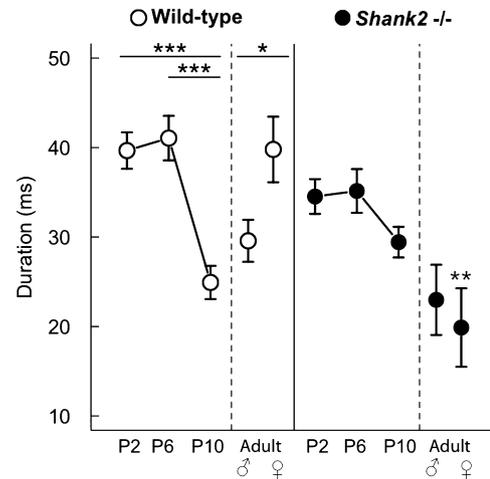
In *ProSAP1/Shank2*<sup>-/-</sup> mice, these age-related differences followed the same developmental pattern in pups. However, during pup development, genotype effects were not observed, except a marginal variation in *ProSAP1/Shank2*<sup>-/-</sup> P10 pups that uttered less short calls in comparison with their wild-type littermates ( $W = 251$ ,  $p = 0.039$ , Wilcoxon non-paired test; Fig. 4A).

Differences in the repertoire among wild-type and *ProSAP1/Shank2*<sup>-/-</sup> mice were significant in adult mice only. Adult *ProSAP1/Shank2*<sup>-/-</sup> males used a repertoire with an increased proportion of short calls ( $X=5.497$ ,  $df=1$ ,  $p=0.019$ ) and unstructured calls ( $X=5.222$ ,  $df=1$ ,  $p=0.022$ , chi-squared test; Fig. 4A) in comparison with wild-type mice. In adult females, *ProSAP1/Shank2*<sup>-/-</sup> mice emitted more short calls ( $X=13.652$ ,  $df=1$ ,  $p<0.001$ ) and unstructured calls ( $X=10.439$ ,  $df=1$ ,  $p=0.001$ ), as well as less complex calls ( $X=5.470$ ,  $df=1$ ,  $p=0.019$ ) and frequency jumps calls ( $X=5.566$ ,  $df=1$ ,  $p=0.018$ , chi-squared test, Fig. 4A) in comparison with wild-type mice. In summary, P2, P6 and P10 pups emitted preferentially three of the five call types, while adult mice (and even more so *ProSAP1/Shank2*<sup>-/-</sup> adult mice) used the five different call types more equally.

### 3.5. Call usage IV: early occurrence of sequence organisation in wild-type and *ProSAP1/Shank2*<sup>-/-</sup> mice

Sequences of ultrasonic vocalisations were considered as Markov chains (i.e., the result of a system that undergoes transitions from one vocalisation to another). We calculated the entropy rate of a zero-order Markov process ( $H1$ ) and the entropy rate of a first-order Markov process ( $H2$ ) for sequences of more than three calls (see Section 2; Fig. 4B). No differences between sexes emerged in pups, and therefore data from male and female pups were pooled. A decrease of the entropy between the two entropic orders  $H1$  and  $H2$  was observed in most age classes in wild-type mice ( $p<0.05$  in P2 pups ( $n=20/20$ ) and P10 pups ( $n=20/20$ ), and in adult mice (males:  $n=15/15$ ; females:  $n=16/16$ ), paired Student's *t*-test with sequential Bonferroni correction) and similarly in *ProSAP1/Shank2*<sup>-/-</sup> mice ( $p<0.01$  in P2 pups ( $n=18/18$ ) and P10 pups ( $n=17/18$ ) and  $p<0.05$  for adult mice (males:  $n=6/13$ , females:  $n=8/16$ ), paired Student's *t*-test with sequential Bonferroni correction), except at P6 ( $p>0.05$ ) in both wild-type ( $n=20/20$ ) and *ProSAP1/Shank2*<sup>-/-</sup> ( $n=18/18$ ) mice. These results indicated that sequences of ultrasonic vocalisations are at least a first-order process throughout development in both wild-type and *ProSAP1/Shank2*<sup>-/-</sup> mice. Within a sequence, the next ultrasonic vocalisation is therefore not independent from the previous one. Finally,  $H1$  entropy is different in adult *ProSAP1/Shank2*<sup>-/-</sup> mice compared with wild-type mice ( $p<0.01$  for males and females). This result is compatible with the previous observations that the repertoire of calls is differently used in adult wild-type mice and in *ProSAP1/Shank2*<sup>-/-</sup> mice.

We next examined the position of specific call types within a sequence (Fig. 4C). Again, no sex-related differences emerged in pups and data from males and females were pooled. A key element is described as a specific call that is not used homogeneously within a sequence. We considered only the three main call categories (simple, complex and with frequency jumps), which were emitted at high rates in all age classes. In wild-type mice, P2, P6 and P10 pups ( $n=20/20$  in each age class) used preferentially simple calls as the first call of a sequence (for instance, the use of simple calls as first call of a sequence was increased by 15.9% in P2 pups, in comparison with what was expected from their proportion within the repertoire; Fig. 4C, left panel). Nevertheless, this preference for simple calls as the first call of a sequence decreased during development, and was no longer significant in adult females. In contrast, calls with frequency jumps were under-used as first calls at P2 ( $p<0.001$ ,  $n=20/20$ ), P6 ( $p<0.01$ ,  $n=20/20$ ), P10 ( $p<0.001$ ,  $n=20/20$ ) and in adult males and females ( $p<0.01$ , males:  $n=15$ , females:  $n=16$ , paired Student's *t*-test with sequential Bonferroni correction), while complex calls were not specifically used as the first call of a sequence whatever the age class ( $p>0.05$  in P2 pups ( $n=20/20$ ), P6 pups ( $n=20/20$ ), and P10 pups ( $n=20/20$ ) and in adult males ( $n=15/15$ ) and females ( $n=16/16$ ); Fig. 4C). Notably,



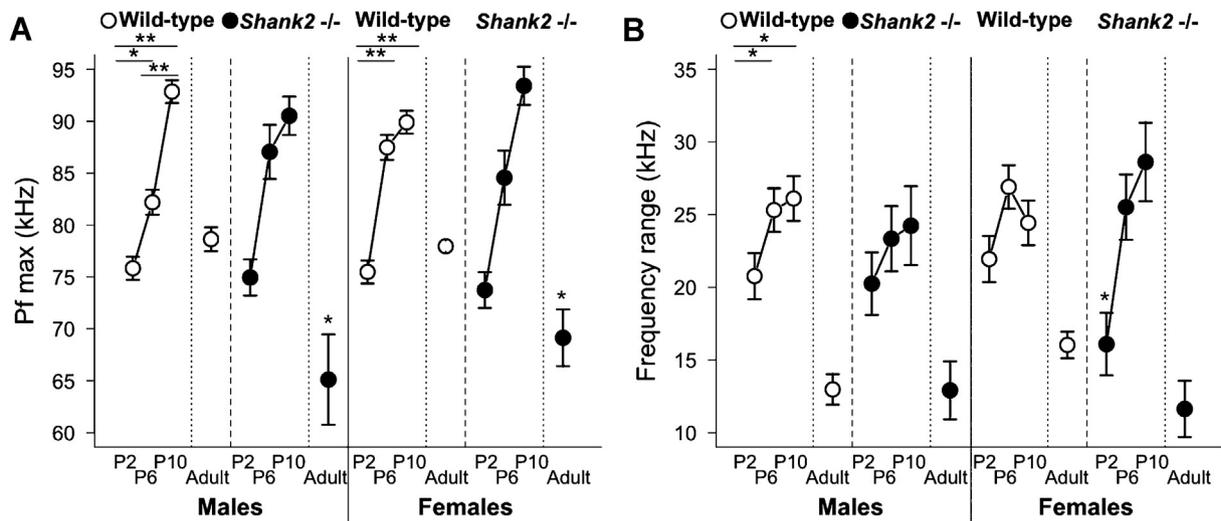
**Fig. 5.** Duration of mouse ultrasonic vocalisations in pups and adult mice. A. Mean duration of ultrasonic vocalisations in P2, P6 and P10 pups (males and females pooled) and in adult males and females for wild-type mice (white) and for their *ProSAP1/Shank2*<sup>-/-</sup> littermates (black). Data are presented as means  $\pm$  SEM (\*:  $p<0.05$ ; \*\*:  $p<0.01$ ; \*\*\*:  $p<0.001$ ; significant differences between genotypes are represented by grey stars; significant differences between age classes and sexes are represented by black stars). Wild-type mice:  $N_{P2}$  = 10 males + 10 females,  $N_{P10}$  = 10 males + 10 females,  $N_{Adult\ males}$  = 16,  $N_{Adult\ females}$  = 15; *ProSAP1/Shank2*<sup>-/-</sup> mice:  $N_{P2}$  = 10 males + 8 females,  $N_{P10}$  = 10 males + 8 females,  $N_{Adult\ males}$  = 16,  $N_{Adult\ females}$  = 13.

these variations were independent of the alteration of the repertoire with age. Proportions of simple and complex calls increased and decreased respectively with age in wild-type mice, while the proportion of the first call of a sequence is constant for complex and decreased for simple calls. An almost similar use of call categories within sequences was observed in *ProSAP1/Shank2*<sup>-/-</sup> mice (Fig. 4C, right panel; P2 pups:  $n=18/18$ , P6 pups:  $n=18$ , P10 pups:  $18/18$ , adult males:  $n=13/13$ , adult females:  $n=16/16$ ).

### 3.6. Call structure I: age- and genotype-related variations in call duration

We measured the duration of each ultrasonic vocalisation for each animal (Fig. 5). In pups, no effect of sex was found, therefore male and female data were pooled and the ANOVA was re-conducted with only age class as within-subject factor and genotype as a between-subject factor. Significant effect of age class ( $F_{(2,72)} = 28.805$ ,  $p<0.001$ ) and a significant interaction of age class with genotype ( $F_{(2,72)} = 6.308$ ,  $p=0.003$ ) were found. In wild-type pups, the mean duration of calls was significantly shorter in P10 pups in comparison with P2 pups ( $V=209$ ,  $p<0.001$ , paired Wilcoxon test) and P6 pups ( $V=209$ ,  $p<0.001$ , paired Wilcoxon test; Fig. 5), mostly because of shorter simple, complex and frequency jumps vocalisations (Suppl. Figure 2A). In wild-type adult mice, ultrasonic vocalisations recorded in female–female interactions were significantly longer in comparison with those recorded in male–oestrus female interactions ( $W=60$ ,  $p=0.019$ , non-paired Wilcoxon test; Fig. 5), because of longer duration of complex, unstructured and frequency jumps calls (data not shown).

The *ProSAP1/Shank2* mutation induced a significant reduction of the duration of ultrasonic vocalisations only in adult female mice ( $W=158$ ,  $p=0.006$ , non-paired Wilcoxon test; Fig. 5). All categories of calls except the short category displayed a decrease in duration in *ProSAP1/Shank2*<sup>-/-</sup> mice in comparison with wild-type mice (Suppl. Figure 2B).



**Fig. 6.** Frequency characteristics of mouse ultrasonic vocalisations in pups and adult mice. A. Mean maximum peak frequency of ultrasonic vocalisations in P2, P6 and P10 pups (males and females pooled) and in adult males and females for wild-type mice (white) and for their *ProSAP1/Shank2*<sup>-/-</sup> littermates (black). B. Mean frequency range of ultrasonic vocalisations in P2, P6 and P10 pups (males and females pooled) and in adult males and females for wild-type mice (white) and for their *ProSAP1/Shank2*<sup>-/-</sup> littermates (black). Data are presented as means  $\pm$  SEM (\*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; significant differences between genotypes are represented by grey stars; significant differences between age classes and sexes are represented by black stars). Wild-type mice:  $N_{P2} = 10$  males + 10 females,  $N_{P10} = 10$  males + 10 females,  $N_{Adult\ males} = 16$ ,  $N_{Adult\ females} = 15$ ; *ProSAP1/Shank2*<sup>-/-</sup> mice:  $N_{P2} = 10$  males + 8 females,  $N_{P10} = 10$  males + 8 females,  $N_{Adult\ males} = 14$ ,  $N_{Adult\ females} = 11$ .

### 3.7. Call structure II: age- and genotype-related variations in frequency characteristics

We manually took four measures of the peak frequency of each call (i.e., the frequency with the highest amplitude): minimum (Pf min), maximum (Pf max), at the beginning of the call (Pf start), and at the end of the call (Pf end). We present in details variations concerning the maximum peak frequency (Fig. 6). Variations in other frequency variables (Pf min, Pf start and Pf end) followed a pattern similar to the one found in maximum peak frequency and are presented in the Suppl. Figures 3–5. During development, the ANOVA with repeated measures on maximum peak frequency highlighted no significant effect of genotype or sex, but a significant effect of age class ( $F_{(2,68)} = 130.733$ ,  $p < 0.001$ ) and a significant interaction of age class  $\times$  genotype  $\times$  sex ( $F_{(2,68)} = 5.410$ ,  $p = 0.007$ ). In wild-type mice, P2 pups uttered ultrasonic vocalisations with significantly lower maximum peak frequency in comparison with P6 pups (males:  $V = 2$ ,  $p = 0.011$ ; females:  $V = 0$ ,  $p = 0.006$ ), and P10 pups (males:  $V = 0$ ,  $p = 0.006$ ; females:  $V = 0$ ,  $p = 0.006$ ; paired Wilcoxon test; Fig. 6A), all call types except frequency jumps being affected (Suppl. Figures 4A and 5A). Wild-type male P6 pups also emitted ultrasonic vocalisations with a significantly lower maximum peak frequency in comparison with P10 pups ( $V = 0$ ,  $p = 0.006$ , paired Wilcoxon test; Fig. 6A), all call types except frequency jumps being affected (Suppl. Figure 4A). In wild-type adult mice, there was no significant difference in maximum peak frequency between males and females (Fig. 6A).

An ANOVA with repeated measures conducted on data related to the frequency range of pup isolation calls revealed a significant effect of age class ( $F_{(2,68)} = 20.392$ ,  $p < 0.001$ ) and of the interaction of age class  $\times$  genotype  $\times$  sex ( $F_{(2,68)} = 4.015$ ,  $p = 0.022$ ). In wild-type male mice, P2 pups uttered ultrasonic vocalisations within a much narrower frequency range in comparison with P6 pups ( $V = 6$ ,  $p = 0.032$ ) and P10 pups ( $V = 6$ ,  $p = 0.032$ , paired Wilcoxon test; Fig. 6B). No significant differences in the frequency range were detected in wild-type female pups during development, as well as in adult male and female mice (Fig. 6B).

Strikingly, while no genotype-related differences emerged in P2, P6 and P10 male and female pups, adult *ProSAP1/Shank2*<sup>-/-</sup> mice uttered ultrasonic vocalisations with significantly lower

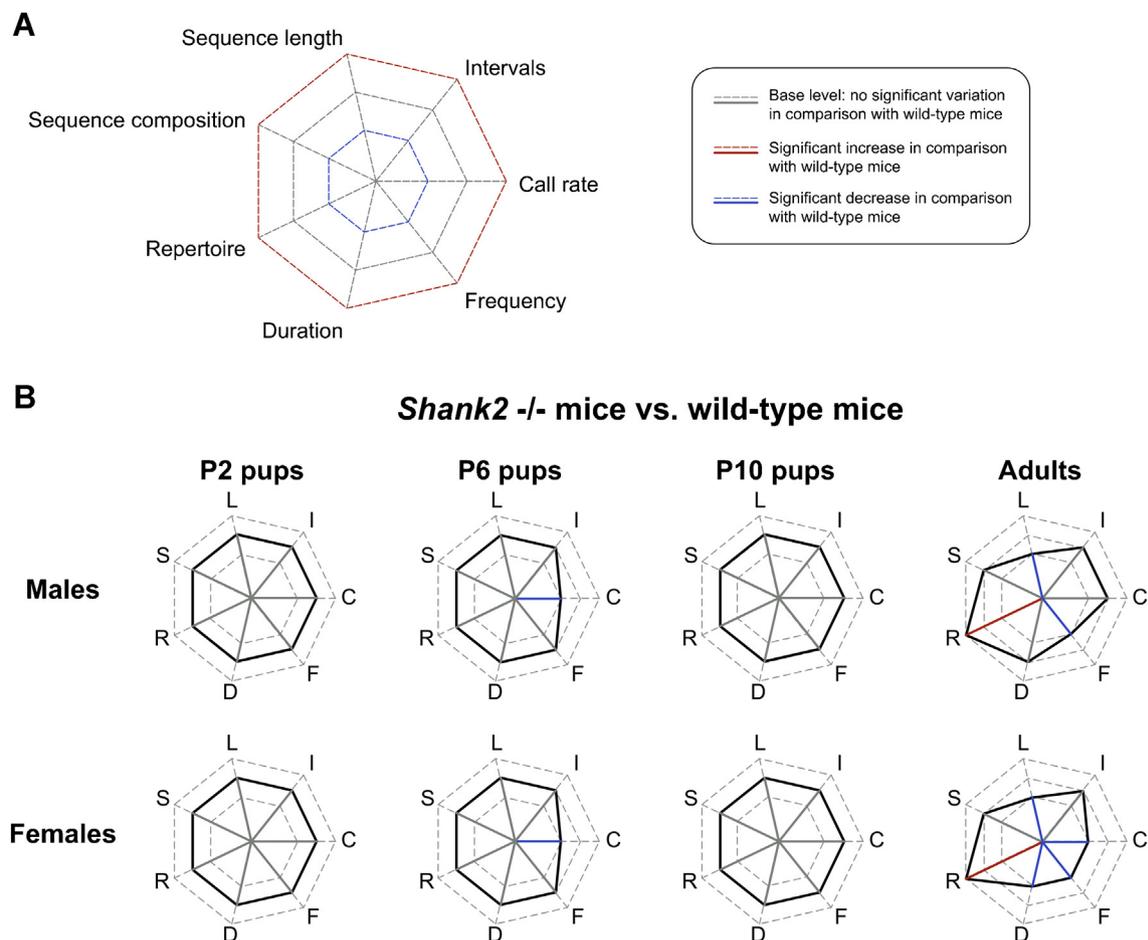
maximum peak frequency in comparison with wild-type littermates (adult males:  $W = 165$ ,  $p = 0.029$ ; adult females:  $W = 126$ ,  $p = 0.026$ , non-paired Wilcoxon test; Fig. 6A). This decrease in peak frequency affected all categories of calls (Suppl. Figures 4B and 5B). Variations in other frequency variables were similar to the one found in the maximum peak frequency (Suppl. Figures 3B, 4B and 5B). *ProSAP1/Shank2*<sup>-/-</sup> mice uttered vocalisations within a similar frequency range as their wild-type littermates. Only female P2 *ProSAP1/Shank2*<sup>-/-</sup> pups uttered ultrasonic vocalisations with a frequency range narrower in comparison with the wild-type littermates ( $W = 65$ ,  $p = 0.029$ , non-paired Wilcoxon test; Fig. 6B).

## 4. Discussion

In this study, we analysed both the call usage and the structure of ultrasonic vocalisations in several age classes in pups and in adults in wild-type and *ProSAP1/Shank2*<sup>-/-</sup> mice of both sexes (Fig. 7A). In wild-type mice, age-related changes affected call rate, call repertoire, temporal and structural organisation of sequences, as well as duration and peak frequency characteristics. Interestingly, most of the differences between *ProSAP1/Shank2*<sup>-/-</sup> mice and wild-type littermates emerged only in adults and occurred mostly for call rate, call repertoire, as well as duration and peak frequency characteristics.

### 4.1. Age-related variations in mouse ultrasonic vocalisations

Our study reports the second analysis of mouse ultrasonic vocalisations in different age classes [42] (Fig. 7B). During pup development, we observed a gradual increase in peak frequency with age, originating from an increase in peak frequency in all call types except frequency jumps. This effect might be correlated with the increasing ability of pups to thermoregulate and should be tested with simultaneous body temperature measures (not available in this study). In contrast, in Grimsley et al. [42], no clear linear correlation between peak frequency and age was observed and the direction of variations changed according to the call category examined. Differences between the present study and Grimsley's study might stem from differences in the strain studied (C57BL/6 vs. CBA/CaJ) or in the way data are considered (repeated measures



**Fig. 7.** Compilation of variations in mouse vocal behaviour during development and in adulthood for *ProSAP1/Shank2<sup>-/-</sup>* mice compared with wild-type littermates. A. Behavioural traits analysed to describe the abnormalities in the emission of ultrasonic vocalisations in mouse models of autism spectrum disorders. B. Variations in the emission of ultrasonic vocalisations in *ProSAP1/Shank2<sup>-/-</sup>* male and female mice in comparison with their wild-type littermates.

over the same individuals with preservation of caller identity in the present study vs. pooling of calls from different individuals in [42]). When considering data from pups and from adults, we observed that variations in peak frequency did not follow body weight variations between age classes, as the mechanisms of vocal production would have predicted (lower peak frequency in larger animals [43]). The context in which calls are emitted might therefore play the major role in these characteristics.

We showed that an increase in call rate in pups and adults might be related to the shortening of the medium inter-call intervals within sequences. The reduction of inter-call intervals observed over age during development might be related to the maturation of the vocal system, with increased lung capacity allowing a more rapid rate of emission of vocal signals. Sequences also appeared to contain less ultrasonic vocalisations with increasing age during pup development, confirming the shortening of bouts between age classes and the increasing call rate related to the increase in the number of bouts [42,44]. This decrease in the number of vocalisations within sequences was not followed up in adults. In summary, rhythm of vocal production reflects contextual differences between isolation calls in pups and social interaction calls in adults, with adult mice displaying shorter time intervals between ultrasonic vocalisations and fewer ultrasonic vocalisations within a sequence, but more sequences. The study of rhythm can be complemented by the study of the call types used at specific positions within sequences. These elements are critical for an animal's vocal characteristics [42]. Bouts from wild-type mice were more likely

to begin with a “simple” vocalisation, similar to the “flat” vocalisation in [42], than predicted from a random model. Moreover, our study highlighted a two-call organisation, predicting the occurrence of one vocalisation with the preceding one. This structure was already present in very young pups (P2). In wild-type mice, we also showed that pups emitted preferentially two categories of calls (complex and frequency jumps calls), while adult mice use call categories in a more balanced manner. The higher entropy in adults compared to pups confirmed the more stereotyped usage of the repertoire during development in pups, probably reflecting the differences in the contexts of vocalisation emissions (social isolation in pups vs. social interactions in adults). In other mouse strains such as CBA/CaJ, mouse pups also used a more repetitive repertoire in comparison with adults [42].

Altogether, our data and those from Grimsley et al. [42] support age-specific features as well as a non-random organisation of the ultrasonic vocalisation system of mice. These aspects provide support to a communicative function for these ultrasonic vocalisations.

#### 4.2. Sexual vs. territorial functionality

Analysing the communicative functions of ultrasonic vocalisations in adult mice was beyond the scope of the present study. Nevertheless, our data provide interesting elements for this discussion. Male ultrasonic vocalisations were first proposed as courtship songs [28]. However, more recently, they were also proposed to be “territorial” or “contact” calls, and only additionally function as

courtship calls in males [40]. In the courtship songs hypothesis, it would be expected that male and female ultrasonic vocalisations have different functions and therefore probably different characteristics. In our study, adult males and females nevertheless emitted ultrasonic vocalisations at a similar rate, with no differences in temporal organisation of sequences, very limited differences in structural organisation of sequences, a similar repertoire and no major differences in the acoustic structure (except call duration).

Very limited sex-related differences in adult ultrasonic vocalisations were also observed in other studies [34,40]. Scattoni et al. [34] examined adult C57BL/6J male–female and female–female interactions and showed minimal differences in the usage of call types between the two contexts of recording. More recently, Hammerschmidt et al. [40] conducted a cluster analysis on ultrasonic vocalisations emitted during male–female and female–female resident–intruder paradigms to compare the distribution of call types between males and females. In the three types of calls considered, they showed that only the category of short upward modulation without frequency jump calls was used with significant difference between sexes [40]. In our present study, the minimal sex-related variations provide support to the “territorial” or “contact” calls hypothesis proposed recently [40]. In this model, ultrasonic vocalisations might be uttered mostly to maintain physical contact or as a marker for arousal when animals are highly motivated to interact socially in C57BL/6 mice. We can nevertheless not exclude that, in other strains (e.g., BTBR *T+tf/J* mouse [34]), sex-related variations might occur.

Additionally, we confirmed that there is no major sex-related variation in the frequency characteristics of adult ultrasonic vocalisations as shown in previous studies [40,45]. Sexual dimorphism in body mass between male and female adult mice (see body weight data in [14]) might not be sufficient to affect vocal production, similar to other species with reduced sexual dimorphism (for instance in New World monkeys; reviewed in [46]).

#### 4.3. *ProSAP1/Shank2*<sup>-/-</sup> mouse model

The relevance of studying ultrasonic vocalisations in mice to understand the communication deficits in humans remains an open question (but see [37]). Nevertheless, our study of the *ProSAP1/Shank2*<sup>-/-</sup> mouse model provides interesting data indicating statistically significant abnormalities in both usage and structure of ultrasonic vocalisations emerging mostly in adulthood (Fig. 7B; see also [14,16]). The only difference we found in comparison with the previous study (we highlighted a genotype-related difference in the call rate of P6 pups, and not of P10 pups as in [14]) probably stems from the reduction of the pup cohort and the different way of analysing the data. Indeed, in the present study, we followed exactly the same individuals over the three age classes (repeated measures). In patients with ASD, abnormalities in social communication can affect gestures as well as vocal communicative skills, both in the comprehension and production levels [47]. Patients carrying *SHANK2* mutations well illustrate the phenotypic heterogeneity of ASD. The three patients presenting a de novo *SHANK2* deletion developed speech, however, two of them presented speech delay, one developing a functional spoken language and the other using dys syntactic sentences. The third patient developed spoken language but at a level below average [8–10]. Our results, combined with those previously obtained from the general characterisation of the *ProSAP1/Shank2*<sup>-/-</sup> mouse [14,16], provide additional support that this mouse is a relevant model of ASD. The fact that vocalisation abnormalities seem to emerge in adulthood might reflect that *Shank2* mutations cause a functional defect rather than a pure neuro-developmental disorder. This possibility was also recently suggested by the reversion of the synaptic and behavioural defects during adulthood in several mouse models

for ASD (*Mecp2*<sup>yl/-</sup> mouse [48]; *Nlgn3*<sup>-/-</sup> mouse [49]; reviewed in [50]).

In *ProSAP1/Shank2*<sup>-/-</sup> adult mice, we showed that the temporal organisation of sequences is affected, with the use of shorter sequences in comparison with wild-type mice. Otherwise, the structural organisation of sequences of ultrasonic vocalisations was not affected in *ProSAP1/Shank2*<sup>-/-</sup> mice in comparison with wild-type littermates. Most importantly, we showed that adult *ProSAP1/Shank2*<sup>-/-</sup> mice display acoustic abnormalities in ultrasonic vocalisations, with decreased peak frequency affecting all call types. This decrease in frequency is not likely to reflect body weight variations between wild-type and mutant mice since *ProSAP1/Shank2*<sup>-/-</sup> adult mice displayed a smaller body weight than wild-type littermates [14]. This decrease in frequency in both males and females was of around 10 kHz, therefore slightly larger than the decrease in frequency shown by mouse pups carrying a humanised version of *Foxp2*, a model for language disorder [51]. These variations nevertheless still remain in the range of normal variability in mice. The *ProSAP1/Shank2*<sup>-/-</sup> mouse is the first mouse model for ASD to display structural variations in ultrasonic vocalisations of adults. Increased peak frequency was found only in P8 pups for both *Shank1*<sup>-/-</sup> [19] and *Fmr1*<sup>-/-</sup> mice [52]. Whether such variations in peak frequency in *ProSAP1/Shank2*<sup>-/-</sup> mice reflect some abnormalities in prosody on patients with ASD remains unknown [53].

The structural abnormalities in ultrasonic vocalisations could be the consequence of a specific defect in the neuronal circuits for vocal motor control [51]. However, we cannot exclude that the structural abnormalities in ultrasonic vocalisations are the consequences of a defect in auditory feedback ([54] if *ProSAP1/Shank2*<sup>-/-</sup> mice (backcrossed on C57BL/6) displayed an earlier hearing loss in comparison with their wild-type littermates (backcrossed on C57BL/6). This explanation is nevertheless unlikely given that: (1) hearing loss in C57BL/6 is minimal up to 12 months of age (our animals were tested at 4–6 months of age [55]); (2) the necessity of auditory feedback to develop ultrasonic vocalisations in mice is still controversial (e.g., [54,56]). Hearing loss could nevertheless be verified through high-frequency audiograms in both mutant and wild-type mice in future investigations. Structural abnormalities might also be a consequence of subtle abnormalities in breathing or laryngeal contraction. To clarify this issue, conditional *ProSAP1/Shank2*<sup>-/-</sup> mice with brain-specific knock-down of *ProSAP1/Shank2* should be used. We could also study the effect of decreasing *ProSAP1/Shank2* expression in specific brain regions to identify the neural circuits sensitive to *ProSAP1/Shank2* and specifically affecting the acoustic structure of ultrasonic vocalisations. Such information would be valuable to unravel some of the neural circuits associated with the control of vocal production, and more particularly with the fine adjustment of the acoustic structure.

An alternative hypothesis to explain the difference in vocalisation structure between *ProSAP1/Shank2*<sup>-/-</sup> mice and wild-type littermates might be that the decrease in peak frequency reflects modulations in the affective state of the calling mice. Indeed, adult ultrasonic vocalisations emitted during aversive situations such as restraint stress have lower peak frequency characteristics in comparison with ultrasonic vocalisations emitted during more neutral or positive situations, such as social interactions after a long period of isolation [33]. In this line, *ProSAP1/Shank2*<sup>-/-</sup> mice might perceive social interactions as aversive and this could trigger the utterance of lower frequency calls. This attractive hypothesis could be tested by rearing *ProSAP1/Shank2*<sup>-/-</sup> mice and wild-type littermates in socially and physically enriched environment, modulating stress levels and stimulating the animals as much as possible. Physiological measurements of stress should be taken during social interactions, to compare the stress level between *ProSAP1/Shank2*<sup>-/-</sup> mice and wild-type littermates.

Finally, an important question is to what extent the variations in *ProSAP1/Shank2*<sup>-/-</sup> mice vocalisations are perceived by other mice. At the moment, it is known that adult *ProSAP1/Shank2*<sup>-/-</sup> mice spent less time in contact with a conspecific during female–female interactions [14]. However, it cannot be said whether the abnormalities in ultrasonic vocalisations are a cause or a consequence (or whether they are independent) of this social atypical behaviour. The perception of these vocal abnormalities by conspecific remains to be tested through playback experiments and analysis of video recordings synchronised with audio recordings.

## 5. Conclusions

In summary, the present study has highlighted three main points. First, in wild-type mice, we showed a high level of organisation in sequences of ultrasonic vocalisations, suggesting a communicative function in this complex system. Second, the very limited sex-related variations in usage and acoustic structure of adult ultrasonic vocalisations suggests that mouse ultrasonic communication might be related to contact maintenance during social interactions and only secondarily to courtship behaviours in adult males. Third, we showed structural abnormalities in ultrasonic vocalisations of adult *ProSAP1/Shank2*<sup>-/-</sup> mice, a model for ASD. These results will trigger further experiment to test whether excitatory synapses containing *ProSAP1/Shank2* might control the fine acoustic structure of vocal production. Which specific brain regions are implicated in this process remains to be characterised. Taken together, these results indicate that an in-depth analysis of ultrasonic vocalisations of mouse models can reveal specific abnormalities in vocal production and therefore open a new area of research on the development of communication skills in animals and their related disorders in humans.

## Conflicts of interest

The authors have declared that no competing interests exist.

## Authors' contribution

EE, TB, and PF conceived and designed the experiments. TMB generated the *ProSAP1/Shank2*<sup>-/-</sup> mouse. EE, CSL, AMLS and NT collected the data. EE, PF and TB analysed the data and wrote the paper.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bbr.2013.08.031>.

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