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Ultrasonic vocalizations (USV) in the three standard laboratory mouse strains: Developmental analysis

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Mice, similarly to some other rodent species, communicate with specialized sounds in the ultrasonic range called ultrasonic vocalizations (USV). Evaluation of this behavioral activity enables estimation of the social interactions in animal models of autistic spectrum disorders (ASD). Because transgenic mouse models are generated, in most cases, on the mixed 129SV/C57BL6 genetic background, we were interested if parameters that characterize USV differ between these two mouse strains. In addition, we wanted to compare these strains with the BALB/c line. In order to analyze USV, we applied the standard isolation test to newborn animals and compared standard parameters. Obtained results indicate clear differences between the 129SV and C57BL6 strains in respect to all analyzed USV parameters. Both strains behave also differently when compared with the BALB/c strain. For this reason in experiments utilizing transgenic animals, contribution of various genetic backgrounds has to be carefully considered.

Key words: USV, laboratory mouse, inbred strains, development

Mice are highly social animals and, similarly to some other rodent species, communicate not only with audible sounds but also with sounds in the ultrasonic range, known as ultrasonic vocalizations (USV) that are emitted as a response to different social situations. Although USV are emitted throughout the mouse lifespan during social play, mating and social investigation, the most known behavior is the pup's USV calling as a response to separation from the mother (Zippelius and Schleidt 1956). This behavioral phenomenon during many years of investigation has been used as a standard test in experiments verifying social interaction between the mother and newborns. Evaluation of this behavioral activity enables estimation of social interactions in animal models of neuropsychiatric disorders e.g. autistic spectrum disorders – ASD (Scattoni et al. 2008a,b, 2009, Scattoni and Branchi 2010, Young et al. 2010). Animal models commonly used in behavioral studies are mostly developed by means of transgenic approaches broadly utilizing the C57BL6 and 129SV strains as donors for genetic manipulations. Obtained transgenic strains and even single animals share often very mixed, not homogenous and sometimes unknown genetic background, which is a mixture of C57BL6 and 129SV strains. Because carried genetic information plays a very important role in modulation of many behavioral activities, including USV, we were interested if the mouse strains C57BL6 and 129SV differ in respect to basic parameters of USV (Bell et al. 1972, Hahn et al. 1987, Roubertoux et al. 1996, Scattoni et al. 2013). Potential differences could results in false interpretation of obtained experimental data, especially when the control group was not properly selected.

Another question addressed in our study was the selection of developmental stage best suitable for USV analysis. Most studies utilizing USV as behavioral parameter focus on a single postnatal day, but previous experiments showed that USV activity of the pups depends on the postnatal phase. Again, if the fluctuations of USV activity during the postnatal development depend on the genetic background, variable genetic information would account for different time course of USV parameters in analyzed strains. For that

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reason, we decided to analyze the first 14 days of postnatal development (Elwood and Keeling 1982, Hahn et al. 1997, 1998, Thornton et al. 2005). The third analyzed mouse strain (BALB/c) was chosen because of its well known behavioral differences when compared with the C57BL6 or 129SV line. According to the data presented by Jackson Laboratories, the BALB/c strain, together with C57BL6 and 129SV, are the most frequently used strains in biomedical research.

All experimental procedures were designed according to the earlier set-up protocol (Hofer et al. 2002) and with permission of the Local Committee for Animal Welfare. All animals used for the experiments were bred in the animal facility of the Center for Experimental Medicine and are offspring of certificated strains provided by Charles River Laboratories (BALB/cAnCrl, C57BL/6NCrl, 129S2/SvPasCrl). To analyze possible differences in USV between selected mouse strains, we applied the standard isolations test. For the test we used 3 to 4 litters from each strain, analyzing at least 15 pups (male and female) from each strain. The number of newborns in analyzed litters was different (4–8). Strains used in our experiments differ in respect to breeding performance. The highest number of pups were born in C57BL6 strain, the lowest in 129SV and BALB/c was the intermediate one. USV was recorded every second postnatal day between P2 and P14. Test duration was 7 min. Vocalizations were recorded with a condenser ultrasound microphone, Avisoft-Bioacoustics CM16/CMPA and the UltraSoundGate 116Hb recording interface (Avisoft Bioacoustics, Germany). Recorded vocalizations were analyzed with Avisoft SASLab Pro program. Statistical analyses of obtained data were performed with the one way ANOVA test. To ensure proper and uniform experimental conditions all trials were performed in accordance with the following procedure. The dam was removed from the home cage and placed in a small cage apart from the litter. The home cage was then placed on a temperature-controlled heating pad set at 34°C, to ensure constant ambient temperature for the pups. Pups were identified with an unscented ink marker and 10 min were allowed for the litter to ensure return to control conditions. For the recording, every pup was transported to the test room and placed carefully in the center of the test cage floor. The microphone was placed 15 cm above the floor of the test cage. After the 7 min recording time elapsed, each pup was removed from the test cage and returned back to the home cage. To compare all three mouse strains with respect to emitted USV we chose 9 parameters (Fig. 1) which characterize the vocalization quantitatively and qualitatively: (1) vocalization rate – number of single USV in one minute, (2) call duration – mean duration of a single USV call, (3) bout rate – number of bouts (group of USV calls) in one minute, (4) bout elements number - mean number of USV calls in a single bout, (5) bout duration – mean duration of bout of USV calls, (6) bout interval – mean interval time between single USV bouts, (7) mean peak frequency, expressed as peak frequency at maximal amplitude, (8) mean bandwidth, expressed as difference between the minimal and maximal frequency in a single USV call, (9) sonogram classification according to Scattoni and coworkers (2008b).

Vocalization rate. Due to the complexity of data statistical analysis of all parameters is summarized in Table I. The first parameter analyzed was the vocalization rate expressed as number of calls emitted in one minute. Similarly to other analyzed parameters, obtained data were recorded during the two first postnatal weeks (starting at P2). The attached graph (Fig. 1) reflects developmental changes during this period. The changes of the parameter during the analyzed period have a very different course in the three mouse strains. Animals from the 129SV strain show much higher values for that parameter in the second postnatal week, whereas animals from the C57BL6 strain reach high values already in the first week with a clear sloping tendency from P10. Similar time course can be estimated for the BALB/c strain, however the values start to drop from P4. Comparing the 129SV strain with C57BL6 we can state the best time point for analysis of that parameter in animals with a mixed 129SV/C57BL6 background would be P10, where the values are very close to each other.

Call duration. The next parameter analyzed in our study was the duration of a single USV call expressed in milliseconds. This parameter seems to be most stable one, and no robust changes during the 14 days of examination have been observed. The mean value of that parameter, however, differs between the three analyzed strains. The highest mean value of this parameter was estimated for the BALB/c pups – 39.63 ms. Stable time course for call duration were also observed for the 129SV strain, with the mean value of 28.96 ms. This value was very close to that of the C57BL6 animals – 26.90 ms.

Bout rate. The first two parameters describe features of single USVs. The next four parameters relate to sets of USVs (bouts). USV calls of newborns are

mostly emitted as bouts and parameters of single calls partially influence parameters of bouts. The first analyzed parameter is the bout rate expressed as the number of bouts in one minute. Estimation of bout rate showed big differences between the strains. In the BALB/c strain, the time course is characterized by a sloping tendency starting already at the first recording day. Bout rate values recorded for 129SV strain have a very similar time course as in the case of the vocalization rate. Low values were recorded in the first postnatal week, whereas in the second week a clear increase was measured, with a peak at P12. Again recordings done in C57BL6 animals resulted in a time course different from that of 129SV strain and similar to vocalization rate curve. The time point where values for bout rate were similar for 129SV and C57BL6 strains was again P10.

Bout elements number. The third selected parameter was the bout elements number – number of single USV calls in one bout. Values measured for the BALB/c strain were higher for the whole 14 days with a clear peak at P4. Curves estimated for the 129SV strain and the C57BL6 strain showed a very similar and stable course during the whole test period without any prominent peak values. Time points with the closest values were P8 and P10.

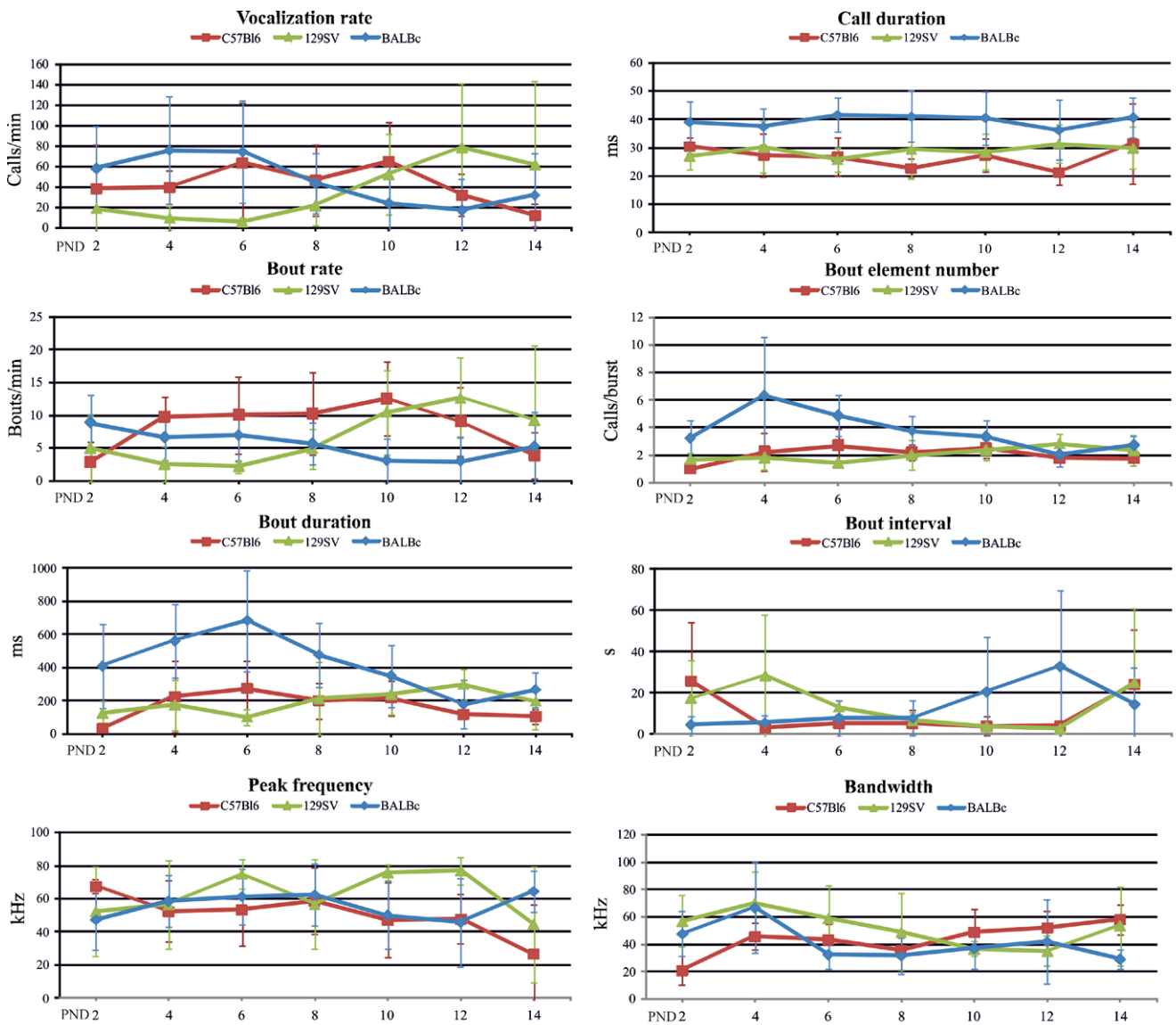

Fig. 1. Developmental analysis of selected USV parameters. C57BL6 – *n*=15. 129SV – *n*=18. BALBc – *n*=25. (PND) Postnatal day. For Statistical evaluation see Table I.

Table I.

Statistical evaluation of differences between tested strains. One way ANOVA was performed for all selected parameters for each recording day (P2–P14). Grey shaded cells show statistically significant differences, where $P<0.05$. (Bc) BALB/c strain, B6 – C57Bl6 strain; (129) 129SV strain. Cells with bold fonts and borders indicate the time points where the most similar values were recorded for the the 129SV and C57BL6 strains.

Vocalization rate	Bc vs. B6	Bc vs. 129	B6 vs. 129
P2	1,52E-01	7,67E-04	1,04E-01
P4	2,56E-02	3,80E-04	8,55E-05
P6	4,86E-01	2,20E-03	2,70E-02
P8	7,76E-01	1,04E-01	1,26E-01
P10	1,23E-02	1,80E-01	5,37E-01*
P12	2,62E-01	1,22E-02	9,66E-02
P14	2,30E-01	3,08E-01	1,35E-01

Call duration	Bc vs. B6	Bc vs. 129	B6 vs. 129
P2	3,75E-05	1,37E-07	1,42E-02
P4	4,99E-04	2,32E-02	4,31E-01
P6	8,35E-11	4,18E-07	7,31E-01
P8	1,82E-08	1,12E-02	4,25E-02
P10	2,42E-04	2,15E-02	7,09E-01*
P12	2,59E-03	3,55E-01	1,01E-02
P14	6,51E-02	1,09E-02	8,20E-01

Bout rate	Bc vs. B6	Bc vs. 129	B6 vs 129
P2	1,81E-05	9,84E-03	1,52E-01
P4	2,63E-02	1,68E-03	3,90E-05
P6	5,25E-02	5,99E-03	5,73E-03
P8	2,04E-02	5,92E-01	1,17E-01
P10	4,11E-04	7,98E-03	7,19E-01*
P12	7,96E-03	8,05E-04	2,90E-01
P14	5,63E-01	3,08E-01	2,57E-01

Bout element n.	Bc vs. B6	Bc vs. 129	B6 vs. 129
P2	6,31E-08	3,69E-05	6,54E-07
P4	1,13E-03	1,84E-03	5,15E-01
P6	1,20E-06	4,00E-06	2,98E-02
P8	9,41E-06	1,09E-03	6,15E-01*
P10	4,60E-02	1,36E-01	7,38E-01*
P12	4,62E-01	9,61E-02	3,18E-03
P14	6,62E-03	4,34E-01	2,49E-01

Bout duration	Bc vs. B6	Bc vs. 129	B6 vs. 129
P2	7,74E-07	4,30E-05	3,09E-05
P4	1,31E-04	5,17E-05	6,68E-01
P6	6,65E-07	5,19E-05	2,07E-02
P8	2,60E-05	8,15E-03	8,57E-01*
P10	3,13E-02	2,45E-01	6,87E-01*
P12	2,94E-01	1,19E-01	6,18E-04
P14	1,20E-03	3,05E-01	1,98E-01

Bout interval	Bc vs. B6	Bc vs. 129	B6 vs. 129
P2	7,52E-04	1,79E-03	3,14E-01
P4	4,04E-02	2,08E-04	6,49E-03
P6	3,12E-01	1,54E-01	1,15E-02
P8	3,30E-01	5,91E-01	7,70E-01*
P10	2,87E-02	1,79E-01	8,56E-01*
P12	5,87E-02	9,24E-02	1,54E-01
P14	3,36E-01	4,10E-01	9,69E-01

Peak frequency	Bc vs. B6	Bc vs. 129	B6 vs. 129
P2	8,65E-05	4,64E-01	3,57E-02
P4	3,17E-01	7,57E-01	7,20E-01
P6	1,49E-01	6,97E-02	2,47E-02
P8	5,45E-01	5,54E-01	8,82E-01*
P10	7,27E-01	1,62E-02	1,24E-02
P12	8,44E-01	2,18E-02	3,01E-03
P14	7,15E-04	8,15E-02	3,59E-01

Bandwidth	Bc vs. B6	Bc vs. 129	B6 vs. 129
P2	1,29E-06	9,20E-02	1,21E-07
P4	3,26E-02	7,72E-01	2,30E-03
P6	9,62E-04	5,32E-05	2,44E-02
P8	3,91E-01	3,07E-02	1,59E-01*
P10	6,85E-02	9,45E-01	1,13E-01*
P12	4,21E-01	6,35E-01	3,30E-02
P14	1,02E-06	9,73E-03	7,08E-01

Bout duration. The next analyzed parameter (bout duration) is resultant of two other parameters: call duration and burst elements number. This dependency was reflected in the values recorded for the three strains. Bout duration recorded for the BALB/c strains was much higher for the most test time, with a very clear peak at P6 and then with clear sloping tendency until P14. Curves estimated for the 129SV and C57BL6 strains had a similar course with the most similar values at P8 and P10.

Bout interval. The last parameter that we estimated for USV bouts was the time interval between the bouts of calls. This parameter varied strongly between

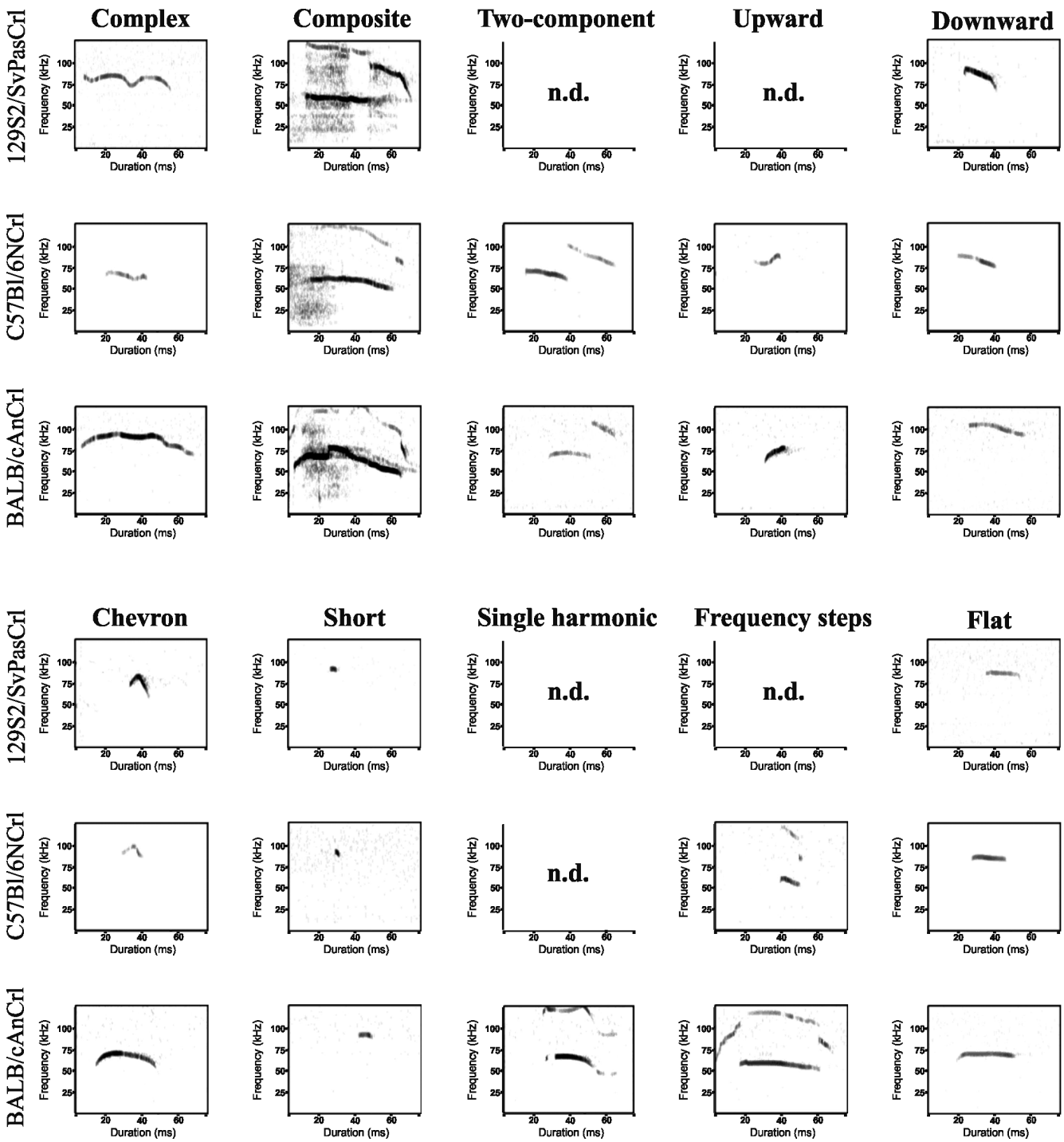

Fig. 2. Classification of obtained sample USV sonograms according to Scattoni and coauthors (2008b). (n.d.) not detected.

examined strains, what was reflected by curves showing the time course for each strain. Because the bout interval shows the time, when mice do not call, the curves resemble, to some extent, an inverse relationship to that of the burst rate, indicating the lower USV activity. In good agreement with that, was the shape of the curves estimated for all three strains. P8 and P10 were the time points, when the values for the 129SV and C57BL6 strains had the most similar values.

Mean peak frequency. The mean peak frequency is expressed as the frequency at the maximal amplitude within a call. analysis of the graph calculated for this parameter shows developmental variability, as in the case of other parameters. Fluctuations of values for all the three strains were very high with similar values at P4 and P8 and the biggest differences at P10–P12.

Mean bandwidth. Mean bandwidth describes the difference between the minimal and maximal vocalization frequency recorded for the pups during each call. At first glance, the curves for all three strains had quite similar course, closer analysis however revealed differences, especially in the first week of experiment. Whereas the values for 129SV peaked at P4 and then were dropping continuously until P12, values for the C57BL6 strain showed almost permanent growing tendency. Curves estimated for these strains crossed between P8 and P10, where the values were most similar.

Sonogram classification. To evaluate strain differences at the sonographic level, we analyzed sonograms obtained during USV recordings. To be consistent with previously published data (Scattoni et al. 2008b), we focused only on recordings made at P8. Spectrograms from at least five different animals from each strain were selected randomly and all calls were classified into ten distinct call categories (Fig. 2). Surprisingly, not all call categories could be found in all three strains. Figure 2 shows the most representative sonograms from all categories for each strain. We were not able to find two-component, upward, single harmonic and frequency steps call types in the 129SV strain. Analysis of sonograms recorded for the C57BL/6 line did not reveal any calls in the single harmonic category.

Data obtained from all recordings allowed establishment of a developmental profiles for all of the selected parameters. Looking at the graphical evaluation presented in Figure 1 and at the statistical analysis summarized in Table I, it becomes obvious that analyzed mouse strains differ substantially in these parameters. The point is that the differences become clear when we look at the time course, while mean values (for the whole recording period) differ statistically significantly only in the case of three parameters: call duration, bout element number and bout duration (data not shown).

Although it seems obvious, we have to stress the fact, that the parameters characterizing USV strongly depend on the genetic background. To determine which parameters analyzed in our study show the strongest differences among selected strains, we analyzed all parameters on a daily basis and compared results by means of the (one-way) ANOVA test. We analyzed all parameters for each day by ANOVA test (Table I). The greatest differences are visible between the BALB/c and C57BL6 strains. Our conclusions are supported by the fact, that for all data points (parameter × day of test = 56), statistically significant differences were obtained in 63% cases for BALB/c vs. C57BL6, in 54% cases for BALB/c vs. 129SV and in 41% cases for C57BL6 vs. 129SV. This result reflects the genetic relationship between the analyzed strains (Atchley and Fitch 1991).

The main goal of our study was to analyze the USV from the point of view of transgenic manipulations, utilizing in most cases animals from the 129SV and C57BL6 strains. As result we obtain transgenic animals with different content (very often not known) of two different genetic backgrounds. Of course analysis of USV in a transgenic mouse line aims to detect USV alterations generated by the introduced transgene and not by the genetic background. To circumvent this issue we can choose following strategies: (i) backcrossing of the transgenic line in to one of the strains used in the transgenic approach, in our case 129SV or C57BL6, for at least 10 generations. Then we can assume, that our transgenic line has a pure genetic background and we can also use a standard strain (129SV or C57BL6) as control, (ii) use as the control group only animals from the same transgenic generation. Then we can assume, that all animals used for the experiment have the same portion of both genetic backgrounds, (iii) selection of the USV parameters with possibly lower dependency on the genetic background and/or (iv) selection of the experimental period (postnatal day), where the differences between animals from both standard strains are the lowest.

According to data from our study only “call duration” and “burst element number” (partially) would fit criteria mentioned in paragraph 3. It is much easier however, to find the appropriate postnatal day, when values obtained

for the 129SV and the C57BL6 are similar. We can assume, that at this time points obtained results would be less influenced by the genetic background. When we analyze data presented in Table I, we can conclude, that the best developmental period for USV analysis is between P8 and P10, however with one exception – sonogram analysis. Recordings for analysis of that trait should be made at a time point with the highest USV activity. To select the proper postnatal day we can look at the curves established for vocalization rate and bout rate, because these two parameters give us a hint on the intensity of vocalization at a given time point. When we look at the charts for vocalization rate and bout rate we will see, that the highest values for the 129SV strain have been recorded in the second postnatal week (Fig. 1). Different results have been obtained for the C57BL6 strain, where the highest values have been recorded in the first postnatal week. To compare our results with data obtained earlier (Scattoni et al. 2008b, Scattoni and Branchi 2010), we analyzed spectrograms obtained on P8. Surprisingly, we were not able to find USV calls from the all ten categories described by these authors. Four of the parameters were missing in the 129SV strain. In reviewing the developmental time course established for vocalization rates in that strain, we can easily see the second lowest value at P8. We can, therefore, hypothesize that missing USV categories were not present on that particular day, but could be found on another one. This possibility needs to be pursued and confirmed in the future.

General conclusions we have drawn from our study are: (i) parameters describing the USV strongly depend on the genetic background and the phase of development, (ii) the most stable parameter during the 14 days of development for all three tested strains is call duration, (iii) the best developmental phase for USV analysis (except sonograms) is between P8 and P10, where the differences between the 129SV and C57BL6 strain are the lowest, (iv) for the sonogram analysis a postnatal day with a high USV activity (high vocalization and bout rate) should be selected.

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