The clock gene *Period1* regulates innate routine behaviour in mice

Philipp Bechstein¹, Nils-Jörm Rehbach³, Gowzakan Yuhasingham¹, Christoph Schürmann², Melanie Göpfert¹, Manfred Kössl³ and Erik Maronde¹

¹Dr. Senckenbergische Anatomie, Institut für Anatomie III, Goethe-University, Theodor-Stern-Kai 7, 60590 Frankfurt, Germany
²Institute for Cardiovascular Physiology, Goethe-University, Theodor-Stern-Kai 7, 60596 Frankfurt, Germany
³Institute for Cell Biology and Neuroscience, Goethe-University, Max-von-Laue-Straße 13, 60438 Frankfurt, Germany

Laboratory mice are well capable of performing innate routine behaviour programmes necessary for courtship, nest-building and exploratory activities although housed for decades in animal facilities. We found that in mice inactivation of the clock gene *Period1* profoundly changes innate routine behaviour programmes like those necessary for courtship, nest building, exploration and learning. These results in wild-type and *Period1* mutant mice, together with earlier findings on courtship behaviour in wild-type and *period*-mutant *Drosophila melanogaster*, suggest a conserved role of *Period*-genes on innate routine behaviour. Additionally, both *per*-mutant flies and *Period1*-mutant mice display spatial learning and memory deficits. The profound influence of *Per1* on routine behaviour programmes in mice, including female partner choice, may be independent of its function as a circadian clock gene, since *Per1*-deficient mice display normal circadian behaviour.

1. Background

Laboratory mice retain a repertoire of innate routine behaviour necessary to survive in natural environments, although housed for decades under artificial conditions [1,2]. However, many of these innate routines are not (or cannot be) performed under standard housing conditions with food and water ad libitum, and are therefore rarely observed or described.

One fundamental influence on innate routine behaviour of most animals is the temporal variation of environmental conditions like the light/dark cycle [3–6]. Current concepts on the usefulness of the circadian clock in terms of evolutionary fitness presume that it serves the anticipation of a rhythmically changing environment, important for proper feeding regulation and other physiological functions [6,7]. In a cold environment, temporal information may serve mice for anticipation of the next night in which temperature lowers, and building of a nest helps to save energy and protect adults as well as offspring. Differences in daytime-dependent behaviour between laboratory and natural conditions have been described in mice, hamsters and fruitflies, suggesting a profound influence of light/dark cycle on the behavioural repertoire of model species [6,8,9].

One gene involved in the circadian clock system in flies and mice is *Period1* (*Per1*) [10–12]. Notably, the insect version of *Per1*, *Per*, is involved in circadian, learning and courtship behaviour [10,13,14].

In this work, C3H mice (wild-type: WT) and animals of the same strain lacking a functional *Period1* gene (*Per1<sup>−/−</sup>*) [12,15,16] were compared regarding body weight, ultrasonic vocalization (USV) and various tests for the comparison of innate routine behaviour [1,17–23].

Data presented here show profound differences between *Per1<sup>−/−</sup>* mice and their WT controls in body weight, USV, habituation, explorative and social behaviour, indicating an important influence of the *Period1* gene on traits regarding the circadian clock and other physiological systems pointing out the pleiotropic properties of the so-called clock gene *Period1*.
2. Material and methods

(a) Animals
Per1-deficient (129S-Per1<sup>tm1Drw</sup>/J) mice [12] and the corresponding WT were both bred back onto a melatonin-proficient C3H/HeN genetic background [15,16]. Health status of the animals was monitored regularly as described previously [24,25].

(b) Housing conditions
Mice were housed under a 12 L/12 D cycle at an ambient temperature of 22 ± 2°C and access to food and water ad libitum. Red light intensity at darkness/night was below 10 lux. Adult male mice were 6–10 months of age during the experiments. Pups tested were 3 days old. Male mice exposed to a female came from standard group housing conditions and had never been in contact with a female post-weaning primary to the start of the experiments. All experiments were conducted with male mice under dim red light using infrared camera detection if not otherwise indicated.

(c) Ultrasonic vocalization
Male mice were housed in sibling groups. For female-induced USV measurements, a male mouse was isolated from the group and placed into an individual cage for at least 24 h prior to the start of the experiment. Females were housed separately and introduced into the experimental context immediately prior to USV recordings. Male and female animals were placed on alternate sides of a clean cage. A translucent plastic plate with holes prevented direct contact and mating but allowed visual, olfactory and some mechanical sensing. All experiments were performed during the dark period, the animal’s active phase, between Zeitgeber time (ZT) 13 and ZT18. Zeitgeber time zero (ZTO) marks the beginning of the light phase, ZT12 the beginning of darkness.

USVs were detected using an ultrasonic microphone (condenser ultrasound microphone Avisoft CM16/CMPA), an amplifier (UltraSoundGate 116 Hb), recording software (Avisoft RECORDER) and sound analysis software (Avisoft SASLAB Pro, all from Avisoft Bioacoustics, Berlin, Germany). The ultrasound microphone was placed at a distance of 20 cm above the male compartment. In this set-up, USVs were recorded continuously for 480 s with a sampling rate of 250 kHz in the male compartment of the test apparatus. The measurements were conducted with the same male–female pairs on 10 consecutive days. To investigate memory retention or extinction an interval of 30 days was introduced before the last USV measurement after the initial 10 days of the experiment. At the end of the recordings, number of calls (‘call rate’), call length, time intervals between calls, as well as minimal and maximal frequencies within each USV element and maximal amplitude of the calls were measured using Avisoft SASLAB Pro with spectrogram parameters as described previously [26]. A high-pass filter with a cut-off frequency of 40 kHz was used because of broadband noise in the lower frequencies. Calls were automatically measured by the function ‘whistle tracking’ with parameters ‘max change’ of frequency modulation set on 32 pixels = 7812 Hz, min duration = 10 ms and hold time 20 ms. Afterwards an experienced experimenter for correct element detection manually screened the spectrograms.

(d) Pup isolation calls
Pups of both genotypes at post-natal day 3 were separated from the mother and placed into a glass beaker padded with nesting material, which was placed on a warming plate. Each pup was then put for 5 min into a sound-attenuated recording chamber equipped with microphone, amplifier and recording software as described above. After the measurements recordings were analysed using Avisoft SASLAB Pro.

(e) Female preference
To analyse the preference of a WT female mouse for a male of either WT or Per1<sup>−/−</sup> genotype, a so-called ‘sociability cage’ (Noldus Information Technology, Wageningen, The Netherlands) was used [27–29]. The sociability cage is a rectangular box consisting of one centre and two side chambers, divided by walls providing just a small passage to the next chamber. In each of the two side chambers, a small cylindrical wire cage was placed containing either a male of the WT or a male of the Per1<sup>−/−</sup> genotype. At the beginning of the test, the female was placed into the centre chamber, thereby given the choice between the different genotypes in the right or in the left chamber. The whole test session of 10 min was recorded on video and later the time spent at each cage was analysed by behaviour evaluation software (Ethovision XT, Noldus Information Technology). After each recording session the apparatus was cleaned and in the next session the location of the WT and Per1<sup>−/−</sup> males was alternated to avoid effects of right or left preference of the WT female.

(f) Nest building
To analyse the nest-building behaviour of WT and Per1<sup>−/−</sup> mice, a standardized five-point scale protocol was used [22]. Since mice build their nest during the dark period [1], a single condensed piece of hemp fibre (Happi-Mat, Scanbur-Nova SCB, Sollentuna, Sweden) was placed in the cage 1 h before onset of darkness. Twenty-four hours later the status of the nest material was evaluated using a standardized scale [22].

(g) Explorative behaviour/marble burying
Initially, we tested the performance of WT and Per1<sup>−/−</sup> mice in the ‘marble burying’ set-up, an experimental situation to test mice for their interest in ‘novelty’ [23,28]. It took advantage of the fact that in a fresh conventional mouse cage (Polycarbonate, type II Euro-standard 267 × 207 × 140 mm) filled with a 50 mm layer of litter (Lignocel, Hygienic Animal Embedding, Rettenmaier & Söhne GmbH & Co KG, Rosenberg, Germany) animals explore the cages in all three dimensions by running, rearing and digging. Objects placed in the cage (like marbles) induce explorative behaviour. This includes prolonged digging eventually leading to burying of the marbles [23]. A video recorded during the 30 min of this experiment together with behaviour evaluation software (Ethovision XT, Noldus Information Technology) was used to analyse differences in mouse behaviour and quantify behavioural parameters such as time spent for running, rearing and digging as well as the latency before starting these activities.

(h) Data analysis
Statistical differences between groups were determined using GRAPHPAD PRISM v. 5.0d.

An ANOVA with Bonferroni post-test was used for comparing the number of USV and the mean and total call duration over the testing interval. The Kruskal–Wallis with Dunn’s multiple post-test was used to analyse the differences in peak frequencies and relative power of the USV. Nest building was analysed by Mann–Whitney U-test. To analyse the burying of marbles, the female preference and the body weight the Student’s unpaired t-test was used. The appropriate tests were chosen according to data structure and quality. A p-value of less than or equal to 0.05 was considered as significant.
mean + frequency was significantly lower in Per1−/− post-test (* Period1 vocalizations (USV) at day 2 in WT (n = 6 animals; total calls = 4031) and Period1-deficient (Per1−/−; n = 16 animals; total calls = 11 675) mice. Both maximal and minimal frequency was significantly lower in Per1−/− compared with WT mice. (b) Mean peak frequency of male USV at day 10 in WT (n = 6 animals; total calls = 206) and Period1-deficient (Per1−/−; n = 16 animals; total calls = 1398) mice. Both maximal and minimal frequency was significantly lower in Per1−/− mice compared with WT mice. Data were analysed by Kruskal–Wallis test with Dunn’s multiple comparison post-test (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001). All values are given as mean ± s.e.m.

3. Results

(a) Ultrasonic vocalization frequency and amplitude

WT (n = 6) and Per1−/− (n = 16) mice showed significant differences in the maximal and minimal peak frequencies (figure 1) and the peak amplitude of USV (figure 2) when confronted with a WT female. At day 2 (figure 1a), the mean maximal peak frequencies of each detected element (USV call) of the WT (78.9 ± 0.2 kHz) differed significantly (p ≤ 0.001) from the Per1−/− (77.7 ± 0.1 kHz) animals. The minimal peak frequencies were also significantly different and, similar to the maximal frequencies, lower in Per1−/− compared with WT (WT: 65.9 ± 0.1 kHz; Per1−/−: 62 ± 0.1 kHz).

At day 10, there was still a significant difference in mean maximal (WT: 80.9 ± 0.1 kHz; Per1−/−: 74.3 ± 0.3 kHz) and minimal (WT: 67 ± 0.8 kHz; Per1−/−: 64.7 ± 0.2 kHz) peak frequencies between WT and Per1−/− animals (p ≤ 0.001) (figure 1b).

This lower frequency in USV calling in male Per1−/− mice occurred despite their lower body weight, compared with WT controls (electronic supplementary material, figure S1).

3. Results

(b) Ultrasonic vocalization habituation behaviour

When male mice of either genotype were confronted with a female for 10 consecutive days, a striking difference in the USV was evident. WT mice displayed the highest number of USV calls at day 2 (558 calls ± 157) of the experiment. Then the call rate declined with every experimental day, reaching a significantly lower number of USV emissions on day 4 (113 calls ± 48) compared with day 2 (p ≤ 0.05), leading to a plateau after 6 days (figure 3).

In Per1−/− males, likewise to WT, the number of USV calls per experimental run was the highest at day 2 (649 calls ± 145). However, the decline in daily call rate in the Per1−/− mice reached a significant difference only at day 6 (284 calls ± 78) when compared with day 2 (p ≤ 0.05) (figure 3). At day 8, the call rate of Per1−/− mice reached the level (89 calls ± 42) that WT males had attained already at day 5 (78 calls ± 65). Nonetheless, the call-rate in Per1−/− mice was never as low as the lowest call-rate observed in the WT (day 10: WT = 19 calls ± 17; Per1−/− = 71 calls ± 42). Figure 3 shows properties of habituation behaviour with a delay of habituation observed in Per1−/− compared with WT animals. After 30 days of no contact of the males to a female, the WT males were still ‘habituated’, as they show no significant difference between the 10th and 40th day (p > 0.05). By contrast, the Per1−/− males
emitted as many USVs as at the first day of the experiment (day 11: WT = 189 calls ± 150; Per1−/− = 496 calls ± 107), however, with a significant difference to the 10th day (all differences determined using one-way ANOVA with Bonferroni post-test, p < 0.01). There were also habituation effects on mean and total call duration (electronic supplementary material, figure S3). Interestingly, the peak amplitude (figure 2) also decreased significantly between day 2 and day 10 in WT but not in Per1−/− mice (Kruskal–Wallis test, p < 0.001). To visualize the structure of USV from WT and Per1−/− mice exemplary spectrograms of day 2 and day 10 are shown (figure 4).

(c) Nest building
For evaluation of nest-building behaviour, we used male WT (n = 9) and Per1−/− mice (n = 17). Only one of the 17 Per1−/− mice built a reasonable nest (score = 4), two reached a score of 3 and one a score of 2, while 11 animals left the nest-building materials unchanged (score = 1; figure 5) over 24 h. Per1−/− mice with score 1, who did not process the nest-building material during the regular 24 h experiment, left nest-building materials unprocessed for up to two weeks. By contrast, the nine WT animals built nests of a high quality with a mean score of 4 of 5 maximal score points. The difference in nest-building score between WT and Per1−/− mice was significant (p < 0.0002).

(d) Explorative behaviour/marble burying
In the set-up for the determination of explorative behaviour, Per1−/− (n = 11) mice buried as many marbles as the WT (n = 6) animals over the time of the experiment (figure 6a). However, both the latency time before the animals started to dig or bury (p < 0.0002) (figure 6c) and the time spent with rearing (figure 6b) were significantly different between WT and Per1−/− mice (p < 0.05).

(e) Female preference test
To investigate a preference of WT females (n = 3) for one of the two genotypes, a female mouse was given the choice between WT and Per1−/− males placed in the right or in the left chamber of a two-chamber sociability cage (figure 7). The time WT females spent at the WT male cage (60.6 ± 9.1 s; n = 12) was significantly shorter compared with the Per1−/− males (98.1 ± 12.9 s; n = 12; p < 0.05).

4. Discussion
(a) Physical differences between wild-type and Per1−/− mice
Our data show that male Per1−/− mice confronted with a WT female vocalize at significantly lower frequencies compared with WT male animals. This is surprising as both female [17] and male (this study; electronic supplementary material, figure S1) Per1−/− mice are lighter than their WT controls. Lighter individuals in a given species normally have a higher voice pitch [30,31]. Interestingly, the lighter Per1−/− male mice displayed a lower USV frequency compared with the heavier WT animals.

The amplitude of the USVs was higher in WT compared with Per1−/− mice on day 2. One possible reason for this observation could be differences in hearing capacity between WT and Per1−/− mice. However, we found no difference in the auditory brain stem response, a measure for hearing capacity, between Per1−/− mice and WT control animals (data not shown), and it has been shown that auditory input is not essential for the development of USV [32].

To evaluate whether the altered properties of the USV of male Per1−/− mice cause a different attraction of the female WT we used the female preference test. Indeed, WT females spent more time exploring the Per1−/− male cage area than that of the WT males. One possible explanation for this preference of the WT female for the Per1−/− males may be their lowered USV frequency. It remains to be determined if other qualitative USV differences, like call structure, or a differing scent [33,34], may also be a reason for the higher attraction of the Per1−/− males to WT females [35]. Interestingly, it has been shown that female mice are able to discriminate USV from siblings and genetically unrelated males [36]. Thus, female choice appears to favour the mutant over the WT male, which consequently would lead, in the case of successful mating, to higher genetic diversity of the offspring, a known principle for the elevation of fitness in a population [29,37].

(b) Innate routine behaviour in laboratory mice
Laboratory mice still exhibit innate routine behaviour when provided with appropriate conditions or environments although kept under rather uniform housing conditions for decades [1,2,38]. This is important in the context that hippocampal learning and memory processes differ between mice held in poor or enriched environments [39]. It is also known that mice show elevated activity during night-time when food and water is available ad libitum [6,41]. Therefore, innate routine behaviour must be tested during the dark period to avoid the influence of stress or arousal owing to waking the animals during their sleep phase [2,38,41].
Deletion of clock genes causes pronounced cognitive and behavioural effects throughout the animal kingdom [41]. 

Period1-deficient mice display alterations in glucocorticoid rhythmicity [42], addiction [43], muscle strength [44], colonic motility [45], fertility [17,46] and memory [16].

One of the propositions of this work was that the clock gene Per1 is important for courtship behaviour and influences reproductive success. Evidence for that conjecture comes from altered courtship behaviour in Per-mutant male fruit-flies (Drosophila melanogaster) [14,47] and the observation of a smaller litter number under homozygous breeding of Per1<sup>−/−</sup> mice [17].

Thus Per-genes appear to be involved in reproductive behaviour of both flies [14,47] and mice, as shown here. Interestingly, in sand flies (Lutzomyia spp.), altered ‘courtship song’ owing to a mutated Period gene has recently been proposed to cause speciation [48].

**Figure 4.** Shown are exemplary spectrograms of USV at day 2 and day 10 in male (a) WT and (b) Per1<sup>−/−</sup> mice. Frequency (kHz) and the relative level of signal intensity (dB) are plotted against time (s). Note the lower intensity (grey scale on the right-hand side) of the calls at day 10 compared with day 2.

**Figure 5.** Shown is the mean nest-building score in WT (white, n = 9) and Per1<sup>−/−</sup> (grey, n = 17) male animals (Mann–Whitney U-test; ***p ≤ 0.001). All values are given as mean ± s.e.m.

**c** Clock genes and behaviour

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Thus Per-genes appear to be involved in reproductive behaviour of both flies [14,47] and mice, as shown here. Interestingly, in sand flies (Lutzomyia spp.), altered ‘courtship song’ owing to a mutated Period gene has recently been proposed to cause speciation [48].

**d** The hippocampus and innate routine behaviour

The hippocampal formation is involved in both learning and the execution of innate routine behaviour [21–23,49]. Examples of such repeated routines tested here include courtship behaviour, social recognition, nest-building and locomotive/explorative aspects of behaviour like latency to start movements, digging or rearing [1,23].

**e** Ultrasonic vocalization-courtship and social behavioural aspects

Under repeated confrontation of a male and a female, the male WT control animals rapidly and significantly reduced the
amplitude in Per1−/− in WT mice decreased from day 2 to day 10, whereas the day 6. In parallel, the amplitude of the single USVs remained habituated, not reacting significantly more than after and reproduction [1,2,39].

which live in large social groups, such behaviour is important stimulus’ in the context of the testing set-up [51,52]. In mice, nition’ or reduced salience of the female as a ‘mating-

radial arm maze test for hippocampus-related memory values are given as mean

per day (day 4), whereas Per1−/− males showed no significant reduction of USV call number before day 6. In parallel, the amplitude of the single USVs in WT mice decreased from day 2 to day 10, whereas the amplitude in Per1−/− mice did not change. Such habituation deficiency suggests a learning and/or memory deficit in Per1−/− mice compared with WT. This is interesting since Per1−/− mice were reported to display a phenotype in the radial arm maze test for hippocampus-related memory [16,50]. It is not clear if this habituation reflects ‘social recognition’ or reduced salience of the female as a ‘mating-stimulus’ in the context of the testing set-up [51,52]. In mice, which live in large social groups, such behaviour is important for both energy conservation (to avoid unnecessary fighting) and reproduction [1,2,39].

After an inter-trial interval of 30 days (day 40), the WT males remained habituated, not reacting significantly more than after day 10, whereas the Per1−/− mice emitted as many calls as in day 2. One interpretation of this finding is that the Per1−/− mice do not recall the experimental situation of not being able to socialize or mate, whereas the WT males show long-term observation that anxious behaviour against the novel object in their environ-

ment. The latter interpretation is compatible with the observation that Per1−/− mice to memorize or recall a specific individual.

Since social recognition is hippocampus-dependent [21], this might explain the deficits of Per1−/− mice regarding the habituation or social recognition in this task. Taken together, these findings point to a fundamental deficit in both a simple (this study) and a more complex, hippocampus-related behaviour [16] in male Per1−/− mice.

Our data from the USV studies raised our interest in natural routine behaviour apart from courtship. Owing to the habituation phenotype discussed above and the known learning deficit of Per1−/− mice [16], we also examined non-reproductive hippocampus-dependent innate routine behaviour.

(f) Nest building

Nest building in rodents serves heat conservation and reproduction, as well as shelter from predators for both the animal itself and its offspring [22,53]. It requires orofacial and fore-limb movement [22,53,54], and is impaired by hippocampal lesions [55]. Both male and female mice build nests, thereby suggesting that the aspects of heat conservation and shelter are at least equally important as reproduction [22]. The overt deficiency of building a proper nest in the Per1−/− mice was somewhat surprising and unexpected, since this behaviour appears fundamental to the animals’ survival [2]. This nest-building deficiency supports our conclusion of hippocampal dysfunction in Per1−/− mice.

(g) Explorative behaviour/marble burying

Longer latency of the Per1−/− mice in the initiation of behav-

iour was a frequently observed phenomenon. Latency to start a behaviour is a hippocampus-dependent feature and is prolonged by lesion [55]. In the test set-ups for marble burying, the Per1−/− mice spent less time rearing and needed longer for the initiation of the digging behaviour (latency time). These findings may be interpreted in different ways: (i) Per1−/− mice are less interested in the marbles and/or in the new environment, and therefore start exploring later; (ii) Per1−/− mice are less well capable of interpreting the set-up as novel than WT mice; or (iii) Per1−/− mice display anxious behaviour against the novel object in their environ-

ment. The latter interpretation is compatible with the observation that Per1−/− mice spend less time rearing, a
phenomenon related to hippocampal defects associated with impaired explorative behaviour [35].

5. Conclusion

In conclusion, WT mice are well capable of exercising innate routine behaviour although having been in captivity and rather uniform housing conditions for decades. Per1−/− mice of the same genetic background display significant differences to their WT controls, suggesting strong genetic influence of this clock gene on innate routine behaviour. The observations described here and the recently reported learning deficits in Per1−/− mice suggest fundamental differences in hippocampal function as a potential explanation for the behavioural discrepancies between WT and Per1−/− mice. Such differences displayed in the wild may constitute a basis for specification in the long run.

All experiments reported here were conducted in accordance to the guidelines of the European Communities Council Directive (89/609/EEC) for humane animal care.

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Data accessibility. Raw data are deposited at Dryad (doi:10.5061/dryad.1ceE3).

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It has been brought to our attention that the data analysis for figures 1 and 2 is not appropriate. The colleague who contacted us correctly remarked that we should have compared the mean frequencies of the two genotypes, instead of all calls of all animals in the genotypes, because the genotype is the decisive parameter ('The decisive number for calculating the mean + s.e.m. for each column is the number of animals, not the number of calls. Therefore, the calculations and the statistics are misleading').

All ultrasound vocalization (USV) data regarding frequency and amplitude have therefore been re-analysed accordingly, the figures were revised and corrected and all frequency and amplitude data are now provided together with the statistical data and the analysis procedure. In brief, we first took the data from the automated analysis provided by AviSoft SASLabPro for USV parameters (start-, peak-, end-frequency, start-, peak-, end-amplitude, etc.) and calculated a mean value for each parameter of each individual mouse (see the electronic supplementary material, tables S1–S3). These mean values were then tested for Gaussian distribution (normality) with the Kolmogorov–Smirnov test with Lilliefors-modification. If the normality-test was passed, we compared the genotypes by Student’s *t*-test, if not by an appropriate non-parametric test, e.g. Mann–Whitney *U*-test. All *p*-values are ‘given per comparison’.

3. Results

(a) Ultrasound vocalization frequency and amplitude

WT (*N* = 6) and *Per1*^−/−* (*N* = 16) mice showed differences in the maximal and minimal peak frequencies (figure 1) and the peak amplitude of USV (figure 2) when confronted to a WT female. At day 2 (figure 1a), the mean maximal peak frequencies of each entire element (USV call) that was detected of the WT (81.1 ± 8.0 kHz) differed from the *Per1*^−/−* (74.0 ± 9.9 kHz) animals. The minimal peak frequencies were significantly different and, similar to the maximal frequencies, lower in *Per1*^−/−* compared to WT (WT: 68.0 ± 4.7 kHz; *Per1*^−/−*: 60.7 ± 7.3 kHz; *p* ≤ 0.02 *t*-test).

At day 10, there was still a difference in mean maximal (WT: 73.1 ± 19.2 kHz; *Per1*^−/−*: 61.9 ± 13.1 kHz) and minimal (WT: 65.2 ± 17.1.8 kHz; *Per1*^−/−*: 53.3 ± 11.8 kHz) peak frequencies between WT and *Per1*^−/−* animals (figure 1b).

This trend towards lower frequency in USV calling in male *Per1*^−/−* mice occurred despite their lower body weight, compared with WT controls (electronic supplementary material, figure S1).

Already 3 days after birth, pup isolation calls (USV emitted by the offspring when separated from their mother) displayed a lower maximal peak frequency in the *Per1*^−/−* animals compared with WT (WT: 88.0 ± 6.7 kHz; *Per1*^−/−*: 76.7 ± 3.9 kHz; *p* ≤ 0.0001 *t*-test; electronic supplementary material, figure S2).

In adult males, peak maximal amplitudes of each entire element (figure 2) exerted a difference between WT and *Per1*^−/−* animals at day 2 (WT: −27.2 ± 6.5 dB; *Per1*^−/−*: −32.1 ± 7.1 dB) as well as at day 10 (WT: −35.3 ± 4.6 dB; *Per1*^−/−*: −31.0 ± 3.7 dB; *p* ≤ 0.05; *t*-test).
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Figure 1. The bar graph shows minimal and maximal peak frequencies of WT (white bars) and Per1<sup>−/−</sup> (grey bars) mice. (a) Mean peak frequency of male ultrasonic vocalizations (USV) at day 2 in WT (N = 6 animals) and Period1-deficient (Per1<sup>−/−</sup>; N = 16 animals) mice. Both maximal and minimal frequency was lower in Per1<sup>−/−</sup> compared to WT mice. (b) Mean peak frequency of male USV at day 10 in WT (N = 6 animals) and Period1-deficient (Per1<sup>−/−</sup>; N = 16 animals) mice. Both maximal and minimal frequency was lower in Per1<sup>−/−</sup> mice compared with WT mice. Genotype comparison was done by t-test (*p ≤ 0.05). All values are given as mean ± s.e.m.

Figure 2. Shown is the relative power (dB) of the mean peak max amplitude of male USV at day 2 and 10 in WT (N = 6 animals; white bars) and Period1-deficient (Per1<sup>−/−</sup>; N = 16 animals; grey bars) mice. The peak amplitude was not different in Per1<sup>−/−</sup> mice compared with WT mice at day 2. By contrast, the mean peak amplitude was significantly higher at day 10 in Per1<sup>−/−</sup> mice compared with WT mice. Genotype comparison was done by t-test (*p ≤ 0.05). All values are given as mean ± s.e.m.