Effects of Genetic Background, Gender, and Early Environmental Factors on Isolation-Induced Ultrasonic Calling in Mouse Pups: An Embryo-Transfer Study

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Abstract Infant rodents emit ultrasonic vocalizations when isolated from dam and littermates. Due to the context of their occurrence and the well described bidirectional modulation by substances known for their capability to influence emotionality, it was postulated that such calls reflect a negative affective state akin anxiety. Comparative studies observed pronounced differences in calling behavior between strains, which were paralleled by differences in maternal care. Therefore, it was recently hypothesized that early environmental factors may have strong impact on call production. Here, the relative contributions of genetic background, gender, and early environmental factors on calling behavior in C57BL/6JOlaHsd and C57BL/6NCrl were studied by using an embryo-transfer procedure. The results show that these sub-strains differ in the amount of calling and specific call features, like call frequency and amplitude. The embryo-transfer procedure indicated that the observed differences in the amount of ultrasonic calling are dependent on the dyadic interaction between mother and pup. Conversely, call features were primarily dependent on the genotype of the pup. Thus, call frequency and frequency modulation were solely dependent on the pup, i.e. its genotype and gender. However, there was one exception, namely call amplitude, which was solely dependent on the genotype of the mother, i.e. on early environmental factors. Furthermore, it was shown that particularly changes in call amplitude might be of high functional relevance, since a sub-strain dependent preference towards pups emitting calls with high amplitudes was observed. In total, it can be concluded that both genomic and nongenomic factors can tune calling behavior in mouse pups.

Keywords Ultrasonic vocalization · Maternal care · Pup retrieval · Individuality · Anxiety · Alpha-synuclein · Embryo-transfer · Strain differences · Inbred · USV · C57BL/6N · C57BL/6JOla

Introduction

Infant rodents emit ultrasonic vocalizations when isolated from dam and littermates (e.g. Zippelius and Schleidt 1956; for review see: Constantin and D’Amato 2006). Such calls play an important role in pup survival, since they can elicit maternal behavior, like retrieval (Allin and Banks 1972; Ehret 1992; Ehret and Haack 1982; Sewell 1970; Smith 1976; Smotherman et al. 1974; Wöhr and Schwarting 2008; for review see: Ehret 2005). Importantly, isolation-induced ultrasonic vocalizations seem to reflect a negative affective state akin anxiety, since they are modulated by anxiogenic and anxiolytic drugs (Gardner 1985; Insel et al. 1986; for review see: Hofer 1996). Also, these pup vocalizations have been proposed as sensitive markers to
evaluate alterations of neurobehavioral development (Branchi et al. 2001). Therefore, they have received increasing experimental attention, for example, to examine the respective roles of genetic, maternal and other environmental influences.

The importance of genetic effects was indicated by early studies where differences between species and strains were observed (Sales and Smith 1978). Within the species Mus musculus, inbred strain differences in call rate and call characteristics have been consistently observed (Bell et al. 1972; Cohen-Salmon et al. 1985; Hennessy et al. 1980; Robinson and D’Udine 1982), and genetic studies have shown, in summary, that call rate and probably all acoustic call characteristics have a multiple genetic background shown, in summary, that call rate and probably all acoustic call characteristics have a multiple genetic background (Hahn et al. 1987, 1997, 1998; Hahn and Schanz 2002; Roubertoux et al. 1996; Thornton et al. 2005). Ehret (2005) explained this observation by the fact that genes in three main areas of the infant development may affect ultrasonic vocalizations, namely genes, which contribute to the perceptual pathways of the nervous system that are responsible for the perception of the releasing stimuli, genes that are involved in the regulation of emotion and motivation, and genes that are linked to the anatomical properties of the breathing system and larynx. The multitude of genetic influences on sound production was also observed in studies on knockout mice. There it was found that mice with demyelization (Bolivar and Brown 1994), mice lacking Foxp2 (Shu et al. 2005), MeCP2 (Picker et al. 2006), oxytocin (Winslow et al. 2000), or different receptors, like mu-opioid (Moles et al. 2004), vasopressin 1b (Scattoni et al. 2007), 5-HT1A (Weller et al. 2003), 5-HT1B (Brunner et al. 1999; El-Khodor et al. 2004; Weller et al. 2003), and CB1 (Fride et al. 2005) show altered calling behavior in infancy.

Besides, numerous environmental variables, in particular maternal care, have also been shown to modulate ultrasonic calling in rodents. Hofer and Shair (1978, 1980; for review see: Hofer 1996) showed that the mere presence of the dam acutely inhibits ultrasonic calling. Moreover, brief interactions of the pup with its dam can induce an intensified vocal response during subsequent isolation (Hofer et al. 1994, 1999; Moles et al. 2004; Muller et al. 2005, 2008; Myers et al. 2004; Shair et al. 1997, 2003; for review see: Shair 2007). Apart from acute and short-term effects, however, there are also data suggesting that maternal behavior can have long-term effects on ultrasonic calling of pups during isolation. Such long-term effects were indicated by genetic analyses, where small but persistent maternal effects on call rate, duration, frequency, and frequency modulation were observed (Roubertoux et al. 1996; Thornton et al. 2005). A possible mechanism for maternal effects on ultrasonic calling was observed by D’Amato and Populin (1987) who found that call rate of normal mouse pups was reduced when reared by deaf mothers, indicating that the absence of an adequate response by the mothers can result in a reduction of calling behavior. However, in pups raised by normal mothers, reduced calling rates may not result from the absence of adequate maternal responses, but instead from a sustained level of maternal care yielding anxiolytic-like effects. Recently, D’Amato et al. (2005) demonstrated that pups raised by mothers from the more responsive C57BL/6 strain elicited fewer isolation-induced calls than those raised by the less responsive BALB/c strain.

Strain differences in mice have been reported for several measures of maternal behavior, like pup retrieval, nest building, nursing, and licking (Carlier et al. 1982; Cohen-Salmon et al. 1985; Champagne et al. 2007; Hennessy et al. 1980). Evidence for maternal effects on offspring development came from reciprocal breeding of inbred mouse strains (Calatayud and Belzung 2001; Calatayud et al. 2004) and cross-fostering studies (Francis et al. 2003; Priebe et al. 2005; Zaharia et al. 1996; for review see: Gordon and Hen 2004). By using an embryo-transfer, Francis et al. (2003) were able to show that early environmental factors hold strong influence on anxiety-related behavior in adult mice. From rat studies it is known that variations in the nursing style affect the development of stable individual differences in emotionality (Caldji et al. 1998; Francis et al. 1999; Menard et al. 2004; Menard and Hakvoort 2007; Zhang et al. 2005), and that isolation-induced calling is a sensitive marker for differences in maternal licking experienced throughout the first week of life (Wöhr and Schwarting 2008).

The objective of the present study was to assess potential causes of individual differences in various characteristics of pup ultrasonic vocalizations in C57BL/6 mice. To dissociate between effects of genetic background and early environmental factors, an embryo-transfer was conducted, where blastocysts of C57BL/6JolaHsd (B6Jola) and C57BL/6NCrl (B6N) were transferred to pseudo-pregnant females either of the same or the other sub-strain. These sub-strains were selected since it is known that they differ in adult anxiety-related behavior, namely the course of extinction of conditioned fear. Thus, C57BL/6Jola develop lower levels of freezing to the context where they have been shocked before, and their maximal fear responses were restricted to a shorter period of time (Radulovic et al. 1998; Siegmund et al. 2005; Siegmund and Wotjak 2007; Stiedl et al. 1999), reflecting a different susceptibility to develop symptoms resembling those in posttraumatic stress disorder (PTSD; Siegmund and Wotjak 2007).
Materials and methods

Experiment I—embryo-transfer

Animals and housing

C57BL/6NCrl (B6N) mice were purchased from Charles River Laboratories (Sulzfeld, Germany) and C57BL/6Jo—laHsd (B6Jola) mice were purchased from Harlan-Winkelmann (Borchen, Germany). All mice were housed in Makrolon type II long cages (36 × 21 × 12 cm) in the specified pathogen free mouse facility of the Gene Centre in Munich. Water and food (Sniff, Germany) were freely available. Room temperature was 25°C with 40% humidity and a 12-h light/12-h dark cycle (lights on at 7 am). All experiments and experimental procedures were approved by the Committee on Animal Health and Care of the local governmental body of the state of Bavaria (Regierung von Oberbayern) and performed in strict compliance with the EEC recommendations for the care and use of laboratory animals.

General methods

By using an embryo-transfer, four developmental conditions were created (Donor strain > Recipient strain): B6Jola > B6Jola (n: males = 17, females = 16; 6 l), B6Jola > B6N (n: males = 7, females = 9; 4 l), B6N > B6N (n: males = 27, females = 18; 7 l), B6N > B6Jola (n: males = 12, females = 8; 3 l). Pregnant females were monitored for birth. Within 12 h of birth [postnatal day (pnd) 0] litters with more than 10 pups were reduced to 10 animals/l by discarding surplus pups. Thereafter, animals remained undisturbed until behavioral tests started. On pnd 7, pups were screened for isolation-induced ultrasonic calling and maternal retrieval behavior was measured. Behavioral tests were conducted between 8 am and 7 pm in a separate room.

Embryo-transfer

For the production of the embryos, 8-week-old females were mated with males of the same mouse sub-strain. The females were screened for vaginal plugs every morning and evening. Females were killed at day 3 after finding a vaginal plug (3.5 dpc) through cervical dislocation. The uterus was removed and flushed with M2 medium containing 0.4% bovine serum albumin (BSA) and the blastocysts were collected under a stereomicroscope with 20× magnification (Nagy et al. 2003). The embryos were transferred to M2 medium with 0.4% BSA microdrops on a culture dish covered with paraffin oil at 37°C until needed. Between 12 and 20 embryos were transferred into the uterus of a pseudo-pregnant female recipient (2.5 dpc) which was prepared by mating 12-week-old females with vasectomized males. The skin and muscles of the anesthetized recipient were cut and the uterus externalized from the peritoneal cavity. Under a stereomicroscope with 20× magnification, the uterus was punched with a needle near the oviduct. A transfer pipette prepared with M2 medium and the embryos was inserted through the punched whole and the embryos were placed into the uterus. Embryos of one mouse sub-strain were transferred to recipients of the same mouse sub-strain and to recipients of the other sub-strain, depending on the experimental group.

Maternal retrieval behavior

To induce maternal retrieval behavior, all pups of a given litter were removed from the nest and placed in the edge most distal from the nest. Similar to most studies on maternal retrieval behavior (Hahn and Lavooy 2005), the test was performed in the home cage (36 × 21 × 12 cm) on pnd 7. The latency to pick up the first pup and the latency to retrieve the first and last pup were measured.

Isolation

To induce ultrasonic vocalization, pups were isolated for 5 min from the mother and nest on pnd 7. Pups were individually removed from the nest in random order and gently placed into a dish (8 × 8 × 3 cm) on a warming plate at 27°C. The dish was placed in a sound attenuating chamber (55 × 65 × 50 cm), which was prepared with sound absorbent foam inside and covered outside with aluminum foil. Ultrasonic vocalization was recorded using an UltraSoundGate Condenser Microphone (CM 16; Avisoft Bioacoustics, Germany) suspended 7 cm from the testing surface. The microphone was sensitive to frequencies of 15–180 kHz with a flat frequency response (±6 dB) between 25 and 140 kHz. It was connected via an Avisoft UltraSoundGate 116 USB Audio device (Avisoft Bioacoustics) to a personal computer, and were recorded with a sampling rate of 300,000 Hz in 16 bit format. Thereafter, ultrasonic vocalization was analyzed using Avisoft SASLab Pro (for details see: “Analysis of ultrasonic vocalizations”).

After recording, the pups were marked for identification by foot tattoo with black drawing ink (Pelikan, Germany). The dish was cleaned with Bacillol AF after each session. After replacing the pup into the cage 5 min were allowed to elapse until going on with the next littermate.
Experiment II—maternal search behavior

Animals and housing

Timed pregnant B6N and B6JOla dams (n = 10, each) were purchased from Charles River Laboratories and Harlan-Winkelmann, respectively, in the last gestation week. A total of 6 B6JOla and 5 B6N dams successfully delivered the offspring and brought them up to pnd 7 to 10. All mice were housed in Makrolon type II long cages (36 × 21 × 12 cm). Water and food (Ssniff, Germany) were freely available.

Playback task

Testing of maternal responses to playback of ultrasonic calling was performed on a white platform (100 × 86 cm), elevated 45 cm above the floor, under white light (about 350 lux) when pups were 7–10-days-old. In the center of the platform, a petri dish (diameter: 15 cm, rim: 2 cm) was situated, which was filled with soiled bedding from the home cage, i.e. from the nest. For playback, two ultrasonic speakers (ScanSpeak, Avisoft Bioacoustics), connected to an external sound card (Fire Wire Audio Capture FA-101, Edirol, UK) were used (for details see: Wöh and Schwarting 2007, 2008). They were placed opposite to each other and 20 cm away from the elevated platform at a height of 45 cm above the floor. One speaker was pseudo-randomly chosen for playback, i.e. counter-balanced for strain of the mother and test order. Playback of acoustic stimuli was verified by using an UltraSoundGate Condenser Microphone (CM 16; Avisoft Bioacoustics), which was placed 20 cm away from the platform and next to one speaker. The microphone was connected via an Avisoft UltraSoundGate 116 USB Audio device (Avisoft Bioacoustics) to a personal computer, where acoustic data were displayed in real time by Avisoft RECORDER (version 2.7; Avisoft Bioacoustics).

The following three acoustic stimuli were presented: (1) white noise, (2) B6JOla ultrasonic vocalizations, and (3) B6N ultrasonic vocalizations. To identify recordings of ultrasonic vocalizations of within-transferred pups, which optimally resembled the mean call characteristics of its strain, hierarchical cluster analyses were applied using call number and duration, total calling time, peak frequency and amplitude, and frequency modulation. Values were standardized to z scores before computing proximities (squared Euclidian distance). By means of the cluster method nearest neighbor, the pup was selected, of which the calling behavior displayed in the first min in isolation resembles best the mean call characteristics of its strain. White noise was generated with Avisoft SASLab Pro (version 4.38; Avisoft Bioacoustics). All stimuli were presented with a sampling rate of 192 kHz in 16 bit format with 65 dB.

A given animal was placed into the petri dish with bedding from the nest. Behavioral recording started as soon as the mouse had left the Petri dish for the first time (all four paws on the platform). After an initial habituation phase (3 min), the mouse was exposed to 3 presentations of acoustic stimuli for 1 min, each followed by an inter-stimulus-interval of 3 min. The first stimulus presented was white noise. The second and third stimuli were ultrasonic vocalizations of the own strain, i.e. B6JOla mothers were exposed to B6JOla calls and B6N mothers to B6N calls. Behavior was monitored by a black/white CCD video camera (Conrad Electronic, Germany) from about 102 cm above the platform, which fed into a video recorder (NV-HS950, Panasonic, Germany). For behavioral analysis, the platform was virtually divided into 3 equally-sized areas (33 × 86 cm), namely (1) proximal to the active loudspeaker, (2) distal from the active loudspeaker and (3) central (including the Petri dish). A trained observer measured the time spent in each of these areas. In addition, the time spent in the petri dish was measured separately. An entry was counted when all four paws crossed the virtual grid line.

Behavioral testing was performed between 9 and 17 h. Prior to each test, behavioral equipment was cleaned using a 1% acetic acid solution followed by drying.

Pup discrimination task

Testing of maternal responses to natural ultrasonic calling was performed on the same platform as used for the playback experiment (for details see: “Playback task”) when pups were 7–10-days-old. In the forward middle of the platform, a petri dish (diameter: 15 cm, rim: 2 cm) was situated, which was filled with soiled bedding from the home cage, i.e. from the nest. Additionally, two smaller petri dishes (diameter: 9 cm, rim: 2 cm) without bedding were situated in the two most distal corners (10 cm away from the edge). In each of them, a stimulus pup from a foreign litter was placed. To avoid differences in age, stimulus pups were littermates. Testing was performed under 22°C.

A given mother was placed into the petri dish with home cage bedding. The experiment was started when the mother left the Petri dish for the first time (all four paws on the platform), and stopped when the first pup was removed from the small petri dish. Ultrasonic vocalization was recorded using two UltraSoundGate Condenser Microphones (CM 16; Avisoft Bioacoustics) suspended 12.5 cm from the testing surface. They were connected via an Avisoft UltraSoundGate 416 USB Audio device (Avisoft Bioacoustics) to a personal computer, and were recorded.
with a sampling rate of 214,285 Hz in 16 bit format. Thereafter, ultrasonic vocalization was analyzed using Avisoft SASLab Pro (for details see: “Analysis of ultrasonic vocalizations”). Behavior was monitored by equipment described above. The time the mother spent in contact with the small Petri dish, in which the pup was placed, was measured.

Behavioral testing was performed between 13 and 17 h. Prior to each test, behavioral equipment was cleaned using a 1% acetic acid solution followed by drying.

Analysis of ultrasonic vocalization

For acoustical analysis, recordings were transferred to SASLab Pro (version 4.38; Avisoft Bioacoustics) and a fast Fourier transformation was conducted (512 FFT-length, 100% frame, Hamming window and 75% time window overlap). Spectrograms were produced at 586 Hz of frequency resolution and 0.427 ms of time resolution.

Call detection was provided by an automatic threshold-based algorithm (threshold: −40 dB) and a hold-time mechanism (hold time: 10 ms). Since no ultrasonic vocalizations were detected below 30 kHz, a lower-cut-off-frequency of 30 kHz was used to reduce background noise outside the relevant frequency band to 0 dB. The accuracy of call detection was verified by an experienced user. When necessary, missed calls were marked by hand to be included in the automatic parameter analysis. Based on previous studies on isolation-induced calling (Wöhr and Schwarting 2008), various parameters, including peak frequency and peak amplitude, which were derived from the average spectrum of the entire element, were determined automatically. Peak amplitude was defined as the point with the highest energy within the spectrum, and peak frequency was defined as the frequency at the location of the peak amplitude. The extent of frequency modulation, i.e. the difference between the lowest and the highest peak frequency within each call was also measured automatically. Temporal parameters determined included call duration, total calling time, and the duration of intervals between subsequent calls. Finally, the total number of calls emitted was measured.

Statistical analysis

For call duration, peak frequency, peak amplitude, and the extent of frequency modulation, the mean of each call parameter served as the statistical unit in each subject. To test whether B6JOla and B6N pups differ in their calling behavior following within-strain transfer, two-way analyses of variance (ANOVAs) with the factors sub-strain of the pup and gender were used. The contribution of genetic predispositions, gender, and early environmental factors to ultrasonic calling behavior in these two sub-strains was determined by using a three-way ANOVAs with the factors sub-strain of the pup, i.e. genotype, gender, and sub-strain of the mother. Since it is known that individual pups within one litter receive variable levels of maternal care and emit highly variable numbers of isolation-induced vocalizations (Wöhr and Schwarting 2008), statistical analyses were based on individual pups. However, to control for potential litter effects, ANOVAs were repeated by using the litter average for males and females (Abbey and Howard 1973; Zorrilla 1997). Maternal retrieval behavior was compared between B6JOla and B6N pups in consideration of the genotype of the mother by using two-way ANOVAs with the factors sub-strain of the mother and sub-strain of the pup. Maternal search behavior was compared by ANOVAs for repeated measurements with the factors sub-strain and test-phase (playback task), or sub-strain and maternal preference (pup discrimination task). ANOVAs for repeated measurements were used for the pup discrimination task, since pups were from the same litter and therefore not independent from each other. Finally, a principal component analysis with varimax rotation using the Kaiser criterion (eigen-values > 1) was calculated to examine patterns of relationships among call parameters. The exact P-values of 2-tailed testing were calculated, except when explicitly noted. A P-value ≤ 0.05 was considered statistically significant. As measures of effect size partial $\eta^2$ and Cohen’s $f$ were calculated. The $\eta^2$ statistic describes the proportion of total variability attributable to a factor. In case of Cohen’s $f$, values of $\geq 0.100$, $\geq 0.250$, and $\geq 0.400$ were considered as small, medium, and large, respectively (Cohen 1988). Data are shown as mean ± SEM.

Results

Experiment I—embryo-transfer: ultrasonic vocalization

Within-strain embryo-transfer

To test whether calling behavior differs between B6JOla and B6N pups, their calling behavior was compared and considerably differences between sub-strains were observed (see Fig. 1). Firstly, B6JOla pups emitted more calls than B6N pups (main effect pup: $F_{1,74} = 5.664$, $P = 0.020$, $\eta^2 = 0.071$, $f = 0.274$, power = 0.651), irrespective of gender (main effect gender: $F_{1,74} = 0.886$, $P = 0.350$, $\eta^2 = 0.012$, $f = 0.105$, power = 0.153). On the other hand, total calling time (main effect pup: $F_{1,74} = 2.157$, $P = 0.146$, $\eta^2 = 0.028$, $f = 0.165$, power = 0.305) and call duration (main effect pup: $F_{1,74} = 1.590$, $P = 0.211$, $\eta^2 = 0.021$, $f = 0.246$, power = 0.238) did not differ between the sub-strains, whereas females generally
spent more time calling than males (main effect gender: $F_{1.24} = 4.155, P = 0.045, \eta^2 = 0.053, f = 0.232$, power = 0.521). This effect was based on a difference in call duration, since female calls were longer than male calls (main effect gender: $F_{1.74} = 7.012, P = 0.010, \eta^2 = 0.087, f = 0.408$, power = 0.743).

B6JOla and B6N pups also differed with respect to peak frequency and peak amplitude, since calls emitted by B6JOla pups were higher in frequency and amplitude (main effect pup: $F_{1.74} = 7.289, P = 0.009, \eta^2 = 0.090, f = 0.314$, power = 0.760 and $F_{1.74} = 18.899, P < 0.001, \eta^2 = 0.203, f = 0.491$, power = 0.990, respectively), whereas gender had no effect (main effect gender: $F_{1.74} = 0.004, P = 0.947, \eta^2 < 0.001, f = 0.007$, power = 0.050 and $F_{1.74} = 0.776, P = 0.381, \eta^2 = 0.010, f = 0.002$, power = 0.140, respectively). Finally, frequency modulation was higher in females (main effect gender: $F_{1.74} = 9.429, P = 0.003, \eta^2 = 0.113, f = 0.350$, power = 0.858), but did not differ between sub-strains (main effect pup: $F_{1.74} = 1.418, P = 0.238, \eta^2 = 0.019, f = 0.128$, power = 0.217). No evidence for an interaction pup × gender was obtained (all $P$-values > 0.100).

When data were reanalyzed by using the litter average for males and females, a similar picture was obtained. Thus, B6JOla pups tended to emit more calls than B6N pups (main effect pup: $F_{1.20} = 3.419, P = 0.079, \eta^2 = 0.146, f = 0.605$, power = 0.421). Calls emitted by B6JOla pups tended to be higher in frequency ($F_{1.20} = 3.258, P = 0.086, \eta^2 = 0.140, f = 0.607$, power = 0.405) and showed higher peak amplitudes ($F_{1.20} = 16.649, P = 0.001, \eta^2 = 0.454, f = 0.908$, power = 0.972). Call duration, total calling time, and frequency modulation did not differ between strains (all $P$-values > 0.100). Furthermore, calling behavior did not differ between males and females (all $P$-values > 0.100), expect for a trend for a more pronounced frequency modulation in females ($F_{1.20} = 4.085, P = 0.057, \eta^2 = 0.170, f = 0.435$, power = 0.486). No evidence for an interaction pup × gender was obtained (all $P$-values > 0.100).

Between-strain embryo-transfer

To test whether the observed differences are due to genetic or early environmental factors cross-fostered pups were added to the analysis. Results indicate that certain call parameters were primarily dependent on early environmental factors, whereas others were primarily dependent on genotype or gender of the pup (see Table 1 and Fig. 2). Thus, the finding that B6JOla emitted more calls than B6N was based on early environmental factors (main effect mother: $F_{1.106} = 4.457, P = 0.037, \eta^2 = 0.040, f = 0.192$, power = 0.553), whereas pup genotype did not directly contribute to the observed difference (main effect pup: $F_{1.106} = 0.583, P = 0.447, \eta^2 = 0.005, f = 0.005$, power = 0.118). Additionally, an interaction between mother and pup genotypes was observed (interaction mother × pup: $F_{1.106} = 11.733, P = 0.001, \eta^2 = 0.100, f = 0.320$, power = 0.924), since pups born and raised by females of the same sub-strain emitted higher rates of ultrasonic calls in comparison to pups born and raised by females of the other sub-strain. This was especially true for B6JOla pups. Remarkably, these effects were evident throughout testing.
Table 1  Ultrasonic vocalization in B6JOla (J) and B6N (N) pups born and raised by either B6JOla (J) or B6N (N) mothers (donor > recipient)

|                        | J > J         | J > N         | N > N         | N > J         |
|------------------------|---------------|---------------|---------------|---------------|
| Calls (n)              | M 325.88 ± 43.69 | 202.71 ± 49.22 | 252.48 ± 31.42 | 216.50 ± 39.67 |
|                        | F 396.38 ± 61.84 | 122.34 ± 45.28 | 263.56 ± 39.07 | 205.25 ± 55.57 |
| Total calling time (s) | M 7.86 ± 1.33  | 5.34 ± 1.21   | 7.59 ± 1.17   | 7.64 ± 1.78   |
|                        | F 13.51 ± 2.79 | 3.79 ± 1.52   | 8.82 ± 1.44   | 7.09 ± 2.59   |
| Call duration (ms)     | M 22.43 ± 1.54 | 25.92 ± 4.73  | 27.35 ± 1.85  | 30.81 ± 2.72  |
|                        | F 30.26 ± 2.44 | 27.67 ± 2.35  | 30.63 ± 2.31  | 28.34 ± 3.37  |
| Peak frequency (kHz)   | M 78.75 ± 1.29 | 78.81 ± 2.27  | 76.12 ± 0.93  | 76.17 ± 1.01  |
|                        | F 79.20 ± 0.99 | 79.01 ± 2.35  | 75.51 ± 1.41  | 76.34 ± 1.38  |
| Call amplitude (dB)     | M 68.46 ± 0.77 | 67.32 ± 1.42  | 64.45 ± 0.60  | 67.39 ± 0.83  |
|                        | F 68.28 ± 0.87 | 64.26 ± 1.59  | 65.91 ± 0.69  | 66.17 ± 1.04  |
| Frequency modulation (kHz) | M 20.91 ± 0.90 | 21.34 ± 2.57  | 25.29 ± 1.19  | 28.50 ± 1.81  |
|                        | F 28.20 ± 1.80 | 25.06 ± 1.86  | 27.51 ± 2.07  | 29.89 ± 2.71  |

Descriptive statistics for B6JOla pups born and raised either by B6JOla mothers (J > J) or by B6N mothers (J > N), and B6N pups born and raised either by B6N mothers (N > N) or by B6JOla mothers (N > J) regarding call number, total calling time (s), call duration (ms), peak frequency (kHz), peak amplitude (dB), and frequency modulation (kHz). M = males; F = females. Values reflect means ± SEM (see Fig. 3). Gender did not directly or indirectly influence call number (main effect gender: $F_{1,106} = 0.005$, $P = 0.944$, $\eta^2 < 0.001$, $f = 0.006$, power = 0.051; all $P$-values for interactions $> 0.100$).

A similar picture was obtained for total calling time. Thus, the genotype of the mother affected the time spent calling (main effect mother: $F_{1,106} = 3.519$, $P = 0.063$, $\eta^2 = 0.032$, $f = 0.173$, power = 0.460), whereas the genotype of the pup did not directly affect total calling time (main effect pup: $F_{1,106} = 0.013$, $P = 0.910$, $\eta^2 < 0.001$, $f = 0.010$, power = 0.051). As for call number, however, total calling time was primarily dependent on an interaction between mother and pup sub-strain, since pups born and raised by mothers of the same sub-strain spent a longer time calling than pups born and raised by the other sub-strain (interaction mother x pup: $F_{1,106} = 6.121$, $P = 0.015$, $\eta^2 = 0.055$, $f = 0.230$, power = 0.689). Gender had no effect on total calling time (main effect gender: $F_{1,106} = 0.725$, $P = 0.396$, $\eta^2 = 0.017$, $f = 0.078$, power = 0.273; all $P$-values for interactions $> 0.100$). Call duration was independent from genetic background, early environmental factors, and gender (main effect mother: $F_{1,106} = 0.001$, $P = 0.971$, $\eta^2 < 0.001$, $f = 0.003$, power = 0.050; main effect pup: $F_{1,106} = 2.043$, $P =
Fig. 3 Time courses of ultrasonic vocalization per minute in B6JOla pups (left) born and raised either by B6JOla mothers (white circles) or by B6N mothers (black circles), and in B6N pups (right) born and raised either by B6JOla mothers (white circles) or by B6N mothers (black circles). Given are means ± SEM.

Thus, call number was dependent on an interaction between mother and pup genotype (main effect mother: $F_{1,28} = 2.232, P = 0.146, \eta^2 = 0.074, f = 0.242, \text{power} = 0.303$; main effect pup: $F_{1,28} = 0.792, P = 0.381, \eta^2 = 0.028, f = 0.142, \text{power} = 0.138$; interaction mother × pup: $F_{1,28} = 5.486, P = 0.027, \eta^2 = 0.164, f = 0.398, \text{power} = 0.618$; apart from this, no evidence for an interaction mother × pup was obtained: all $P$-values > 0.100). Call duration and total calling time were not affected by mother or pup genotype (all $P$-values > 0.100). Peak frequency was dependent on pup genotype (main effect mother: $F_{1,28} = 0.009, P = 0.925, \eta^2 < 0.001, f = 0.016, \text{power} = 0.051$; main effect pup: $F_{1,28} = 5.489, P = 0.026, \eta^2 = 0.164, f = 0.415, \text{power} = 0.619$), whereas peak amplitude was primarily dependent on early environmental factors (main effect mother: $F_{1,28} = 9.116, P = 0.005, \eta^2 = 0.246, f = 0.486, \text{power} = 0.830$; main effect pup: $F_{1,28} = 3.161, P = 0.086, \eta^2 = 0.101, f = 0.269, \text{power} = 0.404$). Finally, frequency modulation tended to depend on the genotype of the pup (main effect mother: $F_{1,28} = 1.919, P = 0.177, \eta^2 = 0.064, f = 0.228, \text{power} = 0.268$; main effect pup: $F_{1,28} = 4.018, P = 0.055, \eta^2 = 0.125, f = 0.319, \text{power} = 0.490$). Calling behavior did not differ between males and females, expect for a trend for a more pronounced frequency modulation in females ($F_{1,28} = 3.001, P = 0.094, \eta^2 = 0.097, f = 0.289, \text{power} = 0.387$; all other $P$-values > 0.100). Furthermore, no evidence for interactions with gender, i.e. mother × gender, pup × gender, or mother × pup × gender, was obtained (all $P$-values > 0.100).

Despite differences in call rate and call features between both sub-strains, individual relationships between call parameters were similar as indicated by factor analyses (see Table 2). Thus, factor analyses revealed two dimensions in all four groups. Remarkably, in all four groups the first dimension was characterized by high positive factor loadings of call duration and frequency modulation, whereas the second dimension was characterized by a high positive factor loading of peak amplitude, but a high negative factor loading of peak frequency.
Furthermore, when analyzing calling behavior of animals with different background, no evidence for qualitative differences in their calling repertoire was obtained (see Figs. 4, 5). Thus, although the scatter plots clearly indicate that the infant mouse calling repertoire contains different call types, the scatter plots show a profound overlap between groups. This means that both, prenatal cross-fostered and non-cross-fostered animals, show call types with an upper peak frequency ranging around 85–105 kHz and a lower peak frequency ranging around 60–70 kHz as a call type which was strongly frequency-modulated, i.e. showing a frequency modulation of about 40–60 kHz, and another one which was less frequency-modulated, i.e. showing a frequency modulation of about 0–20 kHz.

Experiment I—embryo-transfer: maternal retrieval behavior

Retrieval task

No evidence for a difference in retrieval behavior between B6N and B6JOla mothers was obtained, i.e. no differences in the latency to pick up or retrieve the first pup were observed ($F_{1,15} = 1.615, P = 0.223, \eta^2 = 0.097, f =$)
0.276, power = 0.222 and $F_{1,15} = 0.200, P = 0.661, \eta^2 = 0.013, f = 0.204, \text{power} = 0.070$, respectively. However, pup genotype affected the latency to pick up the first pup, since B6JOla were picked up sooner than B6N ($F_{1,15} = 5.127, P = 0.039, \eta^2 = 0.255, f = 0.540, \text{power} = 0.563$). Despite this, pup genotype did not affect the actual latency to retrieve the first pup ($F_{1,15} = 0.018, P = 0.894, \eta^2 = 0.001, f = 0.020, \text{power} = 0.052$), and no significant interactions were obtained for the latency to pick up or retrieve the first pup (interaction mother × pup: $F_{1,15} = 2.464, P = 0.137, \eta^2 = 0.141, f = 0.350, \text{power} = 0.312$ and $F_{1,15} = 2.300, P = 0.150, \eta^2 = 0.133, f = 0.930, \text{power} = 0.295$, respectively). However, it is striking that the picture of the retrieval behavior appears to be inverse to that of call number (see Fig. 6).

Experiment II—maternal search behavior

Playback task

To test whether the emission of ultrasonic vocalizations can affect behavior of B6JOla and B6N mothers, a playback task was performed. It was expected that playback of ultrasonic vocalizations would induce maternal search behavior. During the first playback of ultrasonic vocalizations, mothers spent more time in the petri dish than before and after
playback, irrespective of strain (main effect test phase: \(F_{2,18} = 4.237, P = 0.031, \eta^2 = 0.320, f = 0.943, \text{power} = 0.665\); main effect strain: \(F_{1,9} = 0.004, P = 0.953, \eta^2 < 0.001, f = 0.020, \text{power} = 0.050\); interaction test phase \times \text{strain}: \(F_{2,18} = 0.649, P = 0.535, \eta^2 = 0.067, f = 0.279, \text{power} = 0.142\); see Fig. 7), whereas behavior was unchanged during playback of white noise and the second playback of ultrasonic vocalizations (all \(P\)-values > 0.100). A preference for the area proximal to the speaker was not observed during any test phase (all \(P\)-values > 0.100).

**Pup discrimination task**

To test whether those call parameters, which were affected by early environmental factors, are functionally relevant for the induction of maternal search behavior, a pup discrimination task was performed. It was expected that pups emitting high number of calls with high peak amplitudes will attract the mother more than pups emitting few calls with low peak amplitudes. When calling behavior was compared between the two pups of a given exposure that either attracted the mothers little (B6JOla: 1.04 ± 0.49 s/min) or much (B6JOla: 9.85 ± 4.74 s/min; B6N: 7.12 ± 0.84 s/min), a trend for an interaction of maternal preference \times \text{strain} was observed (main effect maternal preference: \(F_{1,9} = 1.116, P = 0.318, \eta^2 = 0.110, f = 0.378, \text{power} = 0.157\); main effect strain: \(F_{1,9} = 3.287, P = 0.013, \eta^2 = 0.268, f = 0.758, \text{power} = 0.367\); interaction maternal preference \times \text{strain}: \(F_{1,9} = 3.589, P = 0.081, \eta^2 = 0.300, f = 0.868, \text{power} = 0.419\), since B6JOla mothers spent more time in contact with pups, which emitted calls with high peak amplitudes (B6JOla: 63.88 ± 1.97 dB) in comparison to pups, which emitted calls with low peak amplitudes (B6JOla: 59.00 ± 1.51 dB; \(F_{1,5} = 4.453, P = 0.049, \eta^2 = 0.471, f > 0.999, \text{power} = 0.401\); one-tailed testing). In contrast, the preferences shown by B6N mothers were not related to peak amplitude (high contact time: 57.25 ± 0.91 dB and low contact time: 58.71 ± 2.29 dB; \(F_{1,5} = 0.449, P = 0.270, \eta^2 = 0.101, f = 0.340, \text{power} = 0.082\); one-tailed testing). Call number did not differ between pups attracting the mother for a short (B6JOla: 71.77 ± 31.70 calls/min, B6N: 81.92 ± 18.05 calls/min) or long time in either strain (B6JOla: 55.44 ± 11.50 calls/min, B6N: 87.88 ± 8.73 calls/min; main effect maternal preference: \(F_{1,9} = 0.063, P = 0.808, \eta^2 = 0.007, f = 0.084, \text{power} = 0.056\); main effect strain: \(F_{1,9} = 1.057, P = 0.331, \eta^2 = 0.015, f = 0.364, \text{power} = 0.152\); interaction maternal preference \times \text{strain}: \(F_{1,9} = 0.289, P = 0.604, \eta^2 = 0.031, f = 0.182, \text{power} = 0.077\).

**Discussion**

Within-strain embryo-transfer: comparison between B6JOla and B6N

The present results show for the first time that two sub-strains of C57BL/6 mice, namely B6JOla and B6N, differ in their ultrasonic calling behavior when isolated from dam and litter. This is in accordance with a bulk of observations of strain differences in the emission of ultrasonic vocalizations in mice (Bell et al. 1972; Cohen-Salmon et al. 1985; Hahn et al. 1987, 1997, 1998; Hennessy et al. 1980; Robinson and D’Udine 1982; Hennessy et al. 1996; Sales and Smith 1978; Thornton et al. 2005), and adds to other differences between B6JOla and B6N.

Firstly, B6JOla and B6N mice differ genetically, since B6JOla mice carry a spontaneous deletion on chromosome 6 (Chen et al. 2002; Siegmund et al. 2005; Specht and Schoepfer 2001, 2004). This deficit leads to a loss of alpha-synuclein, a presynaptically localized protein that has been implicated in the etiology of Parkinson’s disease (Maries et al. 2003; Polymeropoulos et al. 1997). Alpha-synuclein may have affected call production in infancy, possibly through its regulative function on dopaminergic...
transmission (Abeliovich et al. 2000; Oksman et al. 2006), since dopaminergic transmission itself influences ultrasonic calling in isolation (Cuomo et al. 1987; Dastur et al. 1999; Kehoe and Boylan 1992; Muller et al. 2005, 2008). However, the present gene-dependent findings cannot necessarily be attributed to alpha-synuclein deficits, since other genetic factors may have been critical or may have contributed. Indeed, detailed mapping and sequencing of the breakpoint recently revealed the absence of Mmnr1 gene in addition (Specht and Schoepfer 2004). A role of Mmnr1 for ultrasonic calling is currently unknown.

Secondly, B6JOla and B6N mice differ in their adult anxiety-related behavior, namely the course of extinction of conditioned fear. Thus, B6JOla mice display lower levels of freezing to the context where they have been shocked before and shorter maximal fear responses (Radulovic et al. 1998; Siegmund et al. 2005; Siegmund and Wotjak 2007; Stiedl et al. 1999). Such behavioral differences are usually explained by genetic differences between strains. However, Siegmund et al. (2005) have shown that the difference in the extinction of fear memory in B6JOla and B6N is unlikely to be based on the different expression of alpha-synuclein. Therefore, it can be assumed that environmental factors contribute to such differences as well. Indeed, such factors have proven to hold strong influence on the development of emotionality (Calatayud and Belzung 2001; Calatauyed et al. 2004; Francis et al. 2003; for review see: Gordon and Hen 2004) and out of these, maternal care is a crucial one (Caldji et al. 1998; Francis et al. 1999; Menard et al. 2004; Menard and Hakovoot 2007; Wöhr and Schwarting 2008; Zhang et al. 2005).

Finally, it can be noted that the virtual absence of gender differences in infant mice calling is in accordance with the vast majority of the literature (Hahn et al. 1997, Hahn et al. 2000; Hahn and Schanz 2002; Roubertoux et al. 1996; Thornton et al. 2005) revealed an influence of the genetic background on ultrasonic call emission; a finding, which is supported by studies on knockout mice. For instance, it was shown that several genes are involved in the production of ultrasonic vocalizations, especially Foxp2 (Shu et al. 2005). Disruption of this gene led to a loss of ultrasonic vocalizations. Interestingly, Foxp2 has been considered as a potential susceptibility locus for language disorders in humans (Lai et al. 2001).

However, genetic analyses also indicated maternal effects on call rate, duration, frequency, and frequency modulation (Roubertoux et al. 1996; Thornton et al. 2005). Actually, high levels of variability in call production were found even within lines selectively bred for high or low calling rates in isolation. For instance, Brunelli et al. (1997) observed that call rates ranged between 0 and 700/min in the line selected for high rates of calling. Thus, it seems likely that early environmental factors hold strong influence on isolation-induced calling, and the results of the present embryo-transfer support this assumption.

The finding that early environmental factors can influence calling behavior is in accordance with studies on the effects of prenatal malnutrition (Tonkiss et al. 2003), prenatal stress (Morgan et al. 1999; Williams et al. 1998), perinatal asphyxia (Calmandrei et al. 2004) or pre- and postnatal exposure of various substances, like alcohol (Barron and Gilbertson 2005; Marino et al. 2002; Tatolli et al. 2001), cocaine (Hahn et al. 2000), lead (De Marco et al. 2005), aluminum (Alleva et al. 1998), or carbon monoxide (Di Giovanni et al. 1993) on ultrasonic calling in infant rodents. However, in the natural context, variations in maternal care might be of major importance. This is indicated by studies on
the effects of handling (Bell et al. 1971), maternal separation (D’Amato and Cabib 1987; Zimmerberg et al. 2003a, b), and litter size (Hofer et al. 1993), and adoptions (Darnaudery et al. 2004) on ultrasonic calling in infant rodents. Darnaudery et al. (2004) found that pups raised by fostering dams showed less isolation-induced calling when compared to pups raised by their actual mothers, a finding which is similar to the present observation of lowered calling behavior in prenatal cross-fostered pups. Remarkably, they also observed that this difference in call production was paralleled by a difference in maternal care, namely that fostering dams showed more maternal care than actual mothers, indicating that maternal care can reduce isolation-induced calling. Other evidence that maternal care can tune calling behavior in offspring was provided by D’Amato et al. (2005), who found that mouse pups raised by mothers with higher maternal responsiveness emitted lower call rates than pups of mothers with a comparatively low maternal responsiveness. Furthermore, Wöhr and Schwarting (2008) have shown that rat pups raised by mothers that demonstrated pronounced approach behavior in response to playback of isolation-induced calls called less in isolation than pups raised by mothers with weak or no approach behavior. Further, it was found that maternal licking is strongly linked to isolation-induced infant calling. Thus, rat pups that experienced a comparatively high rate of maternal licking emitted less calls in isolation than pups that were licked less often. A detailed analysis of ultrasonic calls revealed that apart from call number, several call features were affected by maternal care; and it is striking to see in the present mouse study that the call parameters affected by early environmental factors are quite similar to those, which were most predominantly influenced by maternal care in rats, namely call number and peak amplitude, but not peak frequency (Wöhr and Schwarting 2008). The modulation of peak amplitude is particularly interesting, because it was demonstrated that call amplitude can be reduced by anxiolytic drugs (Insel et al. 1986), and in adult rats it was shown that the averseness of the situation is encoded not only in call number but also in peak amplitude (Wöhr et al. 2005). Furthermore, peak amplitude was shown to be a valid predictor of the susceptibility to develop PTSD-like symptoms in response to a traumatic event in adulthood in the B6N sub-strain (Siegmund et al., unpublished observation).

However, the finding that early environmental factors, such as maternal care, are related to isolation-induced calling seems to contradict results of cross-fostering studies in rats (Brunelli et al. 2001) and mice (Hennessy et al. 1980), where no maternal effects on call rates were observed. With respect to the rat study by Brunelli et al. (2001) it has to be mentioned that they bred their animals for high or low calling rates by using a within-litter selection procedure which minimizes maternal effects (Hofer et al. 2001). Despite this selection procedure, however, Rojowsky et al. (2000) found that dams from the line with high calling rates showed reduced maternal responsiveness compared to dams from lines with random or low calling rates. With respect to the mouse study by Hennessy et al. (1980) it has to be noted that the authors reported that only one of the two strains used emitted ultrasonic calls, namely A/J, but not C57BL/6 J. Bearing in mind the high call rates of B6N and B6JOla mice found in the present study, it seems likely that the absence of calls in the study of Hennessy et al. (1980) is based on the recording technology used there. They set their frequency tuner at 68 kHz with a bandwidth of 5 kHz, meaning that they were able to detect only a small proportion of calls according to the present findings. The present findings highlight the importance of using a sophisticated recording technology, which allows covering the frequency range from 50 up to 110 kHz. However, it might be also possible that maternal effects on ultrasonic calling behavior are only clearly evident when rectified pre- and postnatal experiences occur together. This would be in line with an embryo-transfer study in mice where it was shown that enhancing anxiety in otherwise low-anxious C57BL/6 J pups requires both, pre- and postnatal experience with a more anxious dam (Francis et al. 2003). Whether maternal factors alone are sufficient for these differences to occur is currently evaluated by using reciprocal F1 hybrids. Finally, it has to be noted that the strength of early environmental effects, or the weakness of genetic effects, respectively, observed in the present experiment is probably due to the fact the genetically similar material was used, meaning that one would expect more pronounced genetic effects when more diverse genetic material is used.

The present finding that early environmental factors can affect isolation-induced ultrasonic calling is in line with a bulk of evidence showing that maternal factors strongly influence anxiety-related behavior in the offspring. Apart from the embryo-transfer study by Francis et al. (2003), this was indicated in postnatal cross-fostering studies (Priebe et al. 2005; Zaharia et al. 1996) and reciprocal breeding of inbred mouse strains (Calatayud and Belzung 2001; Calatayud et al. 2004). Using backcrosses of hybrids from BALB/c and C57BL/6, i.e. using genetically identical pups which were exposed to different mothering styles, Calatayud et al. (2004) were able to verify their previous finding that maternal care can affect emotional reactivity as measured in the elevated plus maze and a free exploration paradigm. From rat studies, it is known that variations in maternal licking particularly affect the development of stable individual differences in emotionality. Thus, rats licked more often by mothers, showed decreased startle responses (Zhang et al. 2005), increased open field exploration (Caldji et al. 1998; Francis et al. 1999), shorter
latencies to eat food provided in a novel environment (Caldji et al. 1998), fewer defensive responses in a resident-intruder test, and less shock-induced freezing (Menard et al. 2004; Menard and Hakvoort 2007) in adulthood than rats that were licked less often. Interestingly, these behavioral differences are accompanied by alternations in physiological stress reactivity (Liu et al. 1997) and various neural changes in brain areas implicated in anxiety regulation (Caldji et al. 1998; Liu et al. 1997; for review see: Gordon and Hen 2004).

In total, the results of the present embryo-transfer study show that apart from call number several other call parameters differ between the two sub-strains, and that these differences are partly due to early environmental factors, and partly based on the genetic background. Early environmental factors lead to changes in call number and call amplitude. Changes in these call parameters might be of great functional relevance, since call rate, peak amplitude, and variability of calls, e.g. frequency modulation, are assumed to be a primary source of arousal induction in the mother (Ehret 2005). Although the present retrieval data do not allow to satisfactorily answer the question whether such differences are functionally relevant, it is conspicuous that ultrasonic calling is positively related to retrieval behavior, since pup genotype affected the latency to pick up the first pup, i.e. B6JOla pups which emitted high levels of calls in isolation were picked up sooner than B6N pups which emitted fewer calls, whereas the mothers of both sub-strains did not differ significantly in their retrieval performance. Furthermore, the picture of retrieval behavior, i.e. the latency to retrieve pups, is inverse to the picture of call number, also indicating that pup ultrasonic calling plays a role in the induction of maternal behavior. In support of this notion, it was shown that B6JOla and B6N mothers are able to detect ultrasonic vocalizations, i.e. are not deaf to ultrasound, by means of a playback task, where it was demonstrated that isolation-induced infant calling can induce maternal search behavior in both strains. Mothers spent more time in the area with soiled bedding from the nest during playback of calls than before or after playback. The impact of pup odor for the induction of maternal search behavior was demonstrated by Smotherman et al. (1974), who showed that ultrasonic signals were effective cues only when olfactory information was present. Overall, the present finding of playback-induced maternal search behavior is in accordance with several studies in mice and rats (Allin and Banks 1972; Ehret 1992; Ehret and Haack 1982; Sewell 1970; Smith 1976; Smotherman et al. 1974; Wöhr and Schwarting 2008; for review see: Ehret 2005).

In addition to call number, call amplitude seems also to be important to attract the mother. By means of a pup discrimination task, it was shown that B6JOla mothers spent more time near pups emitting calls with high amplitudes. B6N mothers, however, showed no preference related to peak amplitude. This is particularly remarkable, since pups of either strain show lower call amplitudes when born and reared by B6N (when it is functionally less relevant) than when born and reared by B6JOla (when it is of higher functional relevance). The fact that call number was not associated with maternal preference indicates that call amplitude is of particular importance in competing situations. In principle, however, factors other than call amplitude may have caused maternal preference, since pups emitting calls with high amplitudes might have differed not only herein, but also in other features, like odor. A definite answer on the functional relevance of differences in call features can best be obtained by conducting a playback experiment, which provides opportunity to test the communicative impact of specific call parameters without confounding variables, like odor. A playback experiment would also allow testing whether the temporal sequencing and call types are of functional relevance, e.g. whether the different call types observed here convey different information. Playback studies have already shown that lactating mice can distinguish between different call types, and that they prefer certain call types over other if given the choice (Ehret 1992; Ehret and Haack 1982; Smith 1976).

Conclusion

The results of the present embryo-transfer study show that early environmental factors can tune calling behavior in mouse pups. This adds to several other examples, where it was shown that particularly maternal care holds strong influence on anxiety-related behavior in infancy and adulthood.

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