Pediatric RESEARCH

BASIC SCIENCE ARTICLE

Lactobacillus reuteri effects on maternal separation stress in newborn mice

Evelyn S. Park[1], Jasmin Freeborn[1], Venugopal Reddy Venna[2], Stefan Roos[3,4], J. Marc Rhoads[1] and Yuying Liu[1]

BACKGROUND: Probiotic *Lactobacillus reuteri* DSM 17938 (LR 17938) is beneficial to infants with colic. To understand its mechanism of action, we assessed ultrasonic vocalizations (USV) and brain pain/stress genes in newborn mice exposed to maternal separation stress.

METHODS: Pups were exposed to unpredictable maternal separation (MSU or SEP) or MSU combined with unpredictable maternal stress (MSU + MSUS or S + S), from postnatal days 5 to 14. USV calls and pain/stress/neuroinflammation-related genes in the brain were analyzed.

RESULTS: We defined 10 different neonatal call patterns, none of which increased after MSU. Stress reduced overall USV calls. Orally feeding LR 17938 also did not change USV calls after MSU. However, LR 17938 markedly increased vocalizations in mice allowed to stay with their dams. Even though LR 17938 did not change MSU-related calls, LR 17938 modulated brain genes related to stress and pain. Up-regulated genes following LR 17938 treatment were opioid peptides, kappa-opioid receptor 1 genes, and CD200, important in anti-inflammatory signaling. LR 17938 down-regulated CCR2 transcripts, a chemokine receptor, in the stressed neonatal brain.

CONCLUSIONS: USV calls in newborn mice are interpreted as "physiological calls" instead of "cries." Feeding LR 17938 after MSU did not change USV calls but modulated cerebral genes favoring pain and stress reduction and anti-inflammatory signaling.

Pediatric Research (2021) 90:980–988; https://doi.org/10.1038/s41390-021-01374-0

IMPACT:

- We defined mouse ultrasonic vocalization (USV) call patterns in this study, which will be important in guiding future studies in other mouse strains.
- Newborn mice with maternal separation stress have reduced USVs, compared to newborn mice without stress, indicating USV calls may represent "physiological calling" instead of "crying."
- Oral feeding of probiotic *Lactobacillus reuteri* DSM 17938 raised the number of calls when newborn mice continued to suckle on their dams, but not when mice were under stress.
- The probiotic bacteria had a dampening effect on monocyte activation and on epinephrine and glutamate-related stress gene expression in the mouse brain.

INTRODUCTION

Infantile colic affects up to 20% of infants[1] and is defined by the "rule of 3s": episodes of crying lasting more than 3 h a day for more than 3 days a week for more than 3 weeks, resolving during the third month of life, in an otherwise healthy infant.[2,3] Currently, the exact pathogenesis for infantile colic remains elusive. The gut–brain axis suggests a functional link between the gastrointestinal tract and the central nervous system.[4] Infant crying is multifactorial and could be related to gut dysmotility, hormone alterations, behavioral factors, or an increased level of serotonin (causing discomfort) and reduced levels of melatonin.[5] Psychological factors are also important and may include inadequate parent–infant interaction, maternal depression, and parental anxiety.[6]

Colicky infants have gut microbial dysbiosis.[7–12] Probiotics have been widely studied to exert effects on the gut microbiota. Available evidence shows that administration of probiotic *Lactobacillus reuteri* strain DSM 17938 (LR 17938) is safe, tolerable, and beneficial in infants with colic.[13–20] Even though LR 17938 clinically appears to represent an effective treatment for infantile colic, its mechanism of action is still unknown. We intended to gain insight into the mechanism of LR 17938 in improving infantile colic by developing an infantile colic-related animal model. Currently, there are no established animal models for infantile colic. In this study, we used a technique called "unpredictable maternal separation" (MSU or SEP). MSU is considered a more severe stressor than scheduled separation.[21] Additionally, MSU with or without unpredictable maternal stress

[1]Departments of Pediatrics at McGovern Medical School, the University of Texas Health Science Center at Houston, Houston, TX, USA; [2]Departments of Neurology at McGovern Medical School, the University of Texas Health Science Center at Houston, Houston, TX, USA; [3]Department of Molecular Sciences, Uppsala BioCenter, Swedish University of Agricultural Sciences, Uppsala, Sweden and [4]BioGaia AB, Stockholm, Sweden
Correspondence: Yuying Liu (Yuying.Liu@uth.tmc.edu)

Received: 22 October 2020 Revised: 23 December 2020 Accepted: 30 December 2020
Published online: 2 February 2021

(MSUS), consisting of maternal restraint in a restraining tube,[22] was also used to evaluate the "crying" of newborn mice.

Mice produce ultrasonic vocalizations (USVs) in a variety of social contexts throughout development and adulthood. USVs are whistle-like calls between frequencies of 30 and 90 kHz, which are robust and consistent and therefore can be quantitatively analyzed and may be elicited by quantifiable stimuli such as pup isolation.[23] USVs are used for mother–pup retrieval,[24] juvenile interactions,[25] opposite and same sex interactions,[26–28] and territorial interactions.[29] Investigators have used USVs to study neuropsychiatric and developmental or behavioral disorders.[30–32] Analysis of rodent USVs under experimental conditions (we proposed) would give valuable insight into infantile colic. The underlying mechanism for USVs is not well understood, and the motivation behind different patterns of USVs need to be better defined. However, USVs follow a clear ontogenetic profile from birth to the second week of life,[33] which falls within the window for infantile colic in humans. In rat pups, maternal separation can also affect the prefrontal cortical transcriptome, affecting genes related to neurotransmission and circadian rhythms.[34]

The model for using USV to evaluate "crying" as a colic model, once established, not only would provide a tool to understand the mechanism of action for LR 17938 to mitigate colic but also could be quantified. We postulated that one could use the number of calls to compare different probiotics and combinations for optimizing efficacy in pre-clinical trials. In this study, the primary metrics were (a) number and qualities of USVs and (b) expression of pain-related and neuropathic genes in the brain of stressed mouse pups.

METHODS

Mouse models and experimental setup

Mice. Breeding pairs of C57BL/6J mice (Jackson Lab) and their newborns were used in this study. All experimental procedures were approved by the Center for Laboratory Animal Medicine and Care at UTHealth Houston (Protocol # AWC-18-0060).

Separation stress. The procedures for induction of stress included (a) unpredictable maternal separation (MSU or SEP) and (b) MSU combined with unpredictable maternal stress (MSU + MSUS, or S + S).[21,35] Briefly, dams (F0) and litters (F1) were separated for 3 h per day, daily, from postnatal day 5 (d5) to d14. (c) Control mice were left undisturbed except for a cage change once a week. Maternal stress consisted of 20-min of restraint in a cylindrical tube during the 3 h separation, daily.[21] Female and male newborn mice were studied.

Experimental setup for measuring USV

The experimental setup is shown in Fig. 1a. Mounted atop of the chamber is a sensitive microphone to record ultrasonic vocalization. The signals (calls) of USV are amplified by a sound amplifier device and recorded using the Avisoft Bioacoustics Recorder (Avisoft BioAcoustics, Glienicke/Nordbahn, Germany). Each mouse's vocalization was collected daily from d5 to d14. From each mouse, four recordings were taken per 3 h separation, at 0, 1, 2, and 3 h intervals, when we measured for 2 min their USVs in a chamber that attenuates light and sound. We tattooed the feet or tail using the Aramis Micro Tattoo Kit (Braintree Scientific Inc.), in order to identify correctly each newborn mouse for sequential measurements.

Experimental design for probiotic intervention

Probiotic preparation. Human breast milk-derived LR 17938 (ref. [36]) was provided by BioGaia AB (Stockholm, Sweden). The probiotic was prepared as described previously.[37] Briefly, LR 17938 was anaerobically cultured in deMan-Rogosa-Sharpe (MRS) medium at 37 °C for 24 h, plated in MRS agar at serial dilution, and grown anaerobically at 37 °C for 48–72 h to count colonies for generating a standard curve of bacterial colony-forming units (CFUs) per milliliter (mL). Quantitative analysis of bacteria in culture media on CFUs/mL were calculated by comparing absorbance at 600 nm of cultures using a standard curve. Before gavage feeding, the calculated number of probiotic LR 17938 was centrifuged at 4000*g* for 10 min at room temperature, supernatant was removed, and bacteria were resuspended in sterilized phosphate-buffered saline (PBS, pH 7.0).

Probiotic intervention. The newborn mice received daily an oral gavage of either LR 17938 (10^6 CFU/day, 100 µL) or PBS (100 µL) from d5 until d14. Each feeding of either probiotics or PBS was performed after measuring USVs. We used a Nutriline 1252.31G catheter (VYGON GmbH & Co., KG, Germany) to gavage the newborn mice. One set of experiments was performed in newborn mice that were exposed to MSU (SEP), while other dams were also exposed to MSUS. The experiment groups included (a) newborn mice with MSU (SEP, $n = 10$); (b) newborn mice with MSU that were gavaged PBS (SEP + PBS, $n = 3$); and (c) newborn mice with MSU, gavaged with LR 17938 (SEP + LR, $n = 6$). Another set of experiments was performed in newborn mice with MSU and their dams with MSUS (S + S). These experiment groups included (d) newborn mice with MSU and their dams with MSUS (S + S, $n = 5$); (e) newborn mice with MSU and dams with MSUS, gavaged with PBS (S + S + PBS, $n = 7$); and (f) newborn mice with MSU and dams with MSUS, gavaged with LR 17938 (S + S + LR, $n = 7$). The controls included newborn mice with (g) no MSU (Ctrl or NoSEP, $n = 24$); (h) no MSU with gavage with PBS (NoSEP + PBS, $n = 4$); and (i) no MSU with gavage of LR 17938 (NoSEP + LR, $n = 8$).

Experimental timeline

Figure 1b shows briefly the experimental timeline. The experiments were scheduled to take place from d5 to d14 of age. Besides this period falls in infant colic in humans, studies in rodents demonstrated that maternal care and maternal separation stress could affect USV production during this period.[38–40] In the control groups, each newborn mouse was taken out to be measured then put back into the same cage with dams. These pups staying with their dam had no MSU, but did experience 2 min of brief USV measurement. For mice with MSU, the entire litter was separated from the dam for 3 h, during which time we collected and analyze USVs in the various groups. Each pup was then taken out into the measuring chamber for 2 min and put back into the separation cage at each time point (0, 1, 2, and 3 h maternal separation).

Brain tissue pain-related (neuropathic and inflammatory) gene array analysis

At the end of the experiment at d14, brain tissues of mice were collected, fresh frozen immediately, and stored at −80 °C. Brain tissue RNAs were isolated by using RNeasy Lipid Tissue Mini Kit (Qiagen). RT^2 First Strand kit was used to perform reverse-transcript from RNA to cDNA including genomic DNA digestion. RT^2 Profiler PCR Array containing 84 genes that are related to mouse pain, including neuropathic and inflammatory genes (Qiagen PAMM-162D), were measured by reverse transcriptase-PCR using the Bio-Rad model CFX96 PCR machine. Raw Ct values were filled into the designed template, and data were analyzed using the online tool (https://geneglobe.qiagen.com/us/analyze). We analyzed gene expression from $n = 3$ mouse brains for each designated group.

Statistical analysis

USV measurements were performed in a randomized fashion by one investigator who was blinded to treatment conditions to avoid bias. Experimental results are expressed as means ± SE. Statistical analysis was performed using one-way ANOVA

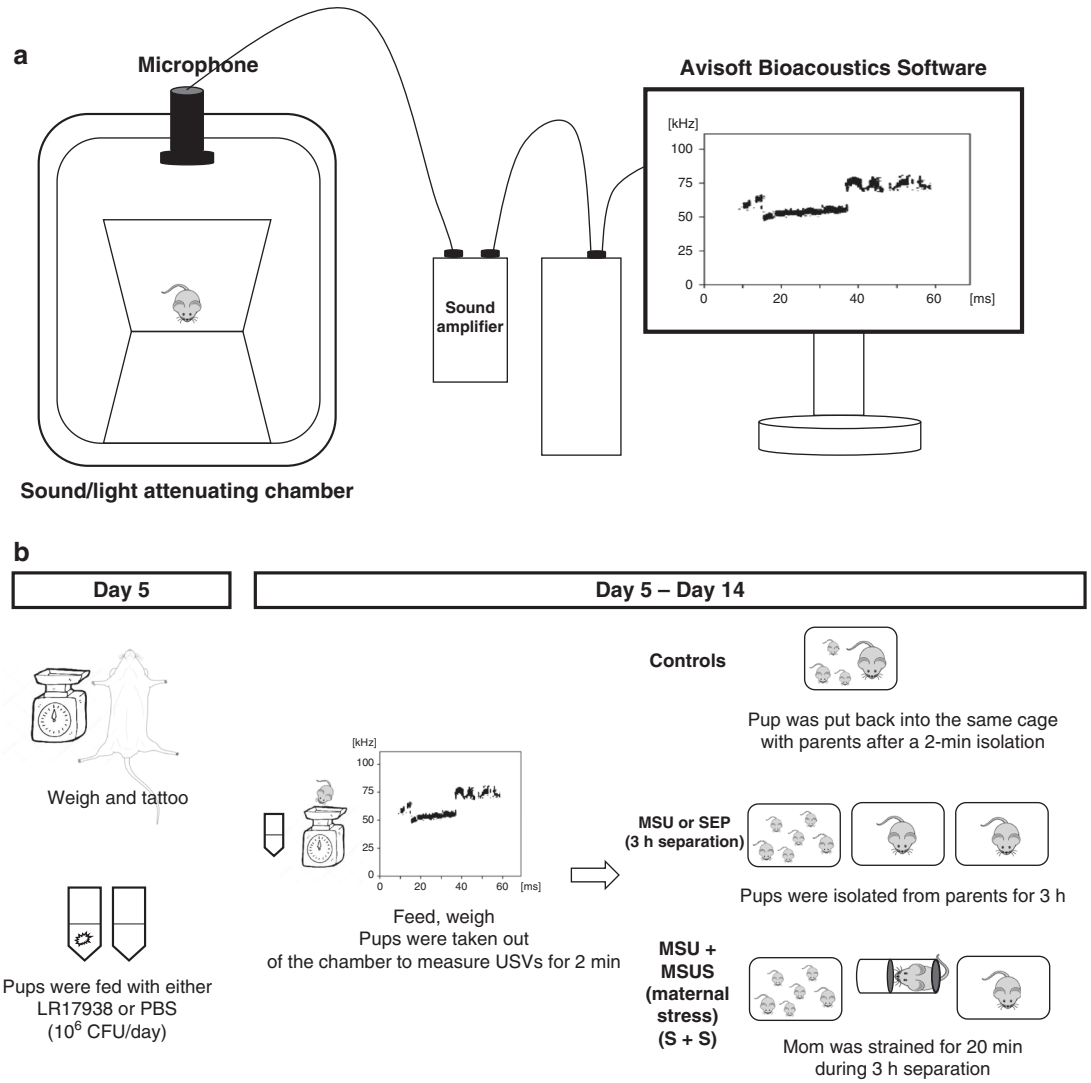

Fig. 1 Experimental setup and timeline. a Experimental setup for measuring USVs. **b** The experimental timeline. Each newborn mouse was weighed and identified by tattooing at day 5 of age. In the control group, each newborn mouse was taken out to be measured then put back into the same cage with dams, indicating that pups staying with their dam with no unpredictable maternal separation (MSU or SEP). For mice with MSU, the entire litter was separated from the dam for 3 h, during which time we collected USV calls in the various groups. These included a group of dams exposed to stress (MSU + MSUS, or S + S) during 3-h maternal separation and groups of pups fed with either PBS or probiotic LR 17938 by gavage (10^6 CFU/day), daily, from d5 to d14 of age. Each pup was taken out into the measuring chamber for 2 min and put back into the separation cage at each time point (0, 1, 2, and 3 h maternal separation) daily.

(Prism 4.0; GraphPad Software, San Diego, CA). Tukey's multiple-comparison tests were used for comparison of multiple groups, and $p < 0.05$ was considered statistically significant.

RESULTS

Exposure of newborn mice to stress reduced USV calls

Our initial analysis of USVs showed that newborn mice vocalized frequently on d7 and d11 of age, with a steep decline by d14. We focused on d7 and d11 of life. On d7, after exposure to MSU for 2 days, mice in the maternal separation (SEP) group had significantly reduced calls compared to control mice without MSU (Control, noSEP, $p < 0.05$ at 0 h) (Fig. 2a). We noticed that during the third hour of separation, calls increased back toward numbers similar to those of control mice (Fig. 2a). At d11 of age, after newborn mice have been exposed to MSU for 6 days, the differences between groups were more prominent: mice in the SEP group showed significantly reduced calls compared to control mice at 0, 1, and 2 h during 3 h separation (all $p < 0.05$), even though the calls gradually increased at 1 and 2 h compared to 0 h (Fig. 2b). Contrary to our hypothesis, these results showed that stress given to newborn mice reduced the numbers of USV calls compared to newborn mice with no stress. However, we noticed that giving MSUS to dams along with infant MSU (S + S) did not have the same effect on USV calls, for unknown reasons (Fig. 2a, b). We also observed that USV calls were not affected by sex of the neonates; therefore, data from both sexes were combined.

The patterns of USVs were not affected by neonatal stress

To further understand USV calls by stressed neonatal mice and their meaningful interpretation, we analyzed multiple different patterns of USVs. We used sound analysis software to classify whistle-like sounds with frequencies between 30 and 90 kHz. We identified many different patterns of USVs based on our recordings of the calls. We took the liberty to define the patterns of USVs as: Single Straight, Single Downsweep, Single Long Downsweep, Big Stub, Short Stub, Overlap, Double Straight, Double Downsweep, Double Long Downsweep, and Triple pattern

(Supplementary Fig. 1A). We observed that Single Downsweep was the most frequent pattern in both controls and separation groups at d7 of age. Even though we observed an increased trend of Single Straight in mice from the separation group (SEP) (Supplementary Fig. 1C) compared to that in mice of the control group without maternal separation (NoSEP control) (Supplementary Fig. 1B), the difference was not statistically significant. In addition, during 3 h separation when we measured USVs at each hour time point (0, 1, 2, and 3 h), there was no difference among the different time-point measurements (Supplementary Fig. 1B, C). Each mouse from each study group displayed various USV patterns at different time points and on different days. Therefore, in trying to correlate the mouse stress calls to human crying during colic, it is not clear what pattern in the mouse actually most closely corresponds to a human infant with colic. However, the patterns defined here may be useful for other studies with different models.

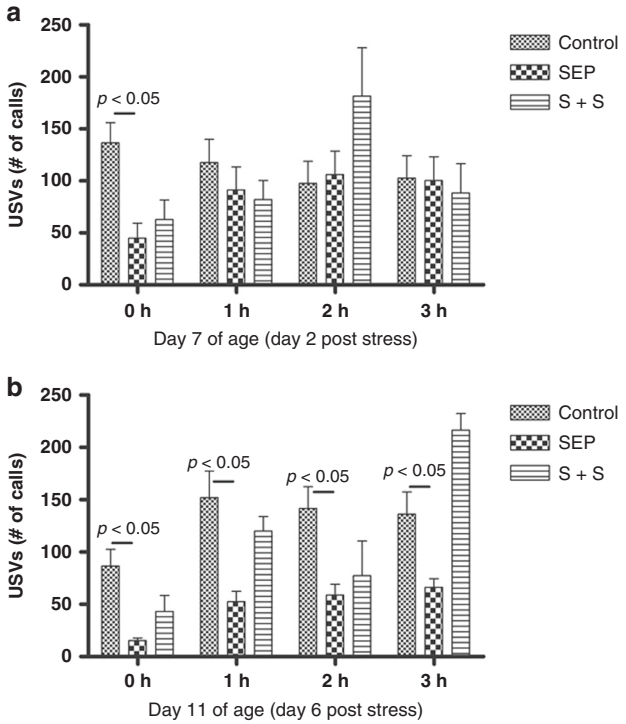

Fig. 2 Total number of USV calls analyzed in newborn mice with unpredictable maternal separation (MSU or SEP) and MSU in combination with MSUS (S + S) compared to the controls with no maternal separation and maternal stress (NoSEP) at d7 and d11 of age. a The number of USV calls of mice in d7 of age after exposure to SEP or S + S for 2 days. **b** The number of USV calls of mice at d11 of age after exposure to SEP or S + S for 6 days. USV calls were collected and analyzed during 3 h MSU at 0, 1, 2, 3 h time point. Control group: $n = 24$, SEP group: $n = 10$, S + S group: $n = 5$. $P < 0.05$ indicates a significant difference between groups.

Probiotic *Lactobacillus reuteri* DSM 17938 differentially affected USV calls under stress conditions

Because LR 17938 has been shown to reduce colicky crying in human babies, we determined the effects of this probiotic on infant mouse calls, under stress conditions. We measured whether orally feeding probiotic LR 17938 to these mice would affect the numbers of calls. We compared results with oral feeding of sterilized saline (PBS), the latter to rule out any stress that might be related to gavage feeding. We compared among three sets of groups at d11 of age when MSU mice were fed with probiotic LR 17938 for 6 days, at which time we had found reduced USV calls (Fig. 2b). Set I first compared non-stressed mouse pups among three groups [NoSEP (Control), NoSEP+PBS, NoSEP+LR]. Set II compared separation stress among three groups [3-h separation only (SEP), SEP + PBS, SEP + LR] (Fig. 3). Finally, Set III measured separation plus maternal restraint stress among three groups [S + S, S + S + PBS, S + S + LR] (Supplementary Fig. 2).

We found that USVs did not differentiate the numbers of calls, comparing the groups of mice exposed to stress (Fig. 3). However, in mice with no separation (Set I), the numbers of calls in mice fed LR (NoSEP + LR) was significantly higher than in mice fed with PBS (NoSEP + PBS) ($p < 0.05$) or NoSEP controls ($p < 0.05$ at 2 h, $p < 0.01$ at 3 h time point). We also compared the effect of probiotic LR in non-stressed mice (Set I, NoSEP+LR) to stressed mice (Set II, SEP + LR) or stressed mice and maternal restraint (Set III, S + S + LR). The number of calls in NoSEP+LR was significantly higher than in SEP + LR ($p < 0.01$ at 3 h time point, $p < 0.001$ at 2 h time point) (Fig. 3),

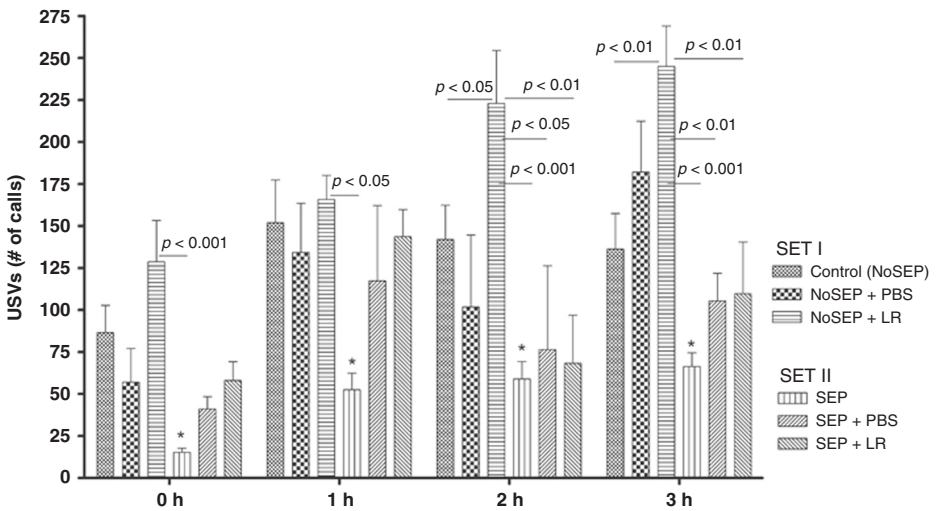

Fig. 3 The effect of oral feeding probiotic *Lactobacillus reuteri* DSM 17938 (LR 17938) on the number of calls of USVs in neonatal mice with MSU (SEP) and mice without MSU (NoSEP) at d11 of aged when mice have exposed to MSU for 6 days. USV calls were collected and analyzed during 3 h MSU at 0, 1, 2, 3 h time point. Control (NoSEP) group: $n = 24$, NoSEP+PBS group: $n = 4$, NoSEP+LR group: $n = 8$; SEP group: $n = 10$, SEP + PBS group: $n = 3$, SEP + LR group: $n = 6$. $P < 0.05$ indicates a significant difference between the groups indicated. * indicates $p < 0.05$, SEP vs. Control (NoSEP) at the same time point.

and higher than in S + S + LR ($p < 0.01$ at 2- or 3 h time point) (Supplementary Fig. 2).

These results demonstrated that orally feeding probiotic LR 17938 markedly increased vocalizations in newborn mice that remained suckling on their dams. However, orally feeding LR 17938 did not increase USV calls in newborn mice exposed to maternal separation. We therefore speculate that the USVs represent "physiological vocalizations" and that these are reduced by stress in our mouse model.

Probiotic *Lactobacillus reuteri* DSM 17938 modulated pain-related genes in the stressed neonatal brain

To further analyze potential beneficial effects of the probiotics on MSU, brain analysis was done in selected groups to quantify objectively the effects of separation stress and LR 17938 on the developing brain.

We measured a panel of 84 pain/stress/neuroinflammation-related genes expressed in the mouse brain to compare mice in the following six groups: control (NoSEP), SEP, SEP + PBS, SEP + LR, NoSEP+PBS, and NoSEP+LR. Quality controls, including a genomic DNA control, reverse-transcription controls, and positive PCR controls, were run, and all samples passed the quality controls. Selected housekeeping genes were beta-actin (Actb), beta-2-macroglobulin (B2m), glyceraldehyde-3-phosphate dehydrogenase (Gapdh), and beta-glucuronidase (Gusb). We defined the cutoff value for up- or down-regulation as ±1.5 with $p < 0.05$ as significant, as demonstrated by Volcano plots (Fig. 4 and Supplementary Figs. 3 and 4). The genes that were significantly up- or down-regulated in the studied groups and their major biological functions are listed in Table 1.

Using multiple group comparisons, we observed significant up-regulation of chemokine (c–c-motif) receptor 2 (CCR2) and prostaglandin-endoperoxide synthase 2 (Ptgs2) when compared neonatal mice with maternal separation (SEP) to mice with no-separation control (Fig. 4a), indicating that maternal separation increased central expression of inflammation-related genes.

Importantly, when we compared brain gene expression from mice with SEP + LR to that of mice with SEP and no probiotics, several other genes were differentially expressed. The greatest change was seen in a gene called *cluster of differentiation 200 antigen* (CD200), which has an anti-inflammatory effect on microglia. In addition, up-regulated were voltage-gated sodium-channel type IX α-subunit (Scn9a) and sodium-channel type III α-subunit (Scn3a); opioid receptor kappa 1 (Oprk1); and preprononociceptin (Pnoc). Genes encoding purinergic receptor P2X4 (P2rX4) and Ptgs2 (up-regulated by SEP) were down-regulated, while CCR2 was no longer increased (Fig. 4b). These findings demonstrated that orally gavage feeding LR 17938 up-regulated opioid peptide and opioid receptor transcripts related to pain relief, while LR down-regulated inflammation-related genes.

The chemokine (c–c motif) receptor 2 (CCR2) gene was also up-regulated in the brain of neonatal mice with maternal separation and oral gavage of saline (SEP + PBS), compared to controls (Supplementary Fig. 3A). However, in mice fed LR, up-regulation of CCR2 was not observed (SEP + LR). Therefore, up-regulated CCR2 might be related to both SEP and gavage feeding, whereas feeding of LR blocked its increased expression. Instead, we found down-regulation of beta-2-adrenergic receptor (Adrb2) and glutamate metabotropic receptor 1 (Grm1) transcripts in the brain of mice with SEP + LR compared to controls (Supplementary Fig. 3B). When we compared the group with SEP + PBS to the group with SEP (and no gavage feeding), no genes were up- or -down-regulated significantly (Supplementary Fig. 3C).

Comparing mice with SEP and LR (SEP + LR) with mice with SEP and PBS (SEP + PBS), three genes were up-regulated, namely CD200 (mentioned above), prodynorphin (Pdyn, known to interact with an opioid receptor to reduce pain), and Scn11a (Supplementary Fig. 3D). These two group comparisons are important. Inasmuch as the effect of LR on gene expression was independent of gavage feeding-induced stress. Results indicated that LR feeding to stressed newborns increased expression of genes associated with reduced microglial inflammation (CD200 antigen) and the relief of pain (Pdyn).

Our data also indicate that when neonatal mice stayed with their dam, gavage feeding-induced stress increased CCR2 expression in the brain; however, the levels of CCR2 expression were higher with PBS feeding (NoSEP + PBS) than with LR feeding (NoSEP + LR) (Supplementary Fig. 4A, 4B); in fact, CCR2 was down-regulated in the group NoSEP+LR vs. NoSEP+PBS (Supplementary Fig. 4C). Feeding of LR also reduced the expression of Grm1 and macrophage-related colony-stimulating factor 1 (csf1) compared to mice without gavage feeding (NoSEP control) (Supplementary Fig. 4B). The probiotic down-regulated dopamine beta (β)-hydroxylase (Dhb) mRNA compared to mice with feeding of saline (NoSEP + LR vs. NoSEP+PBS) (Supplementary Fig. 4C). These findings suggest that LR 17938 reduced the expression of inflammatory genes that were up-regulated due to stress induced by gavage feeding.

Finally, because the number of USV calls decreased when we fed LR to SEP mice, we analyzed the modulation of gene expression in mouse brain by LR — comparing mice with maternal separation stress (SEP + LR) to mice without stress (NoSEP + LR) (Supplementary Fig. 4D). We found that genes for cannabinoid receptor 2 (Cnr2), Pnoc, and Scn9a were up-regulated by oral feeding LR to mice with SEP (SEP + LR) compared to mice that stayed with their dams (NoSEP + LR) group. Six pain/stress/inflammatory-related genes were down-regulated by LR, including cholecystokinin B receptor (CCKbr), 5-hydroxytryphtamine receptor 1A (Htr1a), mitogen-activated protein kinase 14 (Mapk14), interleukin 1 alpha (IL1a), prostaglandin-endoperoxide synthase 2 (ptgs2), and potassium inwardly rectifying channel subfamily J member 6 (Kcnj6) (Supplementary Fig. 4D). Overall, these results indicate that despite the absence of an effect of LR on USV calls in stressed mice, feeding LR to stressed mice changed the expression of more genes in the stressed brain than in the non-stressed brain.

DISCUSSION

Infantile colic is a major burden to families and health professionals. It is strongly associated with maternal depression[41] and is the strongest risk factor for shaken baby syndrome.[42] Colic is also a common cause of early breastfeeding cessation.[43] Crying beyond the usual colicky period has been linked to later sleep problems, allergic disorders, family dysfunction, and behavioral problems.[44–46] The etiology of infantile colic is unknown, but is likely to be multifactorial. Psychosocial factors including inadequate parent–infant interaction, parental anxiety, maternal smoking, and advanced maternal age have been suggested as potential contributors to infant colic,[6,47] in addition to gastrointestinal, hormonal, neurodevelopmental factors.[48]

In mice, maternal separation during early postnatal development induces inadequate and disorganized maternal care, resulting in behavioral deficits that persist into adulthood. MSU is considered a more severe stressor than routine separation.[21] Previous studies used MSU or MSU in combination with MSUS to define an impact of early life stress on a variety of long-term behavioral and physiological effects, with epigenetic transmission in adult offspring.[22,35,49–52]

Before our study, others did not focus on early life stress before weaning at 3–4 weeks of age, the corresponding age of infantile colic in humans. Therefore, in this study, we specifically addressed murine "calls" as measured by USV, in order to differentiate mice with and without stress. As initially described, neonatal USVs were interpreted as a communicative behavior.[53] For example, newborn mice when separated from their dam produce USVs that elicit maternal retrieval behavior and to discourage rough handling by

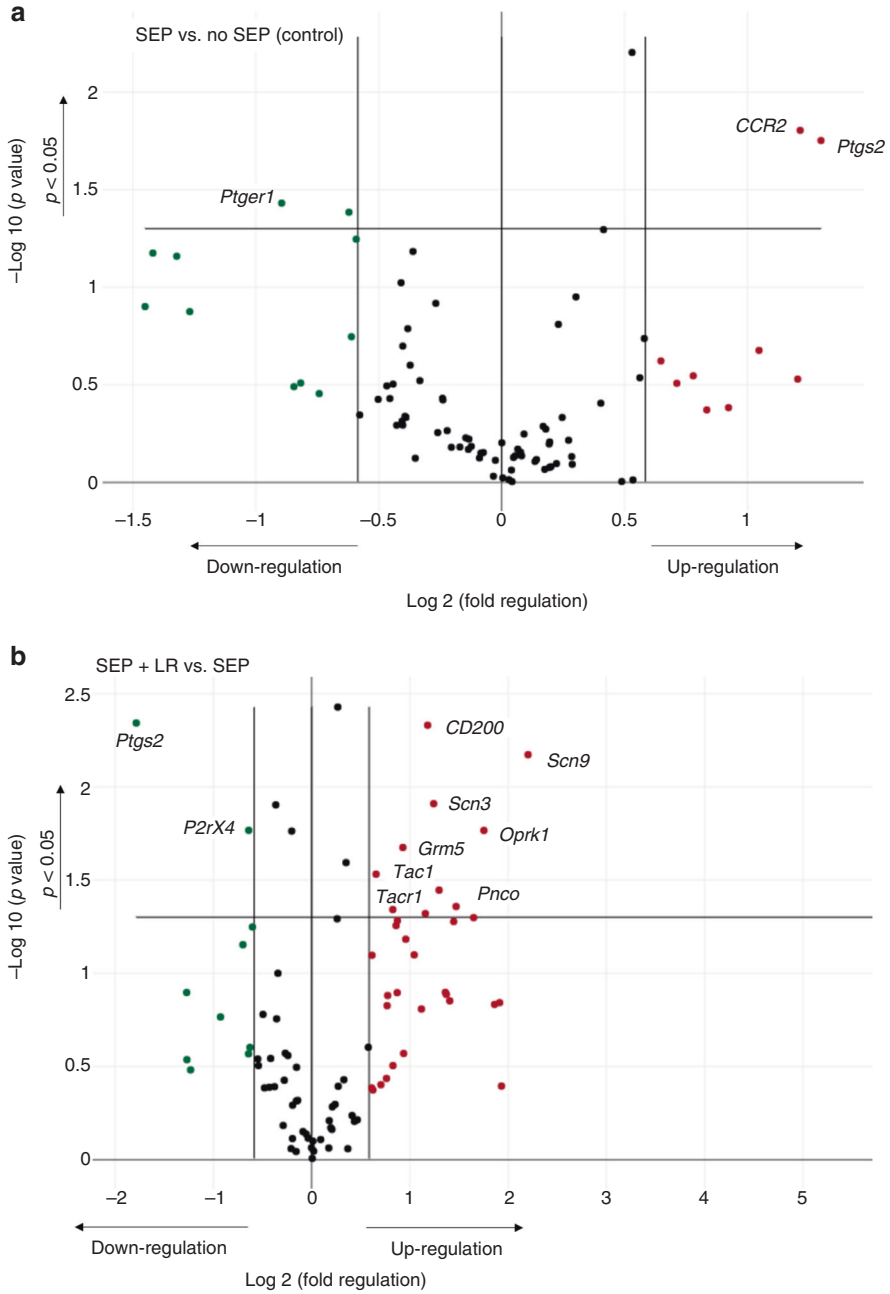

Fig. 4 Volcano plots demonstrating up- or down-regulated genes in the brain. The cutoff value for up- or down-regulation was ±1.5-fold compared to controls, with $p < 0.05$ regarded as significant. *X*-axis represents up-regulated genes (toward the right), or down-regulated genes (toward the left). *Y*-axis represents *p* value, with significance above the line. **a** Up- or down-regulated genes are shown, comparing SEP to controls (NoSEP). **b** Up- or down-regulated genes are shown, comparing groups SEP + LR to SEP.

the dam.[54] We found that the rate of calling was the highest between the 7th and 11th day of life, decreasing markedly at day 14 after birth. This observation is consistent with previous reports.[54–56] Another observation regarding USVs was that in mouse models of autism spectrum disorders, pups that are separated from their dams display an increase in both the number and duration of USV calls.[57–60] Because of these studies, we hypothesized that calls represent "crying", as a signal of infantile colic, predicting that the calls would increase with maternal separation. However, our data clearly demonstrated the opposite: that stress reduces USV calls. Previous studies in older mice yielded contradictory observations. Both increases[58,59,61] and decreases[62,63] in vocalization have been reported, and the meaning of these disparate changes is not understood.

Because we observed that murine USV calls fall into many different patterns, in our study, we described 10 USV patterns from the recordings to characterize them and potentially identify difference between the stress groups compared to the control group. Even though we divided the types of calls, we remained unable to identify differences between the groups. The patterns of USV calls uniquely described in the current study we hope will provide analytic reference data for future studies.

It has been shown that males are more susceptible to the detrimental effects of early stress than females.[64,65] MSUS alters

Table 1. Major up- or down-regulated genes by multiple group comparisons.

Genbank	Symbol	Description	Major biological function
NM_009915	CCR2	Chemokine (c–c-motif) receptor 2	CCL2 receptor, facilitates monocyte infiltration in inflammation
NM_011198	Ptgs2	Prostaglandin-endoperoxide synthase 2	Converts of arachidonic acid to prostaglandin H2, a precursor of prostacyclin, expressed in inflammation
NM_010818	CD200	CD200 antigen	Regulates myeloid cell activity with an inhibitory signal for the macrophages in diverse tissues
NM_011011	Oprk1	Opioid receptor kappa 1	Receptor responding to opioid peptides to reduce pain/stress
NM_010932	Pnoc	Prepronociceptin	Endogenous ligand for opioid receptor-like-1 (ORL1) to reduce pain/stress
NM_011026	P2rX4	Purinergic receptor P2X4	Proliferation and migration of neural stem cells, vascular reactivity, apoptosis, and cytokine secretion
NM_007420	Adrb2	Beta-2-adrenergic receptor	Epinephrine (adrenaline) binds Adrb2 to mediate physiologic responses, e.g., smooth muscle relaxation and bronchodilation
NM_016976	Grm1	Glutamate metabotropic receptor 1	Glutamatergic neurotransmission in normal brain function and in neuropathologic conditions
NM_001088414	Grm5	Glutamate metabotropic receptor 5	Glutamatergic neurotransmission in normal brain function and in neuropathologic conditions
NM_018863	Pdyn	Prodynorphin	Opioid polypeptide hormone
NM_138942	Dhb	Dopamine beta (β)-hydroxylase	Conversion of dopamine to norepinephrine
NM_007778	Csf1	Colony-stimulating factor 1	Macrophage-related inflammatory cytokine
NM_009924	Cnr2	Cannabinoid receptor 2	Immunological activities of T cell, B cells, and microglia (brain)
NM_007627	Cckbr	Cholecystokinin B receptor	A complex regulator of dopamine activity in the brain related to anxiety and depression
NM_008308	Htr1a	5-Hydroxytryphtamine receptor 1A	Serotonin binding receptor 1a associated with pain with the binding of serotonin
NM_011951	Mapk14	Mitogen-activated protein kinase 14	MAPK14/p38α signaling pathway activated by stress and brain inflammation
NM_010554	IL1a	Interleukin 1 alpha	Inflammatory cytokine
NM_010606	Kcnj6	Potassium inwardly rectifying channel subfamily J member 6	Promotes flow of potassium into the cell; related to GABAergic synapse in brain
NM_018732	Scn3a	Voltage-gated sodium-channel type III α subunit	Initiates and propagates action potentials in neurons and other excitable cells, mainly in CNS
NM_018852	Scn9a	Voltage-gated sodium-channel type IX α subunit	Initiates and propagates action potentials in neurons and other excitable cells, mainly in PNS
NM_011887	Scn11a	Voltage-gated sodium-channel type XI α subunit	Initiates and propagates action potentials in neurons and other excitable cells, mainly in DRG

behavior primarily in males, for example, although females also have some behavioral deficits, as shown by deficits in behavioral control and impaired ability to evaluate danger.[35] In our study, we did not identify sexual dimorphism related to infantile USV calls during early life stress; but we cannot rule out that the behavior changes would be different in males and females in later life. We also considered differences among mouse strains. It has been reported that C57Bl/6 mice showed lower calling rates than other inbred mice (BALB/c, DBA, A/J).[66–69] To better understand the effects of probiotics in the developing newborn under stress, further testing other mouse strains with a higher calling rate should be considered.

Maternal separation stress (MS) has been linked to subsequent psychiatric disorders and to irritable bowel syndrome in humans.[70] Gut microbiota plays an important role in mediating gut–brain interaction. Alterations in the composition of the intestinal microbiota are associated with changes in the inflammatory, neuroendocrine, and behavioral responsiveness of the host.[71,72] Early life MS has been shown to disturb the composition of intestinal microbiota and augments the production of inflammatory cytokines.[51,52,73] The *Lachnospiraceae* family, accounting for approximately 40% of total bacteria, appears highly sensitive to early adversity in both sexes, with different operational taxonomic units (OTUs) affected comparing males versus females.[74] Other studies using MS have reported increased *Bacteroides*, *Lachnospiraceae*, and *Clostridium XlVa* spp. in males, in both rats and mice.[75–77]

We chose to study LR 17938 because of clinical trials in infantile colic,[16,78–80] but the mechanism of action of probiotics in colic remains undetermined. We found clear evidence that oral feeding LR 17938 affect stress and pain-related gene expression in the newborn brain. Importantly, LR 17938 increased the expression of opioid peptide *prodynorphin*; endogenous ligand *prepronociceptin*; and *opioid receptor kappa-opioid receptor 1*, all of which when activated would favor reduction of pain and stress signals.[81,82] Ligands such as prodynorphin or prepronociceptin bind to the κ-opioid receptor which regulates dopamine release and inhibits the release of glutamate, GABA, and serotonin (5-HT).[83] Published studies showed that prodynorphin gene deletion increased anxiety-like behavior and increased GABA receptor gene expression in the amygdala.[84] The levels of glutamate, GABA, and serotonin need to be further assessed in the future studies in this model to evaluate probiotic effects in the developing mouse brain.

Finally, we observed that LR 17938 significantly increased the expression of CD200 in brain. Previous research has shown that exposure to stress up-regulated glial activation markers (MHC II, CD11b, Iba-1, and GFAP) while downregulating glial cell regulatory markers CD200 and CD200R, resulting in microglia primed toward inflammation.[85,86] The mechanism of anti-inflammatory effects

through the CD200–CD200R cross-talk between microglia and neurons has been studied in many other diseases such as stroke, multiple sclerosis, Alzheimer's, and Parkinson's disease[85–89] and would be of great interest in further studies. We are not aware of previous studies showing that a probiotic or its product(s) interacts with glial cells or their receptors CD200 or CD200R.

In summary, the current study provides important information on infant stress responses, in particular USV calls which are reduced in our model of maternal–infant separation stress. We also reveal that stress/pain-related genes in brain are modulated by orally feeding probiotic LR 17938.

Other mouse strains with higher calling rates may be used for further evaluation of probiotics. The up- or down-regulated genes of interest expressed in specific brain regions and their related neurotransmitters (including metabolites such as glutamate, serotonin, melatonin, GABA) should be further assessed. Finally, immune modulation by probiotics and their effects on microglia and T cells that are involved in CD200–CD200R signaling should be further investigated.

AUTHOR CONTRIBUTIONS

S.R., J.M.R., and Y.L. have contributed to the conception and design. E.S.P., J.F., and Y.L. have contributed to acquisition of data. All authors have contributed to data analysis and interpretation of data. E.S.P. and Y.L. drafted the article. J.M.R., S.R., and V.R.V. revised it critically for important intellectual content. All authors approved the final version to be published.

FUNDING

The study was supported by the Investigator Initiated Research Fund from BioGaia AB, Stockholm, Sweden. The probiotic *Lactobacillus reuteri* DSM 17938 was obtained as a gift from BioGaia AB.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41390-021-01374-0.

Competing interests: There are no disclosures for authors. S.R. has part-time employment by the company BioGaia AB, Stockholm, Sweden.

Consent statement: This study did not involve human patients.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

REFERENCES

1. Vandenplas, Y. et al. Prevalence and health outcomes of functional gastrointestinal symptoms in infants from birth to 12 months of age. *J. Pediatr. Gastroenterol. Nutr.* **61**, 531–537 (2015).
2. Wessel, M. A., Cobb, J. C., Jackson, E. B., Harris, G. S. Jr & Detwiler, A. C. Paroxysmal fussing in infancy, sometimes called colic. *Pediatrics* **14**, 421–435 (1954).
3. Barr, R. G. Colic and crying syndromes in infants. *Pediatrics* **102**, 1282–1286 (1998).
4. Mayer, E. A. Gut feelings: the emerging biology of gut-brain communication. *Nat. Rev. Neurosci.* **12**, 453–466 (2011).
5. Weissbluth, L. & Weissbluth, M. Infant colic: the effect of serotonin and melatonin circadian rhythms on the intestinal smooth muscle. *Med. Hypotheses* **39**, 164–167 (1992).
6. Hall, B., Chesters, J. & Robinson, A. Infantile colic: a systematic review of medical and conventional therapies. *J. Paediatr. Child Health* **48**, 128–137 (2012).
7. de, W. C., Fuentes, S., Puylaert, P. & de Vos, W. M. Intestinal microbiota of infants with colic: development and specific signatures. *Pediatrics* **131**, e550–e558 (2013).
8. Partty, A., Kalliomaki, M., Endo, A., Salminen, S. & Isolauri, E. Compositional development of Bifidobacterium and Lactobacillus microbiota is linked with crying and fussing in early infancy. *PLoS ONE.* **7**, e32495 (2012).
9. Rhoads, J. M. et al. Altered fecal microflora and increased fecal calprotectin in infants with colic. *J. Pediatr.* **155**, 823–828 (2009).
10. Savino, F. et al. Intestinal microflora in breastfed colicky and non-colicky infants. *Acta Paediatr.* **93**, 825–829 (2004).
11. Savino, F. et al. Molecular identification of coliform bacteria from colicky breastfed infants. *Acta Paediatr.* **98**, 1582–1588 (2009).
12. Savino, F. et al. Comparison of formula-fed infants with and without colic revealed significant differences in total bacteria, Enterobacteriaceae and faecal ammonia. *Acta Paediatr.* **106**, 573–578 (2017).
13. Savino, F., Pelle, E., Palumeri, E., Oggero, R. & Miniero, R. Lactobacillus reuteri (American Type Culture Collection Strain 55730) versus simethicone in the treatment of infantile colic: a prospective randomized study. *Pediatrics* **119**, e124–e130 (2007).
14. Barr, R. G., Kramer, M. S., Boisjoly, C., McVey-White, L. & Pless, I. B. Parental diary of infant cry and fuss behaviour. *Arch. Dis. Child* **63**, 380–387 (1988).
15. Barr, R. G., Rotman, A., Yaremko, J., Leduc, D. & Francoeur, T. E. The crying of infants with colic: a controlled empirical description. *Pediatrics* **90**, 14–21 (1992).
16. Savino, F. et al. *Lactobacillus reuteri* DSM 17938 in infantile colic: a randomized, double-blind, placebo-controlled trial. *Pediatrics* **126**, e526–e533 (2010).
17. Szajewska, H., Gyrczuk, E. & Horvath, A. *Lactobacillus reuteri* DSM 17938 for the management of infantile colic in breastfed infants: a randomized, double-blind, placebo-controlled trial. *J. Pediatr.* **162**, 257–262 (2013).
18. Chau, K. et al. Probiotics for infantile colic: a randomized, double-blind, placebo-controlled trial investigating *Lactobacillus reuteri* DSM 17938. *J. Pediatr.* **166**, 74–78 (2015).
19. Mi, G. L. et al. Effectiveness of *Lactobacillus reuteri* in infantile colic and colicky induced maternal depression: a prospective single blind randomized trial. *Antonie Van Leeuwenhoek* **107**, 1547–1553 (2015).
20. Harb, T., Matsuyama, M., David, M. & Hill, R. J. Infant colic—what works: a systematic review of interventions for breast-fed infants. *J. Pediatr. Gastroenterol. Nutr.* **62**, 668–686 (2016).
21. Franklin, T. B. et al. Epigenetic transmission of the impact of early stress across generations. *Biol. Psychiatry* **68**, 408–415 (2010).
22. Moloney, R. D. et al. Early-life stress induces visceral hypersensitivity in mice. *Neurosci. Lett.* **512**, 99–102 (2012).
23. Fischer, J. & Hammerschmidt, K. Ultrasonic vocalizations in mouse models for speech and socio-cognitive disorders: insights into the evolution of vocal communication. *Genes Brain Behav.* **10**, 17–27 (2011).
24. D'Amato, F. R., Scalera, E., Sarli, C. & Moles, A. Pups call, mothers rush: does maternal responsiveness affect the amount of ultrasonic vocalizations in mouse pups? *Behav. Genet.* **35**, 103–112 (2005).
25. Panksepp, J. B. et al. Affiliative behavior, ultrasonic communication and social reward are influenced by genetic variation in adolescent mice. *PLoS ONE* **2**, e351 (2007).
26. Chabout, J. et al. Adult male mice emit context-specific ultrasonic vocalizations that are modulated by prior isolation or group rearing environment. *PLoS ONE* **7**, e29401 (2012).
27. Moles, A., Costantini, F., Garbugino, L., Zanettini, C. & D'Amato, F. R. Ultrasonic vocalizations emitted during dyadic interactions in female mice: a possible index of sociability? *Behav. Brain Res.* **182**, 223–230 (2007).
28. Yang, M., Loureiro, D., Kalikhman, D. & Crawley, J. N. Male mice emit distinct ultrasonic vocalizations when the female leaves the social interaction arena. *Front. Behav. Neurosci.* **7**, 159 (2013).
29. Kalcounis-Rueppell, M. C. et al. Differences in ultrasonic vocalizations between wild and laboratory California mice (*Peromyscus californicus*). *PLoS ONE* **5**, e9705 (2010).
30. Bishop, S. L. & Lahvis, G. P. The autism diagnosis in translation: shared affect in children and mouse models of ASD. *Autism Res.* **4**, 317–335 (2011).
31. Lahvis, G. P., Alleva, E. & Scattoni, M. L. Translating mouse vocalizations: prosody and frequency modulation. *Genes Brain Behav.* **10**, 4–16 (2011).
32. Scattoni, M. L., Gandhy, S. U., Ricceri, L. & Crawley, J. N. Unusual repertoire of vocalizations in the BTBR T+tf/J mouse model of autism. *PLoS ONE* **3**, e3067 (2008).
33. Branchi, I., Santucci D., Alleva E. Analysis of ultrasonic vocalizations emitted by infant rodents. *Curr. Protoc. Toxicol*. Chapter 13, Unit13 (2006).
34. Zheng, Y. et al. Transcriptome analysis on maternal separation rats with depression-related manifestations ameliorated by electroacupuncture. *Front. Neurosci.* **13**, 314 (2019).
35. Weiss, I. C., Franklin, T. B., Vizi, S. & Mansuy, I. M. Inheritable effect of unpredictable maternal separation on behavioral responses in mice. *Front. Behav. Neurosci.* **5**, 3 (2011).
36. Rosander, A., Connolly, E. & Roos, S. Removal of antibiotic resistance gene-carrying plasmids from *Lactobacillus reuteri* ATCC 55730 and characterization of the resulting daughter strain, *L. reuteri* DSM 17938. *Appl. Environ. Microbiol.* **74**, 6032–6040 (2008).
37. Liu, Y., Tran, D. Q., Fatheree, N. Y. & Marc, R. J. *Lactobacillus reuteri* DSM 17938 differentially modulates effector memory T cells and Foxp3+ regulatory T cells in

a mouse model of necrotizing enterocolitis. *Am. J. Physiol. Gastrointest. Liver Physiol.* **307**, G177–G186 (2014).
38. Gatta, E. et al. Reduced maternal behavior caused by gestational stress is predictive of life span changes in risk-taking behavior and gene expression due to altering of the stress/anti-stress balance. *Neurotoxicology* **66**, 138–149 (2018).
39. Kaidbey, J. H. et al. Early life maternal separation and maternal behaviour modulate acoustic characteristics of rat pup ultrasonic vocalizations. *Sci. Rep.* **9**, 19012 (2019).
40. Myers, M. M., Brunelli, S. A., Squire, J. M., Shindeldecker, R. D. & Hofer, M. A. Maternal behavior of SHR rats and its relationship to offspring blood pressures. *Dev. Psychobiol.* **22**, 29–53 (1989).
41. Vik, T. et al. Infantile colic, prolonged crying and maternal postnatal depression. *Acta Paediatr.* **98**, 1344–1348 (2009).
42. Barr, R. G. Preventing abusive head trauma resulting from a failure of normal interaction between infants and their caregivers. *Proc. Natl Acad. Sci. USA* **109** (Suppl 2), 17294–17301 (2012).
43. Howard, C. R., Lanphear, N., Lanphear, B. P., Eberly, S. & Lawrence, R. A. Parental responses to infant crying and colic: the effect on breastfeeding duration. *Breastfeed. Med.* **1**, 146–155 (2006).
44. Hemmi, M. H., Wolke, D. & Schneider, S. Associations between problems with crying, sleeping and/or feeding in infancy and long-term behavioural outcomes in childhood: a meta-analysis. *Arch. Dis. Child* **96**, 622–629 (2011).
45. Savino, F. et al. A prospective 10-year study on children who had severe infantile colic. *Acta Paediatr. Suppl.* **94**, 129–132 (2005).
46. Sung, V. Infantile colic. *Aust. Prescr.* **41**, 105–110 (2018).
47. Raiha, H., Lehtonen, L., Huhtala, V., Saleva, K. & Korvenranta, H. Excessively crying infant in the family: mother-infant, father-infant and mother-father interaction. *Child Care Health Dev.* **28**, 419–429 (2002).
48. Mai, T., Fatheree, N. Y., Gleason, W., Liu, Y. & Rhoads, J. M. Infantile colic: new insights into an old problem. *Gastroenterol. Clin. North Am.* **47**, 829–844 (2018).
49. Hyland, N. P. et al. Early-life stress selectively affects gastrointestinal but not behavioral responses in a genetic model of brain-gut axis dysfunction. *Neurogastroenterol. Motil.* **27**, 105–113 (2015).
50. Maccari, S., Krugers, H. J., Morley-Fletcher, S., Szyf, M. & Brunton, P. J. The consequences of early-life adversity: neurobiological, behavioural and epigenetic adaptations. *J. Neuroendocrinol.* **26**, 707–723 (2014).
51. O'Mahony, S. M. et al. Early life stress alters behavior, immunity, and microbiota in rats: implications for irritable bowel syndrome and psychiatric illnesses. *Biol. Psychiatry* **65**, 263–267 (2009).
52. O'Mahony, S. M., Hyland, N. P., Dinan, T. G. & Cryan, J. F. Maternal separation as a model of brain-gut axis dysfunction. *Psychopharmacology (Berl.)* **214**, 71–88 (2011).
53. Zippelius, H. M. & Schleidt, W. M. Ultraschall-laute bei jungen mausen (Ultrasonic vocalization in infant mice). *Naturwissenschaften* **43**, 502–503 (1956).
54. Scattoni, M. L., Crawley, J. & Ricceri, L. Ultrasonic vocalizations: a tool for behavioural phenotyping of mouse models of neurodevelopmental disorders. *Neurosci. Biobehav. Rev.* **33**, 508–515 (2009).
55. Elwood, R. W. & Keeling, F. Temporal organization of ultrasonic vocalizations in infant mice. *Dev. Psychobiol.* **15**, 221–227 (1982).
56. Wiaderkiewicz, J., Glowacka, M., Grabowska, M. & Jaroslaw-Jerzy, B. Ultrasonic vocalizations (USV) in the three standard laboratory mouse strains: developmental analysis. *Acta Neurobiol. Exp. (Wars)* **73**, 557–563 (2013).
57. Ey, E. et al. The autism ProSAP1/Shank2 mouse model displays quantitative and structural abnormalities in ultrasonic vocalisations. *Behav. Brain Res.* **256**, 677–689 (2013).
58. Picker, J. D., Yang, R., Ricceri, L. & Berger-Sweeney, J. An altered neonatal behavioral phenotype in Mecp2 mutant mice. *Neuroreport* **17**, 541–544 (2006).
59. Tesdahl, N. S., King, D. K., McDaniel, L. N. & Pieper, A. A. Altered ultrasonic vocalization in neonatal SAPAP3-deficient mice. *Neuroreport* **28**, 1115–1118 (2017).
60. Yang, M. et al. Reduced excitatory neurotransmission and mild autism-relevant phenotypes in adolescent Shank3 null mutant mice. *J. Neurosci.* **32**, 6525–6541 (2012).
61. Lai, J. K. et al. Temporal and spectral differences in the ultrasonic vocalizations of fragile X knock out mice during postnatal development. *Behav. Brain Res.* **259**, 119–130 (2014).
62. Reynolds, C. D., Nolan, S. O., Jefferson, T. & Lugo, J. N. Sex-specific and genotype-specific differences in vocalization development in FMR1 knockout mice. *Neuroreport* **27**, 1331–1335 (2016).
63. Sungur, A. O., Schwarting, R. K. & Wohr, M. Early communication deficits in the Shank1 knockout mouse model for autism spectrum disorder: developmental aspects and effects of social context. *Autism Res.* **9**, 696–709 (2016).
64. Holmes, A. et al. Early life genetic, epigenetic and environmental factors shaping emotionality in rodents. *Neurosci. Biobehav. Rev.* **29**, 1335–1346 (2005).
65. Kikusui, T. & Mori, Y. Behavioural and neurochemical consequences of early weaning in rodents. *J. Neuroendocrinol.* **21**, 427–431 (2009).
66. Hennessy, M. B., Li, J., Lowe, E. L. & Levine, S. Maternal behavior, pup vocalizations, and pup temperature changes following handling in mice of 2 inbred strains. *Dev. Psychobiol.* **13**, 573–584 (1980).
67. Roubertoux, P. L. et al. Vocalizations in newborn mice: genetic analysis. *Behav. Genet.* **26**, 427–437 (1996).
68. Sewell, G. D. Ultrasonic communication in rodents. *Nature* **227**, 410 (1970).
69. Thornton, L. M., Hahn, M. E. & Schanz, N. Genetic and developmental influences on infant mouse ultrasonic calling. III: Patterns of inheritance in the calls of mice 3-9 days of age. *Behav. Genet.* **35**, 73–83 (2005).
70. Ren, T. H. et al. Effects of neonatal maternal separation on neurochemical and sensory response to colonic distension in a rat model of irritable bowel syndrome. *Am. J. Physiol. Gastrointest. Liver Physiol.* **292**, G849–G856 (2007).
71. Burokas, A., Moloney, R. D., Dinan, T. G. & Cryan, J. F. Microbiota regulation of the Mammalian gut-brain axis. *Adv. Appl. Microbiol.* **91**, 1–62 (2015).
72. Mayer, E. A., Tillisch, K. & Gupta, A. Gut/brain axis and the microbiota. *J. Clin. Invest.* **125**, 926–938 (2015).
73. O'Mahony, S. M. et al. Disturbance of the gut microbiota in early-life selectively affects visceral pain in adulthood without impacting cognitive or anxiety-related behaviors in male rats. *Neuroscience* **277**, 885–901 (2014).
74. Rincel, M. et al. Multi-hit early life adversity affects gut microbiota, brain and behavior in a sex-dependent manner. *Brain Behav. Immun.* **80**, 179–192 (2019).
75. De, P. G. et al. Microbiota and host determinants of behavioural phenotype in maternally separated mice. *Nat. Commun.* **6**, 7735 (2015).
76. Gacias, M. et al. Microbiota-driven transcriptional changes in prefrontal cortex override genetic differences in social behavior. *Elife.* **5**, e13442 (2016).
77. Murakami, T. et al. Changes in intestinal motility and gut microbiota composition in a rat stress model. *Digestion* **95**, 55–60 (2017).
78. Savino, F. & Tarasco, V. New treatments for infant colic. *Curr. Opin. Pediatr.* **22**, 791–797 (2010).
79. Savino, F. et al. Antagonistic effect of *Lactobacillus* strains against gas-producing coliforms isolated from colicky infants. *BMC Microbiol.* **11**, 157 (2011).
80. Savino, F., De, M. A., Ceratto, S. & Mostert, M. Fecal calprotectin during treatment of severe infantile colic with Lactobacillus reuteri DSM 17938: a randomized, double-blind, placebo-controlled trial. *Pediatrics* **135**(Suppl 1), S5–S6 (2015) .
81. Jungling, K., Blaesse, P., Goedecke, L. & Pape, H. C. Dynorphin-dependent reduction of excitability and attenuation of inhibitory afferents of NPS neurons in the pericoerulear region of mice. *Front. Cell Neurosci.* **10**, 61 (2016).
82. Katsuyama, S. et al. Antinociceptive effects of spinally administered nociceptin/orphanin FQ and its N-terminal fragments on capsaicin-induced nociception. *Peptides* **32**, 1530–1535 (2011).
83. Votinov, M. et al. A functional polymorphism in the prodynorphin gene affects cognitive flexibility and brain activation during reversal learning. *Front. Behav. Neurosci.* **9**, 172 (2015).
84. Femenia, T., Perez-Rial, S., Uriguen, L. & Manzanares, J. Prodynorphin gene deletion increased anxiety-like behaviours, impaired the anxiolytic effect of bromazepam and altered GABAA receptor subunits gene expression in the amygdala. *J. Psychopharmacol.* **25**, 87–96 (2011).
85. Frank, M. G., Fonken, L. K., Annis, J. L., Watkins, L. R. & Maier, S. F. Stress disinhibits microglia via down-regulation of CD200R: a mechanism of neuroinflammatory priming. *Brain Behav. Immun.* **69**, 62–73 (2018).
86. Frank, M. G., Annis, J. L., Watkins, L. R. & Maier, S. F. Glucocorticoids mediate stress induction of the alarmin HMGB1 and reduction of the microglia checkpoint receptor CD200R1 in limbic brain structures. *Brain Behav. Immun.* **80**, 678–687 (2019).
87. Ritzel, R. M. et al. CD200-CD200R1 inhibitory signaling prevents spontaneous bacterial infection and promotes resolution of neuroinflammation and recovery after stroke. *J. Neuroinflammation* **16**, 40 (2019).
88. Hernangomez, M. et al. Brain innate immunity in the regulation of neuroinflammation: therapeutic strategies by modulating CD200-CD200R interaction involve the cannabinoid system. *Curr. Pharm. Des.* **20**, 4707–4722 (2014).
89. Zhao, X., Li, J. & Sun, H. CD200-CD200R interaction: an important regulator after stroke. *Front. Neurosci.* **13**, 840 (2019).