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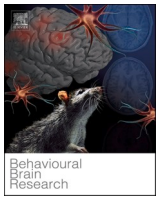

Research article

Ultrasonic vocalization emission is altered following neonatal hypoxic-ischemic brain injury in mice

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ABSTRACT

Neonatal hypoxic-ischemic (HI) brain injury leads to cognitive impairments including social communication disabilities. Current treatments do not sufficiently target these impairments, therefore new tools are needed to examine social communication in models for neonatal brain injury. Ultrasonic vocalizations (USVs) during early life show potential as a measurement for social development and reflect landmark developmental stages in neonatal mice. However, changes in USV emission early after HI injury have not been found yet. Our current study examines USV patterns and classes in the first 3 days after HI injury. C57Bl/6 mice were subjected to HI on postnatal day (P)9 and USVs were recorded between P10 and P12. Audio files were analyzed using the VocalMat automated tool. HI-injured mice emitted less USVs, for shorter durations, and at a higher frequency compared to control (sham-operated) littermates. The HI-induced alterations in USVs were most distinct at P10 and in the frequency range of 50–75 kHz. At P10 HI-injured mouse pups also produced different ratios of USV class types compared to control littermates. Moreover, alterations in the duration and frequency were specific to certain USV classes in HI animals compared to controls. Injury in the striatum and hippocampus contributed most to alterations in USV communication after HI. Overall, neonatal HI injury leads to USV alterations in newborn mice which could be used as a tool to study early HI-related social communication deficits.

1. Introduction

Hypoxic-ischemic (HI) brain injury resulting from perinatal asphyxia is a disabling condition affecting an estimated 1.3–1.7 per 1000 live births in developed countries [1]. Of the surviving infants, 5–10 % develop persistent motor impairments and 20–50 % develop cognitive and social communication impairments, including 17 % developing language disabilities at 2 years of age [2–5]. Worrisomely, children that have been treated with hypothermia, the standard clinical care following moderate to severe perinatal asphyxia, still face lifelong disabilities in IQ, verbal comprehension, verbal reasoning, structural and pragmatic language scores and overall language impairment compared to age-, sex- and social class-matched term-born controls [4,6]. Importantly, studies have shown that language impairments in newborns can be improved by language intervention and parent-directed reading, suggesting opportunities for intervention to target these language impairments [7,8]. Altogether, there is an urgent need to better understand and predict the development of social communication impairments after neonatal brain injury in order to effectively target these impairments with interventions.

In neonatal HI animal models, social communication is currently understudied [9,10]. Interestingly, ultrasonic vocalizations (USVs) have shown potential to be used as a tool to model social disabilities and other consequences of HI injury such as epilepsy or neurodevelopmental disorders like autism spectrum disorder [3,9–16]. Although newborn mouse pups are probably yet to develop the circuitry for hearing, they can already produce USVs from birth to emit distress calls upon isolation to evoke maternal behavior [17]. Calling characteristics are highly influenced by environmental and genetic factors like mouse strain, body weight, sex, temperature, and odor cues [16–18]. USV emission has a clear temporal development with calling rates peaking at postnatal day (P)4–8 and gradually decreasing over time, being nearly absent at two

Abbreviations: HI, hypoxic-ischemic; USV, ultrasonic vocalization; P, Postnatal day; KHz, kilohertz.

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weeks after birth [19]. Moreover, USVs reflect the development of vocal, social and emotional behavior, and calling rate is associated with landmark developmental stages of newborn mice such as eye opening [17,20]. Little is known about the influence of neonatal HI brain injury on USV production in mice. Interestingly, brain regions predominantly affected by HI such as the thalamus, hippocampus, hypothalamus and cerebral cortex, are involved in the emission of USVs [21–24]. Therefore, this study examined whether USV emission was altered after neonatal HI to potentially develop a tool to model impairments in vocal behavior and/or sociability following neonatal HI injury. To this end, HI injury was induced at P9 using the Vannucci-Rice model [21]. From P10 until P12 (i.e. 1–3 days after the HI insult), USVs were recorded daily and audio files were analyzed for USV patterns and distribution of USV classes with the Vocalmat automated tool [25], allowing advanced USV detection and classification.

2. Materials and methods

2.1. Animals and HI injury

All procedures were carried out according to the Dutch and European international guidelines (Directive 86/609, ETS 123, Annex II) and approved by the Experimental Animal Committee Utrecht (University Utrecht, Utrecht, Netherlands) and the Central Authority for Scientific Procedures on Animals (The Hague, The Netherlands) ("Bescherming en herstel van het beschadigde neonatale brein", AVD11500202115334, April 12th 2022). All efforts were made to minimize suffering. This paper is written in accordance with the ARRIVE guidelines [26]. Inbred C57Bl/6 mice (OlaHsa, ENVIGO, Horst, The Netherlands) were kept in standard housing conditions with woodchip bedding, cardboard shelters and tissues provided, on a 12 hr day/night cycle (lights on at 7 am), in a temperature-controlled room at 20–24°C and 45–65 % humidity with ad libitum food and water access. Mice were bred in-house by placing wild type males and females together in a ratio of 1:2 for 10 days. Afterwards, dams were housed solitarily to give birth.

Neonatal HI injury was induced in 9-day-old pups by unilateral carotid artery ligation under isoflurane anesthesia (5–10 min; 5 % induction, 3–4 % maintenance with flow O_2: air 1:1), followed by recovery with their mother for at least 75 min and subsequently systemic hypoxia at 10 % O_2 for 45 min in a temperature-controlled, humidified hypoxic incubator. Control sham-operated (SHAM) littermates were subjected to anesthesia and surgical incision only (no carotid artery ligation, no hypoxia). Subcutaneous 10 mg/kg Xylocaine (#N01BB02, AstraZeneca, Cambridge, UK) and 3.5 mg/kg locally-applied Bupivacaine (#N01BB01, Actavis, Allergan Inc, Dublin, Ireland) were applied to the incision for pre- and post-operative analgesia, respectively.

We strived for an equal contribution of SHAM- and HI-operated animals per litter to control for litter effects. Number of animals used was determined with Power analysis based on an effect size of 1.54, alpha of 0.0083 (alpha of 0.05 corrected for 6 comparisons) and power of 0.9, resulting in a minimum number of animals of 15 per group (G-power 3.1.9). Total number of animals per experimental group and litter are depicted in Table A.1. Number of animals used in this study are described in each figure caption. The exact number of included animals in Figs. 2, 4 and 5 can be found in Table A.2-A.4. Animals from both sexes were used in this study. No *a priori* exclusion criteria were used. Two separate cohorts of animals were used for recordings at P10-P12 or at P10-only to enhance reproducibility (Table A.1).

2.2. Ultrasonic vocalization recording

Between P10 and P12, pups were isolated daily between 12 am-5 pm and placed in the center of a cabinet (EKET, IKEA, Stockholm, Sweden) made soundproof with a layer of foam, for a total duration of 4 min after which the pup was immediately placed back in the home cage. Per litter, animals were recorded in a random order. During the trial, the home cage with mother and littermates was continuously present in the room (distance: 2 m). Ultrasonic vocalisations were recorded at a sampling rate of 384 kHz with an ultrasonic microphone (sensitivity range: 10–160 kHz; M500−384, Pettersson Elektronik, Sweden) mounted in the soundproof cabinet and processed with a freeware sound-recording program (Audacity® 3.2)

2.3. Immunohistochemistry

A subset of the animals in which USVs were recorded (HI: n=6; 3 females, 3 males, SHAM: n=6; 3 females, 3 males) was followed up until P37 (i.e. 4 weeks after induction of HI brain injury) and terminated by overdose of pentobarbital followed by transcardial perfusion with PBS and subsequently 4 % paraformaldehyde (VWR, Radnor, Pennsylvania, USA). Another group of animals (HI: n=6, 3 females, 3 males, SHAM n=6, 3 females, 3 males), in which no USVs were recorded were terminated similarly at postnatal day 10/11 to study early brain injury patterns. Brains were collected and post-fixed for 24 h in 4 % paraformaldehyde. Fixed brains were dehydrated in increasing ethanol dilution from 30 % until 100 %, followed by embedment in paraffin. Coronal sections (8 μm) were cut at hippocampal level (bregma −1.70 for adult mouse brain). Sections were deparaffinized and endogenous peroxidase was blocked with 3 % H_2O_2 (VWR) in methanol for 20 min. Sections were hydrated and antigen retrieval was performed in 10 mM citrate buffer (pH 6.0) for 3 min at 95°C. Next, sections were cooled on ice for 30 min and washed with PBS. Non-specific binding of the antibody was blocked with 5 % normal horse serum (26050088, Thermofisher Scientific, Landsmeer, The Netherlands) in PBS for 30 min at 37 °C. Primary antibody incubation with mouse anti-MAP2 (1:1000, M4403, Merck KgA, Darmstadt, Germany) was performed overnight in 2 % normal horse serum in PBS. The next day, slides were washed with PBS. Secondary biotin-conjugated horse-anti-mouse antibody (1:100, BA2000, Vector Laboratories, Newark, USA) was diluted in PBS and incubated for 45 min at 37 °C. After incubation, sections were washed in PBS and incubated with vectastain ABC kit (PK-6100, Vector Laboratories) for 30 min at RT followed by incubation in 200 mL 3,3′-diaminobenzidine (DAB) solution with 60 μL 30 % H_2O_2 in 0.05 M Tris-HCl (pH 7.6, Roche, Basel, Switzerland) for 3 min. Lastly, slides were dehydrated in increasing ethanol dilutions until 100 % followed by xylene and embedded with DEPEX. Full-section images were captured with a Nikon D1 digital camera (Nikon, Tokyo, Japan). Different brain areas in the ipsilateral and contralateral hemispheres were manually delineated with FIJI 1.53 (National Institutes of Health, NIH, Bethesda, Maryland, USA, Fig. 1A). These regions included the thalamus, the hypothalamus, the striatum (including the dorsal striatum and the striatum-like amygdalar nuclei), the hippocampus and the cerebral cortex (including the motor cortex, somatosensory cortex, auditory cortex, ectorhinal, perirhinal and entorhinal cortex). MAP2 area loss was calculated as [1-(MAP2-positive area ipsilateral side/MAP2-positive area contralateral side) x 100 %].

2.4. USV analysis

Audio files were converted into wav format and imported into Vocalmat [25] for MATLAB analysis (MathWorks, Eindhoven, The Netherlands). For more detailed description of the methods see [25]. Experimenters were blinded for experimental group during data analysis. As USVs with a frequency lower than 50 kHz were not observed, which is in line with literature [18,27], we set a lower threshold of 50 kHz to limit background detection. All USVs detected above 175 kHz were checked and manually deleted from data when identified as an artefact (i.e. no clear USV appearance). Because clear clusters of USVs were observed (Figure A.1), total USVs per animal were analyzed separately and data were split up into a low frequency range (50–75 kHz) and a high frequency range (75 kHz – higher). From P13 onwards, in both SHAM- and HI-treated groups less than 50 % of

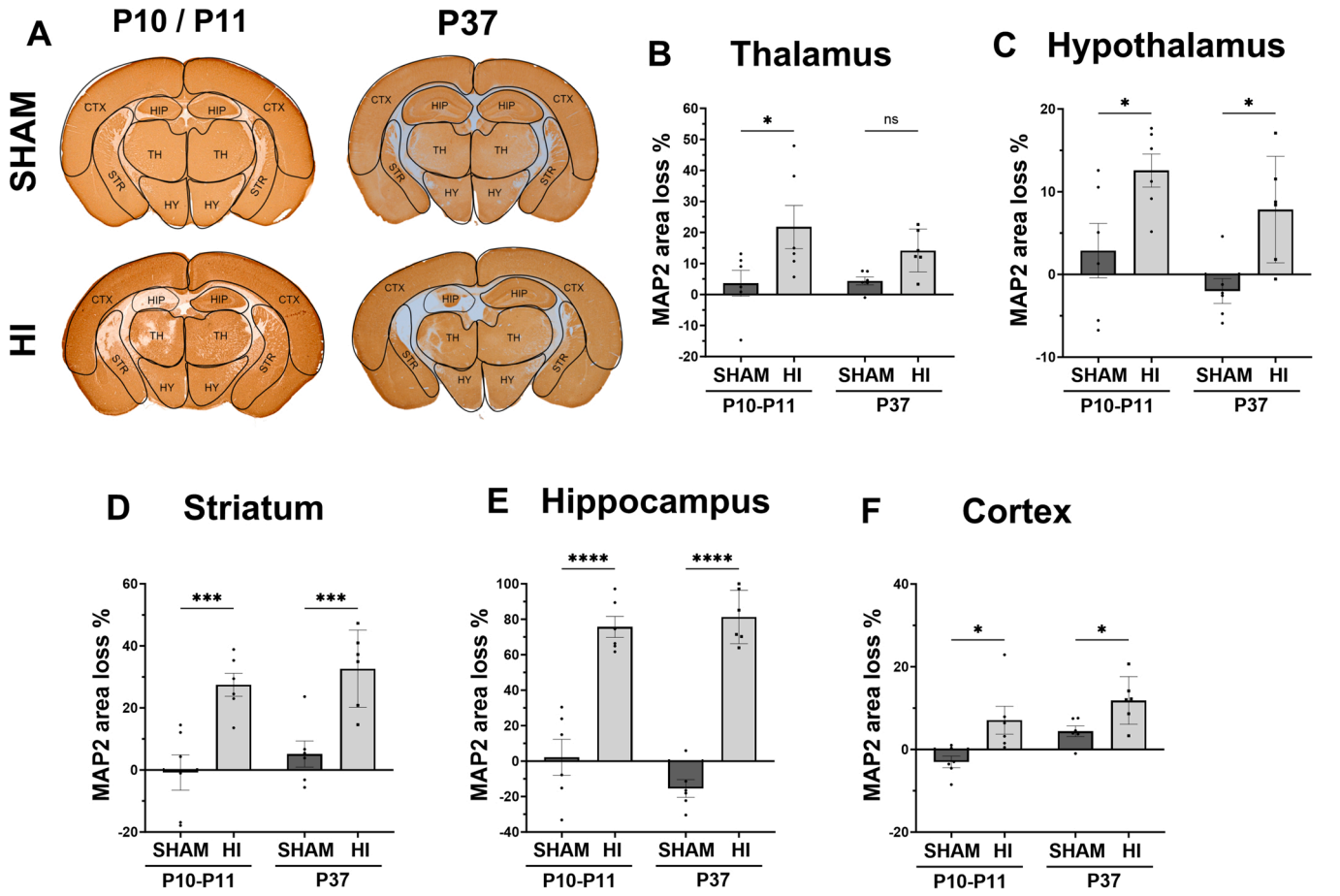


Fig. 1. HI induces neuronal loss in brain regions related to USV production. A) Representative examples of MAP2- stained brains of SHAM-operated animal and HI-injured animal at postnatal day 10/11 and 37 with delineated brain regions at bregma level −1.70 mm; CTX: Cortex, STR: Striatum, TH: Thalamus, HY: Hypothalamus, HIP: Hippocampus. Loss of MAP2 staining indicates neuronal injury. **B)** Percentage of ipsilateral MAP2 loss in SHAM-operated and HI-injured animals at P10/11 or P37 in the thalamus, **C)** hypothalamus, **D)** striatum, **E)** hippocampus and **F)** cortex. *p<0.05, **p<0.01, ****p<0.0001. Data is presented as mean + SEM. SHAM: n=6, HI: n=6.

animals produced USVs. To harbor reliability of the results, data were therefore included until P12 (Fig. 2B-D). No sex differences were found in this dataset, so analysis was performed in a mixed population of males and females (see Table A.1).

2.4.1. *USV parameters*

The USV parameters measured are depicted in Table 1. The experimental unit is animal. Max USV interval was set at 15 (A.U.) and maximum size of a USV was set at 20 detection points in Vocalmat.

2.4.2. *USV classification*

After USV detection, USVs were classified based on 11 distinct categories [25] (Table 2). Accuracy of Vocalmat classification of USVs in HI and SHAM animals was confirmed by manual classification of USVs from a subset of HI and SHAM animals (Table A.5). At P11 and P12, a considerable proportion of the animals in both experimental groups produced none or less than 10 USVs per 4 min, therefore solely data from P10 was analyzed for classification of USVs to prevent biased outcome. In Figs. 3 and 4, percentages of USVs produced in different classes were tested for statistical outliers. Afterwards, the values were normalized to 100 %.

2.5. *Statistical analysis*

Outliers were removed using the Rout method (Q=1 %), number of outliers per figure are depicted in Table A.2-A.4. Data were checked for normal Gaussian distribution. For HI and SHAM comparisons at P10, a Mann-Whitney test was performed. For brain region analysis One-way ANOVA was performed. For multiple timepoint analysis, each parameter was analyzed separately with a Mixed model of Restricted maximum likelihood model with factor postnatal day, injury, and postnatal day x injury, in GraphPad Prism 8.3 software (GraphPad Software, Boston, MA, USA). For correlation analysis, Pearson's correlation coefficient was calculated in GraphPad Prism 8.3 software. Datapoints were matched and Geisser-Greenhouse correction was performed to correct for lack of sphericity. Post-hoc comparisons were performed using the Holm-Šidák correction. Differences of $p<0.05$ were deemed as statistically significant. All statistical details per figure can be found in Table A.6-A.8.

3. Results

3.1. *HI affects multiple brain regions associated with USV production*

To examine if brain regions involved in USV emission were affected by the HI procedure [21–24,28], gray matter damage was assessed early (P10/11) and later (P37) after induction of the lesion in multiple regions as depicted in Fig. 1A. Early after HI injury, ipsilateral neuronal damage, assessed by MAP2 loss, was already observed in the thalamus, hypothalamus, striatum, hippocampus and cortex when compared to SHAM littermates (Fig. 1A-F, B: p=0.02, C: p=0.02, D: p=0.0008, E: p<0.0001, F: p=0.01, respectively). At 4 weeks after injury, similar patterns of

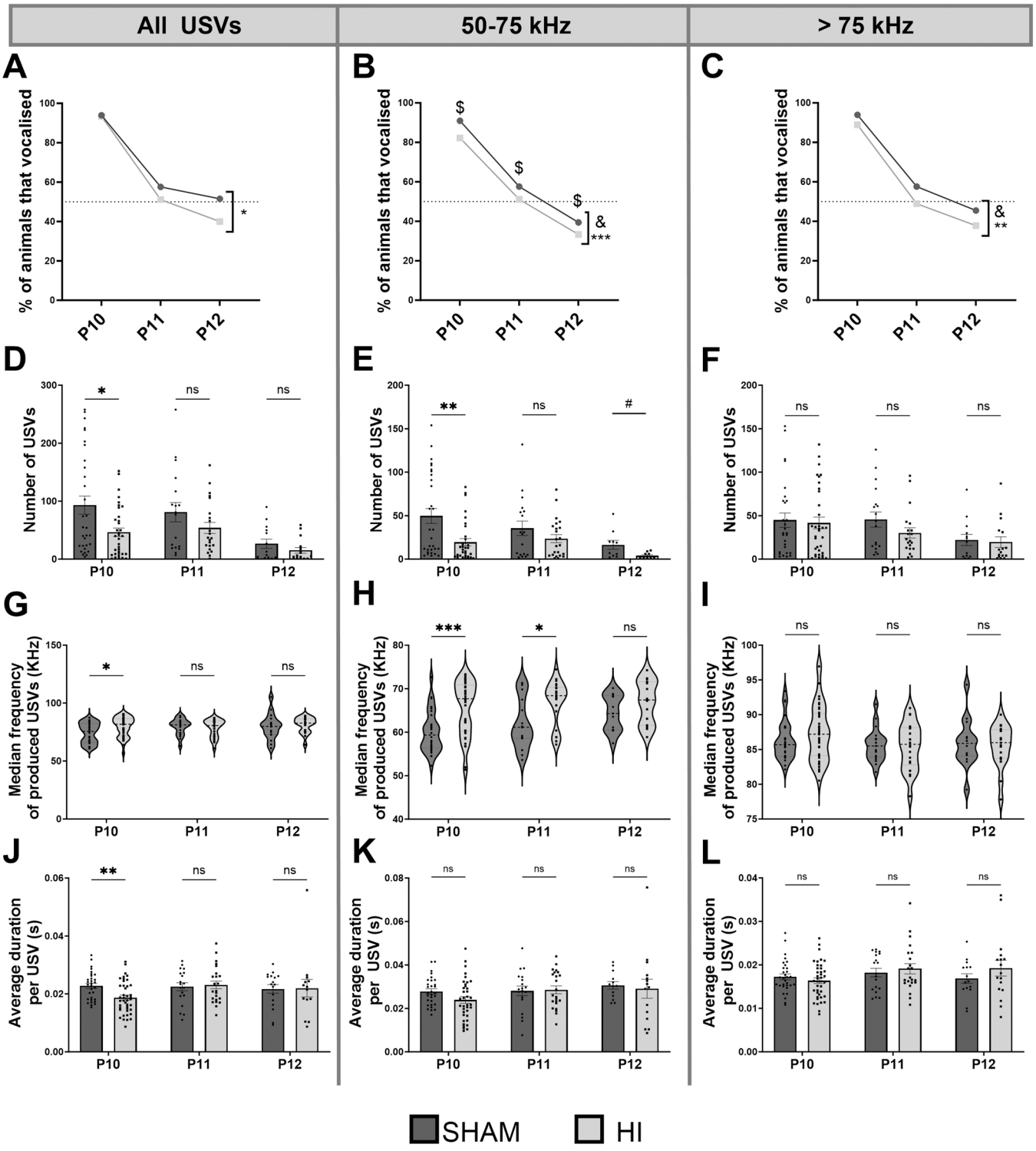


Fig. 2. HI-injured animals produce less USVs, and USVs of a higher frequency and lower duration compared to SHAM animals. A) Percentage of SHAM and HI animals that vocalize at P10-P12 in total frequency range, or separated in **B)** frequency range of 50–75 kHz and **C)** in the range >75 kHz. D) Number of USVs produced per animal in HI and SHAM groups at P10–12 in total frequency range, or separated in E) frequency range of 50–75 kHz and F) in the frequency range >75 kHz. G) Distribution of median frequency of USVs per animal in HI or SHAM groups in kilohertz (kHz) at P10-P12 in total frequency range, or separated in H) frequency range of 50–75 kHz and I) in the frequency range >75 kHz. J) Average duration per USV in seconds, per animal in HI and SHAM groups at P10-P12 in the total frequency range, or separated in K) frequency range of 50–75 kHz and L) in the frequency range >75 kHz. B-D: Data show mean per timepoint. & is main effect of injury. */**/*** is main effect of time. $ is significant difference between HI and SHAM groups, D-L: # trend: p=0.1–0.05, *p<0.05, **p<0.01, ***p<0.001, ns: not significant. Data is presented as (D-F, J-L) mean + SEM or (G-I) median + IQR. A-L: number of animals in each figure can be found in Table A.2.

Table 1
Parameters used for analysis of USV profile per animal.

Parameters	Definition
Count	Total number of USVs produced per animal in 4 min recording
Average duration (s)	Average duration of all USVs produced per animal in 4 min recording
Median frequency (kHz)	Median of lowest frequency of each USV produced per animal in 4 min recording

Table 2
Definitions of classes of USVs (see also Fig. 3A). [25].

Class of USVs	Definition
Short	Constant frequency syllables with modulation ≤5 kHz and duration ≤12 ms.
Flat	Constant frequency syllables with modulation ≤5 kHz and duration ≥12 ms.
Step down	Two-notes syllables in which the second element was ≥6 kHz lower from the preceding element and there was no more than 10 ms between steps.
Step up	Two-notes syllables in which the second element was ≥6 kHz higher from the preceding element and there was no more than 10 ms between steps.
Two steps	Three-notes syllables, in which the second element was ≥6 kHz or more different from the first, the third element was ≥6 kHz or more different from the second and there was no more than 10 ms between elements.
Down-frequency modulation	Downwardly frequency modulated with a frequency change ≥6 kHz.
Up-frequency modulation	Upwardly frequency modulated with a frequency change ≥6 kHz.
Chevron	Shaped like an inverted U in which the peak frequency was ≥6 kHz than the starting and ending frequencies.
Reverse chevron	Shaped like a U in which the peak frequency was ≥6 kHz than the starting and ending frequencies.
Complex	One-note syllables with two or more directional changes in frequency >6 kHz.
Multiple steps	Four-notes syllables or more, in which each element was ≥6 kHz or more different from the previous one and there was no more than 10 ms between elements.

HI-induced neuronal damage were observed in the hypothalamus, striatum, hippocampus and cortex when compared to SHAM littermates (Fig. 1B-F, C: p=0.02, D: p=0.0008, E: p<0.0001, F: p=0.03, respectively). The largest neuronal damage was observed in the hippocampus (P10/P11: 76 %±12.27, P37: 81 %±15.05) (Fig. 1E).

3.2. Fewer animals vocalize in the HI group compared to SHAM group

To examine if HI injury affects early USV emission, the number of vocalizing animals, number of USVs per animal, and USV frequency and duration were quantified at 1–3 days after HI induction (i.e. P10-P12). Importantly, at this early phase after the insult HI animals already showed unilateral neuronal injury as assessed by MAP2 loss, which was observed primarily in the ipsilateral thalamic, hypothalamic, striatum hippocampal and cortical regions [21] (Fig. 1). At P10, no significant changes in body weight were found between HI and SHAM animals. From P10 to P12, the percentage of animals that produced ultrasonic calls decreased in both groups (main effect of postnatal day: p=0.01, Fig. 2A). A lower percentage of HI pups was vocalizing compared to SHAM littermates in the frequency range of 50–75 kHz (main effect of injury: p=0.01 SHAM versus HI, Fig. 2C) and above 75 kHz (main effect of injury: p=0.02, Fig. 2D). Post-hoc comparisons revealed that specifically in the frequency range of 50–75 kHz, less HI-injured animals vocalized compared to SHAM animals at all timepoints (post-hoc: p=0.039, Fig. 2C).

3.2.1. HI animals produce fewer USVs compared to SHAM littermates

A main effect of injury (p=0.035) and time (p<0.0001) was found on the number of USVs produced in HI animals compared to SHAM animals (Fig. 2D). Post-hoc comparisons revealed that at P10, HI animals produced fewer USVs (p=0.029) compared to SHAM animals (Fig. 2D). This reduction was primarily due to less USVs produced in the lower frequency range of 50–75 kHz (main effect HI: p=0.011 and post-hoc: p=0.009, Fig. 2E-F) in HI animals compared to SHAM animals. Additionally, at P12 HI animals show a trend towards the production of fewer USVs compared to SHAM animals (p=0.068, Fig. 2E).

3.2.2. The median USV frequency of HI animals is higher than in SHAM littermates

At P10, HI animals produced USVs at a higher median frequency than SHAM animals (p=0.034, Fig. 2G). This increase in frequency was mainly observed at P10 and P11 in the lower frequency range of 50–75 kHz (main-effect injury: p<0.0001, Fig. 2H-I; P10: p=0.0003, P11: p=0.011, Fig. 2H). These data indicate that HI-injured animals produce less USVs of the lowest frequency ranges.

3.2.3. HI animals produce USVs of shorter duration compared to SHAM littermates

HI animals produced USVs with a shorter average duration per USV than SHAM animals at P10 (p=0.0043, Fig. 2J). No statistical differences in duration were found between HI and SHAM animals in specific frequency ranges or at P11 or P12 (Fig. 2J-L). No differences were found in average bandwidth (P10 HI: 49.98 ± 2.56, SHAM: 51.69 ± 1.58), amplitude (P10 HI: −89.48 ± 0.50, SHAM: −88.41 ± 0.29) or total vocalization duration (P10 HI: 2.16 ± 0.51, SHAM: 2.37 ± 0.45) between HI and SHAM groups (data not shown).

3.2.4. Animals that vocalize less have more striatal neuronal damage

When we correlated MAP2 loss in different brain regions to USV characteristics in HI injured animals, we found a negative correlation between MAP2 loss in the striatum and the number of USVs produced in the whole frequency range as well as the higher frequency range (Fig. 3, All USVs: p=0.025, >75 kHz: p=0.044). Specifically in the higher frequency range, we found a significant positive correlation between hippocampal MAP2 loss and median frequency of USVs in the higher frequency range (>75 kHz, p=0.041). This indicates that injury in the striatum and hippocampus might contribute to the altered USV communication in HI injured mice.

3.3. HI animals produce different USV class ratios compared to SHAM animals

To examine if HI and SHAM animals produce different classes of USVs, all USVs produced at P10 were allocated into 11 different classes (Fig. 4A). The USV classification was specifically performed at P10 since a considerable proportion of animals (~50 %) did not produce USVs or produced a low number of USVs at P11 and P12, which could introduce bias into the classification data (Fig. 2B-G). Notably, *short, flat and down frequency modulation* calls are the most prominent USV classes emitted in both SHAM and HI animals (Fig. 4B,E; yellow, red and black*). Two steps, chevron, reverse chevron, complex* and *multiple steps* calls were almost or completely absent in this study (Fig. 4B-G). When splitting into frequency ranges, all mouse pups produced relatively more *short* (p=0.0002) and *up frequency modulation* (p<0.0001) calls at frequencies > 75 KHz compared to the range of 50–75KHz (Fig. 4C, D, F, G). Conversely, *step up* (p<0.0001), *step down* (p<0.0001) or *two step* calls (p=0.0001) were more often produced at the lower frequency range of 50–75KHz (4B-C, E-F) than >75 KHz. Furthermore, USV classes were differently distributed between HI and SHAM animals at both the lower and the higher frequencies (Fig. 4B-G and Fig. 5 below).

To delineate whether HI affected certain distinctive USV classes compared to SHAM, separate comparisons of each USV class were

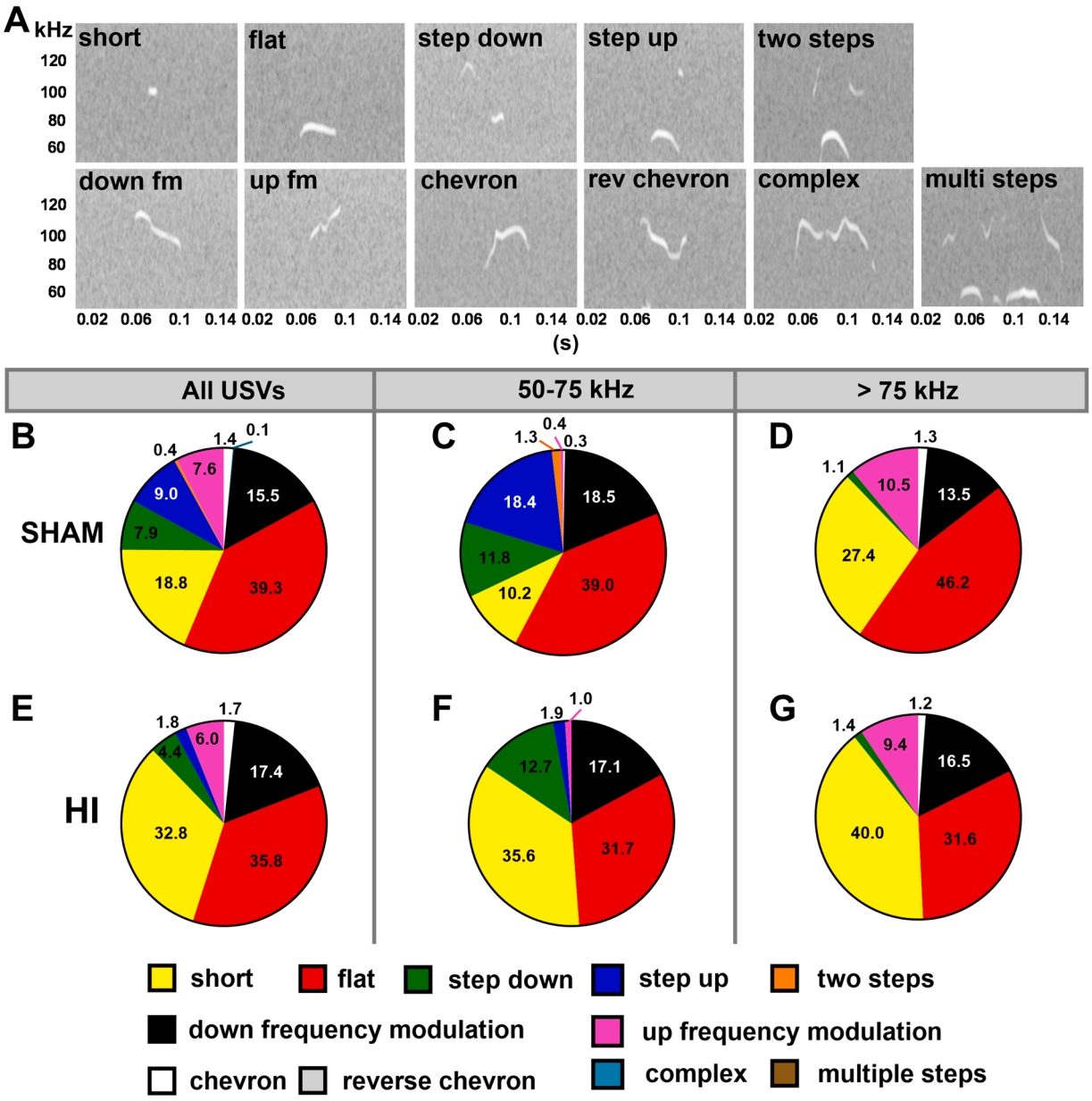


Fig. 4. Differences in USV classes produced by HI pups and SHAM littermates at postnatal day 10. A) Representative images of the different classes of USVs produced by neonatal mice in this study, (s): seconds, (kHz): kilohertz, down fm = down frequency modulation, multi steps = multiple steps, rev chevron = reverse chevron, up fm = up frequency modulation. **B)** Normalized percentual distribution of USV classes produced by SHAM animals at P10 in total frequency range, **C)** in frequency range of 50–75 kHz and **D)** in the frequency range >75 kHz. **E)** Normalized percentual distribution of USV classes produced by HI animals at P10 in total frequency range, **F)** in frequency range of 50–75 kHz and **G)** in the frequency range >75 kHz.

conducted (Fig. 5). Since *two steps, chevron, reverse chevron, complex* and *multiple steps* USVs were almost or completely absent in this study, they were not further analyzed. HI animals produced relatively more *short* USVs compared to SHAM animals in the complete frequency range (All USVs: $p<0.0001$; 50–75KHz: $p<0.0001$; >75 KHz: $p=0.0075$, Fig. 5A-C). Furthermore, HI animals produced less *flat* USVs in the higher frequency range compared to SHAM animals (All USVs: ns, 50–75KHz: ns, >75KHz: $p=0.0006$, Fig. 5D, E, and F, respectively). The multiple syllable USVs (*step down* and *step up* calls) were less prevalent in HI animals compared to SHAM animals when all USV ranges were analyzed. The reduction in *step up* calls in HI animals was primarily caused by less *step up* calls in the range of 50–75 kHz (step down all USVs: $p=0.0166$; step up all USVs: $p<0.0001$, step up 50–75 kHz: $p<0.0001$, Fig. 5G-L). In the higher frequency range of >75KHz, in which little of these classes were produced, no changes were found (Fig. 5I, L). No changes were found in the percentage of *down or up frequency modulation* calls produced by HI or SHAM animals (Fig. 5M-R). The lower duration per USV in HI animals (Fig. 2K) could likely be attributed to higher production of *short* USVs (Fig. 5C) and the diminished production of *flat* calls (Fig. 5F), *step down* and *step up* calls (Fig. 5G, J, K) as these USV classes have a higher average duration and are often emitted. In summary, HI animals produce relatively more simple *short* calls and less *flat* and multiple syllable calls compared to SHAM animals.

3.4. Characteristics of flat, down frequency modulation, step down and step up calls are altered after HI

Next, to examine whether HI animals also express distinctive characteristics within the USV classes, duration and frequency per USV class was assessed. We observed a trend towards a shorter duration of the *flat*

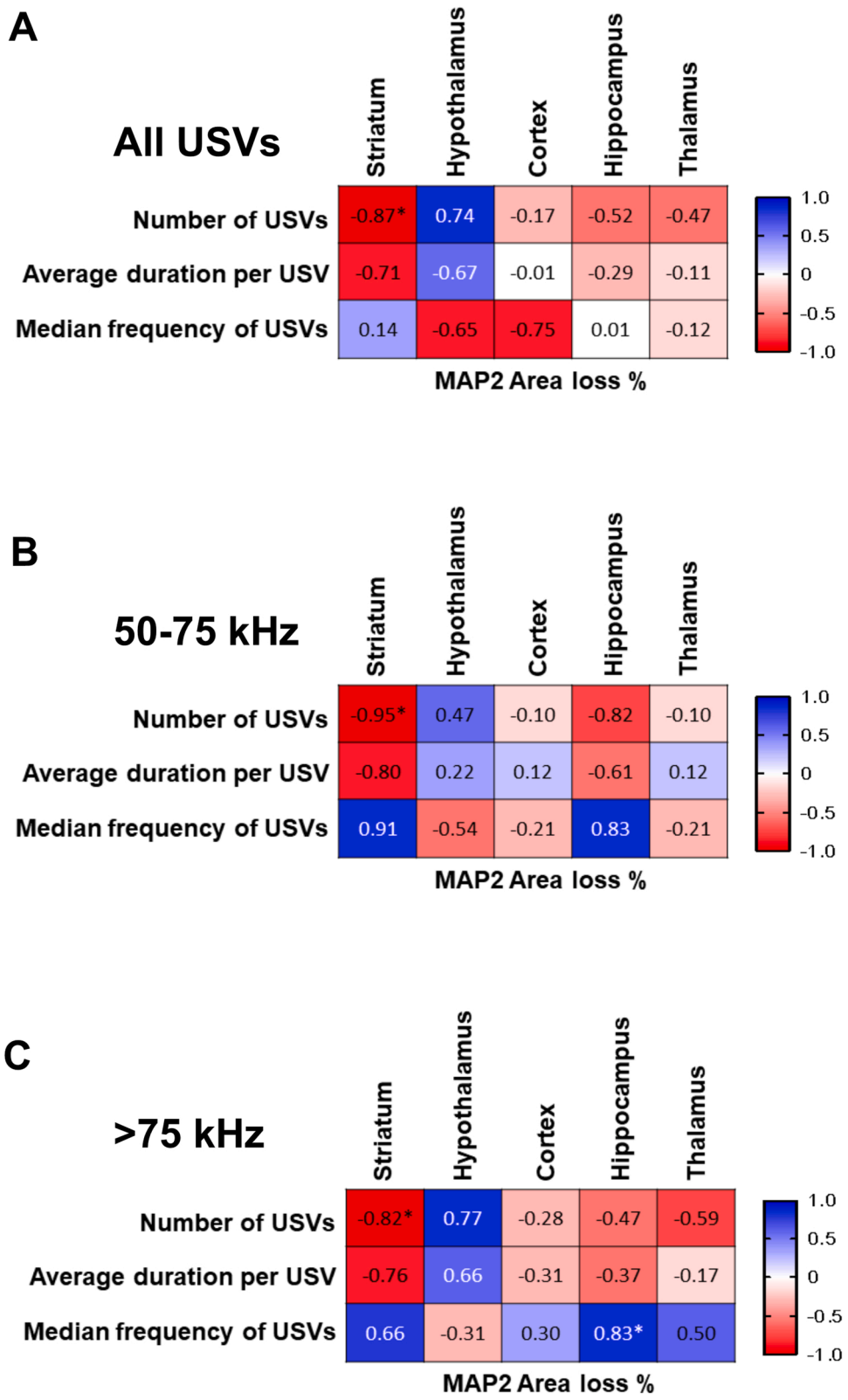
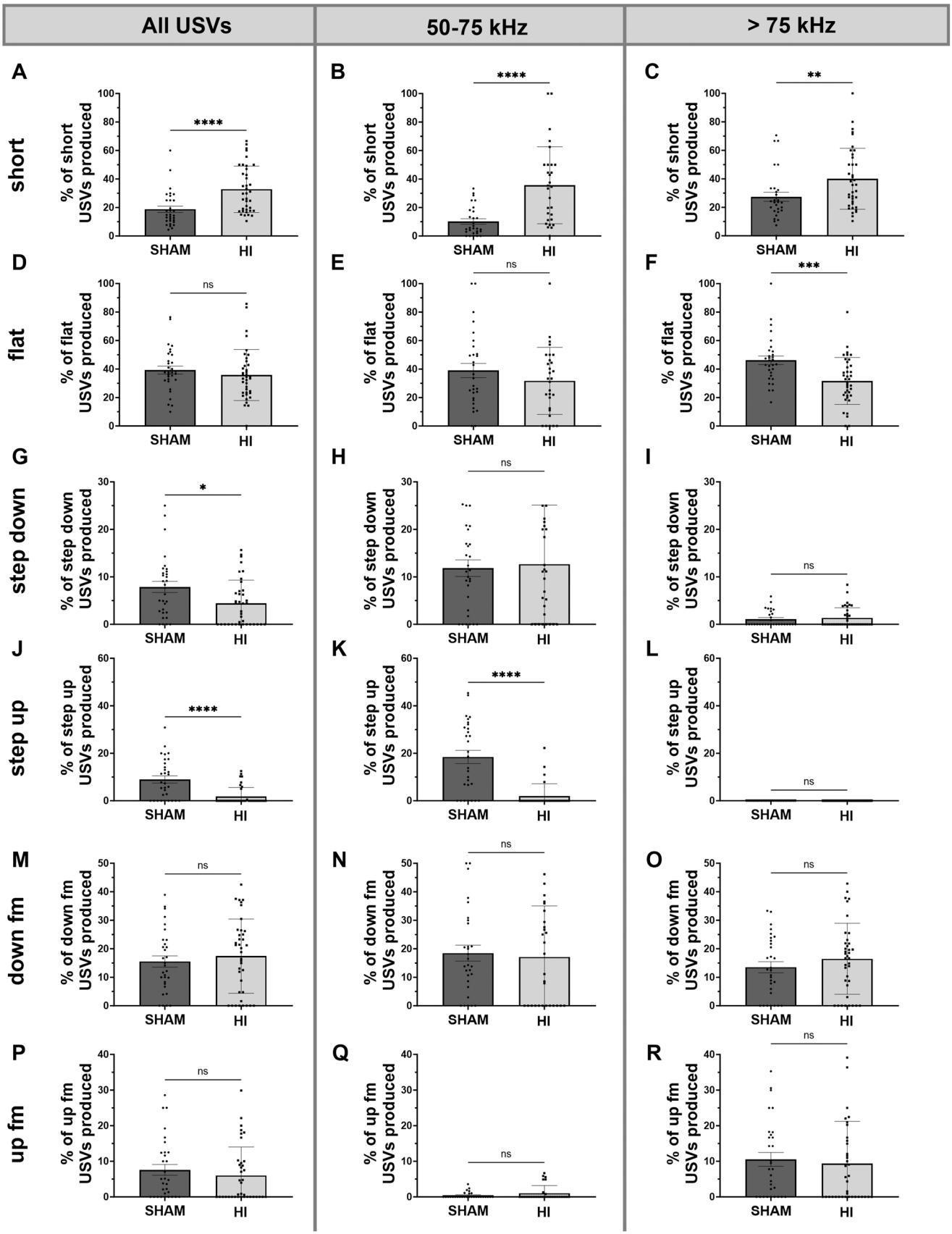


Fig. 3. Correlation matrix of brain region MAP2 loss and USV characteristics in HI injured mice late after injury (P37). A) Correlation matrix (Pearson r) of brain region MAP2 loss of Striatum, Hypothalamus, Cortex, Hippocampus and Thalamus and USV characteristics in total frequency range, **B)** in the 50–75 kHz frequency range and **C)** in the frequency range >75 kHz. n=6. *p=0.05.



(caption on next page)

Fig. 5. HI animals produce relatively more short USVs and less flat, step down, step up and down fm USVs at P10. A) Percentage of short USVs produced per animal in SHAM and HI groups in total frequency range, **B)** in the 50–75 kHz frequency range and **C)** in the frequency range >75 kHz. **D)** Percentage of flat USVs produced per animal in SHAM and HI groups in total frequency range, **E)** in the 50–75 kHz frequency range and **F** in the frequency range >75 kHz. **G)** Percentage of step down USVs produced per animal in SHAM and HI groups in total frequency range, **H)** in the 50–75 kHz frequency range and **I)** in the frequency range >75 kHz. **J)** Percentage of step up USVs produced per animal in SHAM and HI groups in total frequency range, **K)** in the 50–75 kHz frequency range and **L)** in the frequency range >75 kHz. **M)** Percentage of down frequency modulation USVs produced per animal in SHAM and HI groups in total frequency range, **N)** in the 50–75 kHz frequency range and **O)** in the frequency range >75 kHz. **P)** Percentage of up frequency modulation USVs produced per animal in SHAM and HI groups in total frequency range, **Q)** in the 50–75 kHz frequency range and **R)** in the frequency range >75 kHz. Down fm = down frequency modulation, Up fm = Up frequency modulation. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data is presented as mean + SEM. Number of animals in each figure can be found in Table A.3.

and *down frequency modulation* calls in HI animals compared to SHAM littermates (A: p=0.054, B: p=0.056, Fig. 6A-B). Additionally, *step down* and *step up* calls showed an significantly increased median frequency (C: p=0.0004, D: p=0.015, Fig. 6C-D) in HI animals compared to SHAMs. The same trend towards an increased median frequency was observed for *down frequency modulation* calls (E: p=0.065, Fig. 6E). No changes were found in the characteristics of the other classes (data not shown). Our data indicate that not only the composition of USV classes is altered in HI animals, but also characteristics of specific USV classes like duration and median frequency are affected, which could add to the overall frequency and duration differences in USVs observed between HI and SHAM animals (Fig. 2H-M).

4. Discussion

In human neonates, HI brain injury is associated with cognitive impairments including social communication disabilities at 2 years of age [2,3]. In this study we examined whether HI brain injury induced different USV patterns and characteristics, as measures for social communication, in neonatal mice compared to SHAM-operated littermates.

Neonatal USV production is the most important form of infant-to-mother communication and is mostly used by the pups to emit distress calls [17]. The production of mouse USVs is associated with various neural circuits. Mice have a vocalization circuit consisting of a laryngeally connected M1 motor cortex linked to brainstem vocal motor neurons involved in controlling the vocal organ and to parts of the anterior striatum and thalamus [28]. Furthermore, different brain regions related to the limbic system such as the amygdala, striatum and hippocampus engage in USV production [29]. In this study we showed that HI brain injury induced neuronal loss in the cortex, hippocampus, thalamus, striatum and hypothalamus already early after injury. More MAP2 loss in the striatum correlated with less USVs produced and more MAP2 loss in the hippocampus correlated with USVs produced of a higher frequency respectively, indicating that injury in the striatum and hippocampus might contribute to the altered USV communication in HI injured mice. Therefore, the changes in vocalization repertoire observed in this study might directly reflect acute injury in the vocal circuitry following HI brain injury. Although neuronal injury is a major hallmark of hypoxia-ischemia (HI), activated microglia and astroglial scarring are also characteristics of HI brain injury in this model [30]. Therefore, future investigation of glial cell involvement in the pathophysiology of vocalization abnormalities following HI would contribute to further understanding of the development of these vocalization impairments after neonatal HI.

Our study shows that HI-injured mouse pups produce fewer USVs,

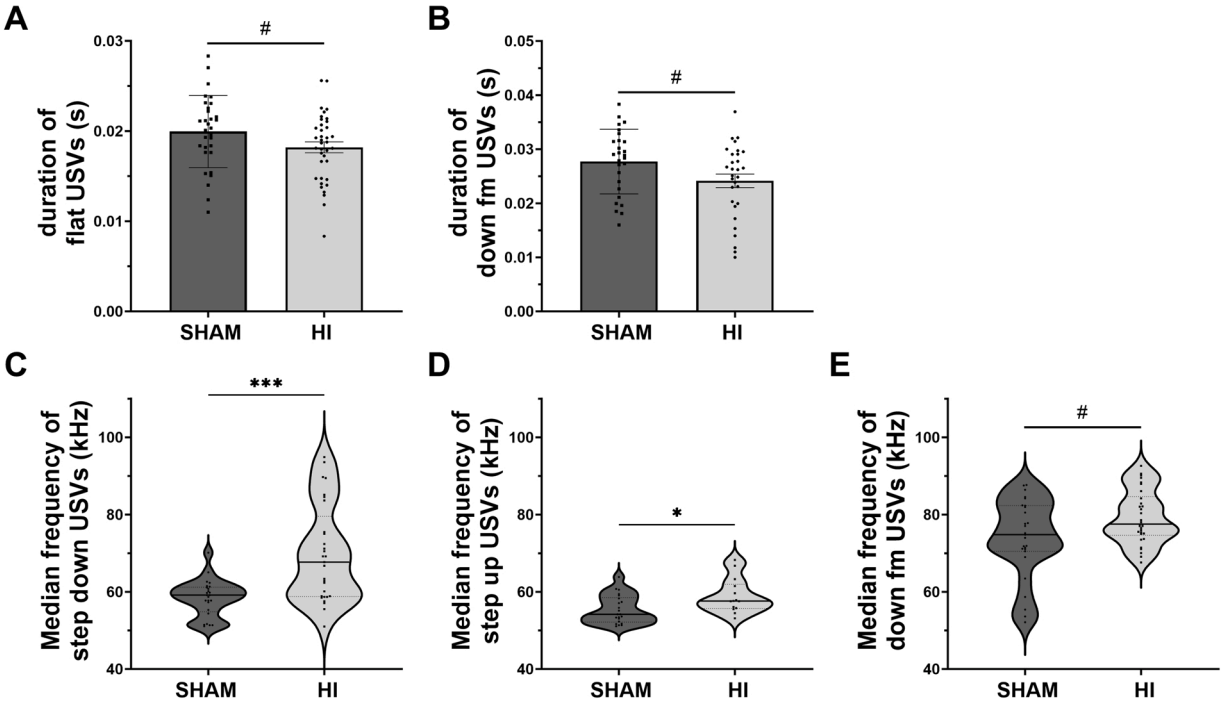


Fig. 6. USV classes with changed characteristics in HI animals compared to SHAM animals. A) Duration of flat calls per animal in seconds (s) in SHAM and HI groups at P10. Data presents mean + SEM. **B)** Duration of down frequency modulation calls per animal in seconds (s) in SHAM and HI groups at P10. Data presents mean + SEM. **C)** Distribution of median frequency in kHz of step down calls per animal in SHAM and HI groups. **D)** Distribution of median frequency in kHz of step up calls per animal in SHAM and HI groups. **E)** Distribution of median frequency in kHz of down frequency modulation calls per animal in SHAM and HI groups. *p=0.05, ***p=0.001. # significant trend: p=0.05–0.1. **C-E**: Data presents median + IQR. Down fm: down frequency modulation. Number of animals in each figure can be found in Table A.4.

particularly evident at P10. Our results align with a neonatal HI rat study in which a similar decrease in USVs was found in rat pups with HI brain injury [10], although this study did not assess other features of USVs. Conversely, our results contradict the study by Doran et al., who showed no differences in USV numbers between HI and controls early after HI at P11 and P13 [9]. Doran et al. induced HI injury at P7, two days earlier than our present study. The brain is rapidly developing in this time window which may affect both brain injury patterns and subsequent USV production, indicating that assessing USV patterns might be timely and dependent on postnatal age [9,21]. In line with Caruso et al., we show a sharp developmental decrease in USV production and number of animals that vocalize from P10 to P12, in both HI and SHAM animals [19]. Moreover, our data show that HI animals do not show a specific developmental delay in USV emission early after the insult, as HI animals do not catch up in USV emission at P11 or P12, rather the USV deviations observed in this study after HI seem acute and injury-specific.

This study is the first to show a comprehensive analysis of USVs in a rodent model of neonatal HI. We demonstrated that HI mice produce vocalizations at a higher median frequency than controls. A study by Wöhr et al. suggested that call features, e.g. frequency, depend on the pup's genotype and is independent of mother-pup vocal interaction [27]. This implies that call frequency could be an inherent biomarker for HI injury. While functional frequency categories are well characterized in rats, the significance of a shift to higher frequency USVs observed in injured neonatal mice remains unexplored [19]. In rodents, pup isolation calls are well defined to be of 30–60 kHz in rats and 30–90 kHz in mice [20]. Rat calls of different frequencies are believed to communicate distinct types of information regarding the emotional state of the emitter, with lower frequency calls communicating negative emotional states [20]. Thus, the loss of calls from a lower frequency in HI-injured mice might reflect impaired ability to make distress calls to the mother.

Although it is unclear whether the changes in vocalization patterns reflect auditory impairments or acute brain injury in HI animals, they might affect adequate mother-pup interaction [17,31]. Dams prefer to respond to longer calls over shorter ones and respond more strongly to calls with a frequency between 45 and 65 kHz than with higher frequency between 55 and 75 kHz [32]. Therefore, HI-injured pups might be at risk for receiving less proper maternal care which could subsequently affect proper development. It is of great interest to investigate mother-pup interactions in the neonatal HI model to demonstrate that the experimental HI paradigm does not only model the direct effects of cerebral injury in the offspring but also mimics indirect adverse environmental effects of reduced maternal care. This would model human newborns with HI brain injury being treated in the neonatal intensive care unit where proper attachment between critically ill newborn infants and parents is a daily challenge [33]. Future behavioral experiments, including auditory functioning tests, home cage observations and pup retrieval tests, should be performed to assess the important of auditory functioning and maternal care behavior in pup USV communication following HI [27,31,34].

Our study also showed that HI animals have a different USV class distribution than controls. An increased production of *short* USVs and a decreased production of *two syllable* USVs and *flat* USVs was evident in HI animals when compared to control littermates. Mouse pups have an innately ill-defined vocal signature early in life which develops into a more complex repertoire when reaching adulthood [19]. This development of vocal repertoire reflects the development of murine emotional state [20]. Frequency modulations, often found in two syllable USVs, are associated with social arousal and competence in adult mice [20]. The altered vocal repertoire in HI animals indicates a less competent and complex vocal phenotype early in life, resembled by increased production of simple short calls and decreased production of two syllable calls. This vocal phenotype potentially might reflect emotional deficits resulting from HI brain injury or damage to vocal circuitry in the brain or both [20]. Interestingly, we also found changes in USV characteristics such as duration and median frequency of *down frequency modulation* calls, *flat* calls and *multiple syllable* calls which might indicate that HI animals have altered speech production capabilities. The overall vocal phenotype of USVs observed in HI-injured animals closely resembles earlier described mouse autism spectrum disorder (ASD) USV vocal phenotypes [15,16]. Similarly to ASD animals, HI animals have been shown to display impaired social interaction and more anxious behavior compared to SHAM animals [35–37]. In the current study we focused exclusively on *neonatal* vocalizations. Whether early deficits in complexity of vocal phenotype as observed in HI animals translate into long-lasting social communication problems and/or emotional deficits should be focus of future behavioral studies. In this way, postnatal USV alterations could develop into an early biomarker tool to gain additional insights into long-lasting behavioral deficits in HI animals.

A limitation of this study is that part of the mouse pups produced less than 10 vocalizations. Low basal levels of USV production might be due to the use of the C57BL/6 strain mice that generally produce fewer calls than CD1 or FVB strains [19]. Additionally, because HI injury is induced at P9, recording of USVs can only start from P10 onwards, when the number of USVs already decline [19]. In total 9.6 % of SHAM animals and 23.8 % of HI animals produced less than 10 vocalizations at P10. It is crucial to acknowledge that the USV class analysis in the low vocalizing animals needs to be interpreted with caution. However, when class analysis was assessed excluding the low vocalizers (<10 USVs), overall differences in USV classes between SHAM and HI animals remained (data not shown).

In summary, in the HI mouse model we can detect early injury-related USV deficits which might serve as a tool to assess very early effects of new neuroprotective treatment strategies. Since the comprehensive USV analysis is most reliable at P10, USV assessment could be used for therapies applied within an early window after HI. In the future, USV patterns might also be used as an early marker for overall development in HI-injured animals. By using the neonatal HI model one could furthermore unravel the neural basis of USV development in healthy conditions and after early life adversity. USV emission appears to reflect vocal, social and emotional behavior and might be used as a versatile tool to study both language impairments as emotional dysregulation disorders. In conclusion, alterations in USV emission in HI-injured mice provides a paradigm for early HI-related communication deficits which might aid in gaining more insights into emotional and communication deficits in infants suffering from HI brain injury.

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Marijke Achterberg: Writing – review & editing, Resources. **Cora H Nijboer:** Writing – review & editing, Resources, Methodology. **Caroline G.M. de Theije:** Writing – review & editing, Resources, Methodology. **Eva C Hermans:** Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of Competing Interest

All authors have no conflicts of interest to disclose.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbr.2024.115113.

References

[1] J.J. Kurinczuk, M. White-Koning, N. Badawi, Epidemiology of neonatal encephalopathy and hypoxic-ischaemic encephalopathy, Early Hum. Dev. 86 (2010) 329–338, https://doi.org/10.1016/j.earlhumdev.2010.05.010.

[2] M. Martinez-Biarge, J. Diez-Sebastian, C.J. Wusthoff, S. Lawrence, A. Aloysius, M. A. Rutherford, F.M. Cowan, Feeding and communication impairments in infants with central grey matter lesions following perinatal hypoxic-ischaemic injury, Eur. J. Paediatr. Neurol. 16 (2012) 688–696, https://doi.org/10.1016/j.ejpn.2012.05.001.

[3] L.J. Millar, L. Shi, A. Hoerder-Suabedissen, Z. Molnár, Neonatal hypoxia ischaemia: mechanisms, models, and therapeutic challenges, Front. Cell Neurosci. 11 (2017), https://doi.org/10.3389/fncel.2017.00078.

[4] T.J. Robb, J. Tonks, A.P.C. Spencer, S. Jary, C.K. Whitfield, M. Thoresen, F. M. Cowan, E. Chakkarapani, Communication skills in children aged 6–8 years, without cerebral palsy cooled for neonatal hypoxic-ischemic encephalopathy, Sci. Rep. 12 (2022), https://doi.org/10.1038/s41598-022-21723-1.

[5] N. Marlow, S. Shankaran, E.E. Rogers, N.L. Maitre, C.D. Smyser, Neurological and developmental outcomes following neonatal encephalopathy treated with therapeutic hypothermia, Semin Fetal Neonatal Med. 26 (2021), https://doi.org/10.1016/j.siny.2021.101274.

[6] R. Lee-Kelland, S. Jary, J. Tonks, F.M. Cowan, M. Thoresen, E. Chakkarapani, School-age outcomes of children without cerebral palsy cooled for neonatal hypoxic-ischaemic encephalopathy in 2008-2010, Arch. Dis. Child Fetal Neonatal Ed. 105 (2020) F8–F13, https://doi.org/10.1136/archdischild-2018-316509.

[7] E.C. McGowan, M. Caskey, R. Tucker, B.R. Vohr, A randomized controlled trial of a neonatal intensive care unit language intervention for parents of preterm infants and 2-year language outcomes, J. Pediatr. 264 (2024), https://doi.org/10.1016/j.jpeds.2023.113740.

[8] J. Mayne, E.C. McGowan, A. Chiem, O. Nwanne, R. Tucker, B.R. Vohr, Randomised controlled trial of maternal infant-directed reading among hospitalised preterm infants, Acta Paediatr., Int. J. Paediatr. 111 (2022) 1921–1932, https://doi.org/10.1111/apa.16445.

[9] S.J. Doran, M. Jandzinski, A. Patrizz, C. Trammel, R. Sharmeen, A.A. Mamun, L. A. Capozzi, V.R. Venna, F. Liu, L.D. McCullough, Deficits in ultrasonic vocalization development and production following neonatal hypoxic ischemic insult, Behav. Brain Res. 369 (2019), https://doi.org/10.1016/j.bbr.2019.111931.

[10] D.M. Saucier, C.A. Ehresman, A.J. Keller, E. Armstrong, A. Elderkin, J.Y. Yager, Hypoxia ischemia affects ultrasonic vocalization in the neonatal rat, Behav. Brain Res. 190 (2008) 243–247, https://doi.org/10.1016/j.bbr.2008.02.025.

[11] M.L. Scattoni, J. Crawley, L. Ricceri, Ultrasonic vocalizations: a tool for behavioural phenotyping of mouse models of neurodevelopmental disorders, Neurosci. Biobehav Rev. 33 (2009) 508–515, https://doi.org/10.1016/j.neubiorev.2008.08.003.

[12] H. Gardener, D. Spiegelman, S.L. Buka, Perinatal and neonatal risk factors for autism: a comprehensive meta-analysis, Pediatrics 128 (2011) 344–355, https://doi.org/10.1542/peds.2010-1036.

[13] S.O. Nolan, S.L. Hodges, S.M. Condon, I.D.A. Muhammed, L.A. Tomac, M.S. Binder, C.D. Reynolds, J.N. Lugo, High seizure load during sensitive periods of development leads to broad shifts in ultrasonic vocalization behavior in neonatal male and female C57BL/6J mice, Epilepsy Behav. 95 (2019) 26–33, https://doi.org/10.1016/j.yebeh.2019.03.037.

[14] C.D. Reynolds, S.O. Nolan, J.L. Huebschman, S.L. Hodges, J.N. Lugo, Early-life status epilepticus acutely impacts select quantitative and qualitative features of neonatal vocalization behavior: spectrographic and temporal characterizations in C57BL/6 mice, Epilepsy Behav. 72 (2017) 58–62, https://doi.org/10.1016/j.yebeh.2017.04.025.

[15] M.S. Binder, J.N. Lugo, NS-Pten knockout mice show sex- and age-specific differences in ultrasonic vocalizations, Brain Behav. 7 (2017), https://doi.org/10.1002/brb3.857.

[16] A. Caruso, L. Ricceri, M.L. Scattoni, Ultrasonic vocalizations as a fundamental tool for early and adult behavioral phenotyping of Autism Spectrum Disorder rodent models, Neurosci. Biobehav Rev. 116 (2020) 31–43, https://doi.org/10.1016/j.neubiorev.2020.06.011.

[17] S. Capas-Peneda, Y. Saavedra Torres, J.B. Prins, I.A.S. Olsson, From mating to milk access: a review of reproductive vocal communication in mice, Front Behav. Neurosci. 16 (2022), https://doi.org/10.3389/fnbeh.2022.833168.

[18] N. Jourjine, M.L. Woolfolk, J.I. Sanguinetti-Scheck, J.E. Sabatini, S. McFadden, A. K. Lindholm, H.E. Hoekstra, Two pup vocalization types are genetically and functionally separable in deer mice, Curr. Biol. 33 (2023) 1237–1248.e4, https://doi.org/10.1016/j.cub.2023.02.045.

[19] A. Caruso, M.A. Marconi, M.L. Scattoni, L. Ricceri, Ultrasonic vocalizations in laboratory mice: strain, age, and sex differences, Genes Brain Behav. 21 (2022), https://doi.org/10.1111/gbb.12815.

[20] N. Simola, S. Granon, Ultrasonic vocalizations as a tool in studying emotional states in rodent models of social behavior and brain disease, Neuropharmacology 159 (2019), https://doi.org/10.1016/j.neuropharm.2018.11.008.

[21] S.J. Vannucci, S.A. Back, The Vannucci Model of Hypoxic-Ischemic Injury in the Neonatal Rodent: 40 years Later, Dev. Neurosci. 44 (2022) 186–193, https://doi.org/10.1159/000523990.

[22] P.S. Bobba, A. Malhotra, K.N. Sheth, S.N. Taylor, L.R. Ment, S. Payabvash, Brain injury patterns in hypoxic ischemic encephalopathy of term neonates, J. Neuroimaging 33 (2023) 79–84, https://doi.org/10.1111/jon.13052.

[23] J.L. Wisnowski, P. Wintermark, S.L. Bonifacio, C.D. Smyser, A.J. Barkovich, A. D. Edwards, L.S. de Vries, T.E. Inder, V. Chau, Neuroimaging in the term newborn with neonatal encephalopathy, Semin Fetal Neonatal Med 26 (2021), https://doi.org/10.1016/j.siny.2021.101304.

[24] L. Mai, H. Inada, N. Osumi, Whole-brain mapping of neuronal activity evoked by maternal separation in neonatal mice: an association with ultrasound vocalization, Neuropsychopharmacol. Rep. 43 (2023) 239–248, https://doi.org/10.1002/npr2.12337.

[25] A.H.O. Fonseca, G.M. Santana, G.M. Bosque Ortiz, S. Bampi, M.O. Dietrich, Analysis of ultrasonic vocalizations from mice using computer vision and machine learning, Elife 10 (2021), https://doi.org/10.7554/eLife.59161.

[26] N. Percie, V. Hurst, A.A. Id, S.A. Id, T.A. Id, M. Baker, W.J.B. Id, A.C. Id, I.C.C. Id, U.D. Id, M. Emerson, P.G. Id, S.T. Holgate, W.H. Id, N.A.K. Id, S.E. Lazic, K. Lidster, J.M. Id, M.M. Id, E.J.P. Id, O.H.P. Id, F.R. Id, P.R. Id, K.R. Id, E.S.S. Id, The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research, PLoS Biol. 18 (2020) 1–12, https://doi.org/10.1371/journal.pbio.3000410.

[27] M. Wöhr, M. Dahlhoff, E. Wolf, F. Holsboer, R.K.W. Schwarting, C.T. Wotjak, Effects of genetic background, gender, and early environmental factors on isolation-induced ultrasonic calling in mouse pups: An embryo-transfer study, Behav. Genet 38 (2008) 579–595, https://doi.org/10.1007/s10519-008-9221-4.

[28] G. Arriaga, E.P. Zhou, E.D. Jarvis, Of Mice, Birds, and Men: The Mouse Ultrasonic Song System Has Some Features Similar to Humans and Song-Learning Birds, PLoS One 7 (2012), https://doi.org/10.1371/journal.pone.0046610.

[29] L. Mai, H. Inada, N. Osumi, Whole-brain mapping of neuronal activity evoked by maternal separation in neonatal mice: An association with ultrasound vocalization, Neuropsychopharmacol. Rep. 43 (2023) 239–248, https://doi.org/10.1002/npr2.12337.

[30] P. Greco, G. Nencini, I. Piva, M. Scioscia, C.A. Volta, S. Spadaro, M. Neri, G. Bonaccorsi, F. Greco, I. Cocco, F. Sorrentino, F. D'Antonio, L. Nappi, Pathophysiology of hypoxic–ischemic encephalopathy: a review of the past and a view on the future, Acta Neurol. Belg. 120 (2020) 277–288, https://doi.org/10.1007/s13760-020-01308-3.

[31] M.M. McClure, S.W. Threlkeld, R.H. Fitch, Auditory processing and learning/memory following erythropoietin administration in neonatally hypoxic-ischemic injured rats, Brain Res. 1132 (2007) 203–209, https://doi.org/10.1016/j.brainres.2006.11.006.

[32] J.C. Smith, Responses of adult mice to models of infant calls, J. Comp. Physiol. Psychol. 90 (1976) 1105–1115, https://doi.org/10.1037/h0077287.

[33] M.H. Pennestri, H. Gaudreau, A.A. Bouvette-Turcot, E. Moss, V. Lecompte, L. Atkinson, J. Lydon, M. Steiner, M.J. Meaney, Attachment disorganization among children in Neonatal Intensive Care Unit: Preliminary results, Early Hum. Dev. 91 (2015) 601–606, https://doi.org/10.1016/j.earlhumdev.2015.07.005.

[34] O.V. Burenkova, A.A. Averkina, E.A. Aleksandrova, I.Y. Zarayskaya, Brief but enough: 45-min maternal separation elicits behavioral and physiological responses in neonatal mice and changes in dam maternal behavior, Physiol. Behav. 222 (2020), https://doi.org/10.1016/j.physbeh.2020.112877.

[35] B.S. Reinboth, C. Köster, H. Abberger, S. Prager, I. Bendix, U. Felderhoff-Müser, J. Herz, Endogenous hypothermic response to hypoxia reduces brain injury: Implications for modeling hypoxic-ischemic encephalopathy and therapeutic hypothermia in neonatal mice, Exp. Neurol. 283 (2016) 264–275, https://doi.org/10.1016/j.expneurol.2016.06.024.

[36] N. Dupré, A. Arabo, C. Orset, J. Maucotel, Y. Detroussel, M. Hauchecorne, B. J. Gonzalez, S. Marret, D. Vivien, P. Leroux, Neonatal cerebral hypoxia-ischemia in mice triggers age-dependent vascular effects and disabilities in adults; implication of tissue plasminogen activator (tPA), Exp. Neurol. 323 (2020), https://doi.org/10.1016/j.expneurol.2019.113087.

[37] V. Donega, C.T.J. van Velthoven, C.H. Nijboer, F. van Bel, M.J.H. Kas, A. Kavelaars, C.J. Heijnen, Intranasal mesenchymal stem cell treatment for neonatal brain damage: long-term cognitive and sensorimotor improvement, PLoS One 8 (1) (2013) 7, https://doi.org/10.1371/journal.pone.0051253.