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A pilot study: Auditory steady-state responses (ASSR) can be measured in human fetuses using fetal magnetoencephalography (fMEG)

Dorothea Niepel[1,2]*, Bhargavi Krishna[3], Eric R. Siegel[4], Rossitza Draganova[5,6], Hubert Preissl[7], Rathinaswamy B. Govindan[8], Hari Eswaran[1]

1 Ob/Gynecology Department, SARA Research Center, University of Arkansas for Medical Sciences, Little Rock, AR, United States of America, **2** Department of Internal Medicine I, University Hospital Tübingen, Tübingen, Germany, **3** Oak Ridge National Laboratory, Oak Ridge, Tennessee, United States of America, **4** Department of Biostatistics, University of Arkansas for Medical Sciences, Little Rock, AR, United States of America, **5** fMEG-Center, University of Tübingen, Tübingen, Germany, **6** Department of Neurology, University Clinic Essen, Motoric Labor, University of Duisburg-Essen, Essen, Germany, **7** fMEG-Center, German Center for Diabetes Research (DZD), Institute for Diabetes Research and Metabolic Diseases of the Helmholtz Center Munich at The University of Tübingen, Tübingen, Germany, **8** Division of Fetal and Transitional Medicine, Children's National Hospital, Washington, DC, United States of America

* dorothea.niepel@med.uni-tuebingen.de

Abstract

Background

Auditory steady-state responses (ASSRs) are ongoing evoked brain responses to continuous auditory stimuli that play a role for auditory processing of complex sounds and speech perception. Transient auditory event-related responses (AERRs) have previously been recorded using fetal magnetoencephalography (fMEG) but involve different neurological pathways. Previous studies in children and adults demonstrated that the cortical components of the ASSR are significantly affected by state of consciousness and by maturational changes in neonates and young infants. To our knowledge, this is the first study to investigate ASSRs in human fetuses.

Methods

47 fMEG sessions were conducted with 24 healthy pregnant women in three gestational age groups (30–32 weeks, 33–35 weeks and 36–39 weeks). The stimulation consisted of amplitude-modulated (AM) tones with a duration of one second, a carrier frequency (CF) of 500 Hz and a modulation frequency (MF) of 27 Hz or 42 Hz. Both tones were presented in a random order with equal probability adding up to 80–100 repetitions per tone. The ASSR across trials was quantified by assessing phase synchrony in the cortical signals at the stimulation frequency.

Results and conclusion

Ten out of 47 recordings were excluded due to technical problems or maternal movements. Analysis of the included 37 fetal recordings revealed a statistically significant response for

the phase coherence between trials for the MF of 27 Hz but not for 42 Hz. An exploratory subgroup analysis moreover suggested an advantage in detectability for fetal behavioral state 2F (active asleep) compared to 1F (quiet asleep) detected using fetal heart rate. In conclusion, this pilot study is the first description of a method to detect human ASSRs in fetuses. The findings warrant further investigations of the developing fetal brain.

Introduction

The auditory cortex undergoes significant maturational changes during the third trimester [1]. Studies of the auditory system are therefore also suited to examine basic principles of fetal brain maturation in general. Currently, two main methods for direct assessment of fetal brain function are established: fetal functional MRI [2] and fetal MEG (fMEG) [3]. Functional MRI measures cerebral blood flow associated with brain activity, and can be mapped with very good spatial, but not temporal, resolution. In terms of the fetal auditory system, a recent functional MRI study in fetuses at the beginning of the third trimester detected a response to auditory stimuli in the primary auditory cortex, indicating sound processing beyond the reflexive, subcortical level [2].

fMEG is completely noninvasive, silent, and has high temporal resolution. We and others have shown successful measurement of transient auditory event related responses (AERRs) [4,5]. The detection rate of AERRs is approximately 50% [4,5] but could be increased to 80% in serial measurements [6]. Fetal magnetocardiogram (fMCG) is a major by-product of fMEG data [7] that can be used to define fetal behavioral states [8]. Previous studies on AERRs to pure tones have shown that AERR latencies decrease with increasing gestational age [9] and increase in fetuses with clinical risk factors such as growth restriction [10] or maternal insulin intolerance [11]. In addition, AERRs have been used to further understand basic principles of fetal learning, such as sound discrimination [12], habituation [13] and numerosity discrimination [14]. AERRs were also recorded to white noise [15] and to simple syllables ('ba' and 'bi') [16].

ASSRs are another kind of auditory evoked brain response that play an important role in auditory processing of complex sounds and speech perception. To our knowledge, ASSRs had never been recorded in the human fetus. The major clinical applications are objective electrophysiological hearing tests in adults and young infants [17,18] and monitoring the level of consciousness in general anesthesia [19]. ASSRs are evoked by repetitive auditory stimuli or continuous tones with an amplitude and/or frequency modulation at a specific modulation frequency (MF) [20]. The cortical response is a continuous waveform at the MF that remains constant in amplitude and phase of its constituent frequency (Fourier) components over a long period of time [21].

AM tones are interesting because of their similarity to phonetic elements that are important for speech perception. Adult MEG studies have shown that stimulation with AM tones can simultaneously elicit two auditory event-related responses: AERRs and ASSRs [22,23]. While the present study focuses on fetal ASSRs, a different study at the collaborating fMEG center in Tübingen [24] investigated AERRs to AM tones very similar to the ones in this study. It was shown that the latencies of AERRs to the onset of AM tones decrease with increasing MF indicating, that the fetus can differentiate between different MFs [24].

The interaction between AERRs and ASSRs is complex. Early studies suggest that ASSRs at 40 Hz are a result of the superimposition of the middle latency components in AERRs [25].

However, not all phenomena observed for ASSRs can be explained with this theory [20] and adult MEG studies revealed that ASSRs and AERRs are localized to different parts of the auditory cortex [22,23]. The intrinsic oscillation hypothesis suggests that the ASSR at 40 Hz is generated by oscillation in thalamocortical networks that can synchronize higher auditory associative areas and non-auditory cortices [20,26]. The distinction between AERRs and ASSRs is important because it implies different functional applications [26]. In the setting of this study, new aspects of the maturation of the fetal auditory system, sound processing and language learning could be investigated. On the other hand, the fact that AERRs have already been recorded using fMEG indicates that the detection of auditory evoked potentials in general is feasible using fMEG.

In awake and relaxed adults, the amplitude of the ASSR decreases with increasing MF in a non-linear pattern [20,27]. It is considerably larger for MFs below approximately 50 Hz compared to the higher frequencies. Furthermore, an enhancement of the ASSR can be seen in the range around 40 Hz, and a second (2–5 fold smaller) enhancement occurs between 80 Hz–100 Hz [20,28]. The EEG background activity decreases with increasing frequency [20,27,29]. Taken together, these facts explain why the most favorable signal-to-noise ratio can be achieved using a MF of 40 Hz–50 Hz, followed by the range between 80 Hz–100 Hz [20,27,29].

It is widely accepted that the ASSR in the region around 40 Hz contains mostly activity from the auditory cortex with only a small proportion of signal coming from the brain stem, whereas the smaller ASSR around 80 Hz predominantly derives from the brain stem [20,26]. A common hypothesis is that the higher components of the central auditory nervous system may have less ability to sustain a rhythmic response at higher frequencies [20,26]. In line with this, a study using EEG in adults has been shown to localize the ASSR at 88 Hz predominantly to the brain stem with minor subsequent cortical activity. When using a MF of 39 Hz, brainstem activation remained, but a significant cortical activation also occurred [30].

The state of sleep or wakefulness has profound effects on human brain activity, particularly for higher cortical functions. The sleeping state attenuates the ASSR at 40 Hz (predominantly containing signal from the auditory cortex) by approximately 50% [19,20,29]. Moreover, the ASSR at 40 Hz decreased in correlation with delta and theta waves, which occur in deeper stages of sleep [31]. In contrast, the effect of sleep was much less pronounced in ASSRs to a MF above 70 Hz (predominantly containing signal from the brain stem) [20,29]. An advantage of studies of sleeping states is that the EEG background noise is generally lower [20,29]. The resulting most efficient MFs varied according to study design and carrier frequency (CF) [32]. For CFs of 1000 Hz or below, MFs in the range of 40 Hz or in the range of 90 Hz were reported to be most efficient [29].

Maturational effects also play a role for ASSR testing [20,33,34]. In neonates, ASSR studies are usually conducted in sedated or unsedated sleep for practical reasons [32,33] so that the results are most comparable to adult studies also conducted during sleep. The amplitude of the ASSR in young infants decreases monotonically with increasing MF [20,35,36]. In contrast to adult data [20,29], an enhancement of the ASSR in the range of 40 Hz has not been described [37–39]. Instead, the response at 40 Hz is only half the response at 10 Hz [20] and cannot reliably be detected in newborns [40]. It was hypothesized that the neonatal auditory cortex was still too immature to sustain a rhythmic response at higher rates [20,33] and that cortical responses may be present at even lower frequencies [37]. Previous studies using different methods to detect ASSRs in neonates and young infants propose an advantage for the higher MFs between 60 Hz–100 Hz that predominantly contain signal from the brain stem [39,41].

The main goal of the current study was to investigate if fetal ASSRs can be measured using fMEG. A thorough literature search did not reveal any previous publications of a successfully recorded ASSR in the human fetus using any technique. We aimed to develop a novel data

analysis method taking into account the specific characteristics of the ASSR and the challenges of the fMEG setting. Another focus was to compare the exemplary MFs of 27 Hz and 42 Hz in their suitability for detection of the fetal ASSR. Based on previous fMEG studies using AERRs and previous ASSR studies in neonates and adults, we also hypothesized that maturational effects and effects based on fetal behavioral states may be detectable.

Materials and methods

Participants

We recruited 24 healthy women with an uncomplicated singleton pregnancy. Three groups for gestational age were defined, and each woman could participate once per age group.

- **Group 1:** 30 weeks and zero days until 32 week and six days

- **Group 2:** 33 weeks and zero days until 35 week and six days

- **Group 3:** 36 weeks and zero days until 38 week and six days

　Seven women took part in all three fetal sessions, nine women in two fetal sessions, and eight women only participated in one fetal session. This resulted in a total number of 47 fetal sessions. A detailed sonography exam was performed in preparation for each session to confirm fetal wellbeing and to document the intrauterine position. The study was approved by the local institutional review board of the University of Arkansas for Medical Sciences (UAMS). Written consent was obtained from every subject.

Data acquisition

All data of this study were recorded using a designated fMEG device called the SARA system at the department of Obstetrics and Gynecology at the University of Arkansas for Medical Sciences (SARA = SQUID Array for Reproductive Assessment; CTF Systems Inc., Port Coquitlam, Canada). The surface of the sensor array is shaped in a concave manner to fit the gravid abdomen, and contains 151 primary magnetic SQUID sensors spaced approximately 3 cm apart with a noise level below 5 fT/sqrt (Hz). The sampling rate is 312.5 Hz [5,6]. The SARA system is installed in a magnetically shielded room (MSR) (Vakuumschmelze, Hanau, Germany). For the fetal recordings, the pregnant woman was seated in front of the SARA device and advised to lean forward into the sensor array [9]. Just prior to every session, a second quick sonography was performed with a portable device to determine the exact position of the fetal head. A fiducial system consisting of four coils was used to mark the position of the fetal head in relation to the SQUID sensors. Before each recording, these coils were activated in a specific frequency and the position of the head coil was calculated [5]. All women were instructed to be as still as possible during the recordings, but if a woman became uncomfortable on SARA, the measurements could always be stopped.

Stimulation

The auditory stimulation of the study was computed using the software Presentation (www. neuobs.com). To avoid magnetic signals correlated to the stimulation, the auditory stimulation was created by speakers outside of the MSR. The tones were then transmitted inside the MSR using an air-filled plastic tubing with an inflated plastic balloon attached to the distal end. During the recording, this balloon was placed between the upper part of the sensor array and the upper abdomen of the participating woman.

The recordings used in this study had a duration of ten minutes. They were conducted in the beginning of an fMEG session and followed by recordings from different studies up to a maximum duration of 30 minutes per session. Our stimulation paradigm uses AM tones with a modulation depth of 100%, a CF of 500 Hz and a MF of either 27 Hz or 42 Hz. MFs with a known high environmental noise level were avoided (e.g. the power line frequency/first sub-harmonic in the US at 60 Hz/30 Hz, in the EU at 50 Hz/25 Hz). The (relatively low) CF at 500 Hz has the advantage that it reportedly evokes a larger ASSR at 40 Hz than higher CFs do [42]. In addition, lower CFs are attenuated less by the maternal abdomen [43]. The volume of the stimulation was set to 94 dB at the surface of the maternal abdomen. Taking into account an estimated attenuation of 20–30 dB by the maternal abdomen [43,44], the volume reaching the fetus is expected to be between 64–74 dB. This value is well above hearing threshold but low enough, not to harm the fetus. Similar volumes had already been established in preceding fMEG studies [13, 14, 16].

The duration of each tone was set to be 1.0 second while the inter-stimulus interval varied randomly between 3.0 seconds and 3.5 seconds, resulting in a stimulus-onset asynchrony of 4.0–4.5 seconds. A randomized process selected a MF of either 27 Hz or 42 Hz with a 50 percent probability prior to every stimulation. This resulted in approximately 80–100 repetitions of each stimulus during a ten-minute recording. Fig 1 visualizes the algorithm as described.

This protocol is designed to simultaneously evoke AERRs and ASSRs as previously described for adults [22,23]. It is very similar to the protocol of a complementary study at the fMEG center in Tübingen that investigated AERR to the onset of AM tones and was conducted during a similar time span [24].

Data analysis

The preprocessing of the data was done using CTF DataEditor (version: 5.4.0, VSM Med Tech, Coquitlam, BC, Canada). We bandpass filtered the data between 1–50 Hz using the eighth order Butterworth filter with zero-phase distortion. A first-gradient noise reduction was added. The maternal and fetal cardiograms were removed using orthogonal projection [45]. Then, the data were divided into one second epochs just prior to the AM tone to define a baseline ('pre-trigger') and one second epochs during presentation of the AM tone ('post-trigger'). A post-trigger paired with its corresponding pre-trigger was defined as trial. S1 Fig shows fMEG data averaged over all trials for an exemplary recording.
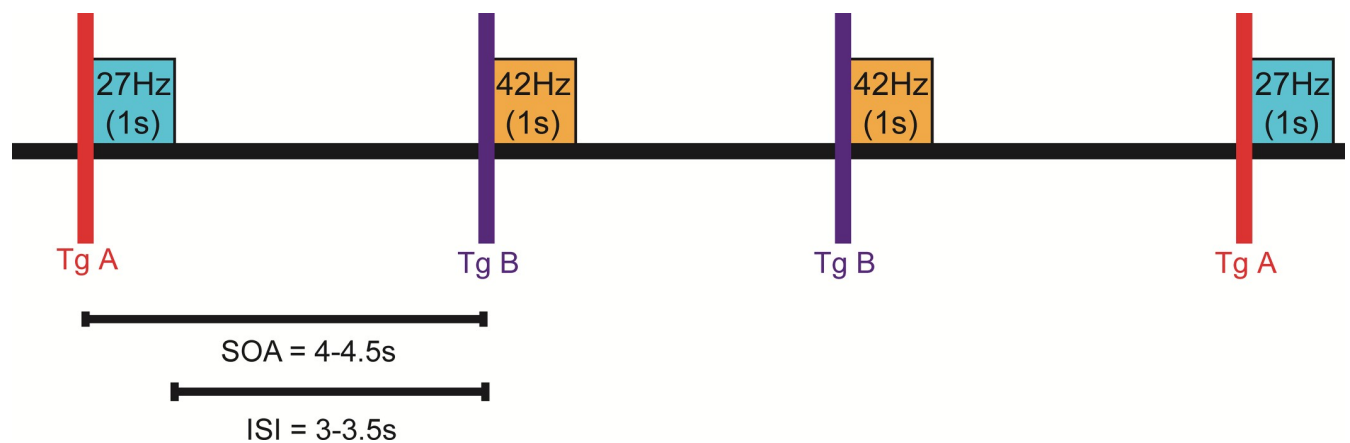

Fig 1. Stimulation paradigm. Illustration of the stimulation paradigm using AM tones with a MF of 27 Hz or 42 Hz in a random sequence. The specific frequency was encoded by a unique trigger value (Tg A or Tg B). The inter-stimulus interval (ISI) and the stimulus-onset asynchrony (SOA) are displayed.

The further data processing was done using MATLAB (version 8.0.0.783(R2012b), The MathWorks, Inc., Natick, MA, USA). Our data analysis utilizes the phase coherence aspect of the ASSR: If the ASSR stimulation elicited a response, the phase of the ASSR at the MF will be synchronous or coherent among all post-trigger segments. The values of phase coherence were calculated for each sensor as follows: The post-trigger and the pre-trigger data were considered for every trial and every sensor and their means were subtracted. A Fourier transform was calculated for MEG in each sensor for each trial and the phase at the investigated MF was calculated using the arctangent transform of the Fourier coefficients. To this, the synchronization index was calculated with a first mode of Fourier transform of the phase. The synchronization index of pre-trigger data was then subtracted from that of the post-trigger data. The rationale of choosing one-second MEG in the pre-stimulus period was to estimate the phase coherence spectrum with the same frequency resolution as that of the MEG in the post-stimulus period. The assumption behind this is that the ASSR lasts only for one second and any response elicited by the previous stimulus will not be present in the pre-stimulus period of the current stimulus. These phase coherence values were color-coded and plotted as contour maps of the SARA sensor array coordinates. The location of the head coil and the center of activity, a virtual integration of coherence values and their location on the sensor array, were also displayed. In a separate approach, power spectral density was estimated via the periodogram using a using a Hamming window. The amplitude values at the investigated MF for pre-trigger, post-trigger and post-trigger minus pre-trigger difference were also plotted on contour maps. Controls were created within the stimulation recordings by interchanging the markers of the two MFs. That way, the ASSR at 27 Hz was measured during 42 Hz stimulation and vice versa.

For the analysis of fetal behavioral states, the maternal heartbeat was removed using orthogonal projection [45] or a Hilbert Transform algorithm [46]. First, the fetal actogram, that displays fetal gross movement, was calculated by tracking the changes in the R-wave amplitude measured from all of the SARA sensors. Then, the cardiogram, that visualizes the fetal heart rate over time, was plotted in a CTG-like fashion. The combined fetal actocardiogram was used to determine the fetal behavioral states by visual inspection. The criteria was based on the Nijhuis criteria [47] and modified according to previous fMEG studies [48,49] as summarized in S1 Table. In fetuses 32 weeks or older, behavioral states were divided in four groups: 1F (passive sleep), 2F (active sleep), 3F (quiet awake) and 4F (active awake). In fetuses with a gestational age below 32 weeks, behavioral states could only be distinguished between 'passive state' and 'active state'. A fetal behavioral state could be identified only if an observed pattern lasted for a minimum of three minutes. This method was well-established at the fMEG center in Tübingen at the time of our study [8,50–52] and all of our results were verified by an expert from the fMEG center in Tübingen.

Our statistical analysis was performed using SAS (version 9.4., The SAS Institute, Cary, NC). For each recording, the pre-trigger and post-trigger values of the phase coherence were considered for all sensors within a 10 cm radius around the head coil. Data were analyzed using a mixed-models repeated-measures approach. In the mixed model for the primary analysis, the "subject" was the study participant during a particular session, the within-subjects fixed effect was 'type of stimulus' (stimulation or control) delivered during the session, the random effect was the participant considered over all of her sessions, and the outcome variable was the post-trigger minus pre-trigger difference under each type of stimulus. For purposes of expressing the post-trigger minus pre-trigger differences as standardized values, the mixed model's SD estimate was calculated as the square root of the sum of the mixed model's variance components. In the mixed models for the subgroup analyses, a second fixed effect was added for 'gestational age group' or 'fetal behavioral state', respectively. In both the primary and subgroup analyses, 90% confidence intervals were calculated for the post-trigger minus pre-trigger

differences. A one-sided hypothesis test with an alpha = 0.05 significance level was then employed for the positive direction of the post-trigger minus pre-trigger difference. This correlates with our main hypothesis is that the fetal ASSR can be detected using fMEG by significantly increased phase coherence at the MF during the post-trigger data. The high noise scenario of the fMEG setting has been considered for the selection of the confidence intervals and significance levels (alpha). In a different context, the same test was also calculated for the negative direction. This accounts for the theoretical possibility that the phase coherence may be larger at baseline than during stimulation and would tend to refute our main hypothesis. To evaluate individual responses, we applied a one-sided one-sample t-test at alpha = 0.05 to classify each subject's response, and continued the practice of investigating significant positive as well as significant negative responses. Because both the stimulus and the control condition were delivered to the same subjects, the effect of stimulus vs control on the proportions of significantly positive, significantly negative, and insignificant responses was evaluated using the chi-square test of symmetry for paired data.

Results

Number of recordings

A total number of 47 recordings was conducted within this study. Ten out of the 47 recordings were excluded: three due to maternal discomfort or extensive movements and seven due to technical problems (wrong or missing head coil information, wrong or missing stimulation or problems in saving the recording). Table 1 provides an overview of the distribution of recordings between gestational age groups.

Group analysis

Fig 2 visualizes the results of the overall group analysis for the post-trigger minus pre-trigger phase-coherence values using a MF of 27 Hz or 42 Hz. The exact statistical values are provided in S2 and S3 Tables.

A statistically significant positive result of the post-trigger minus pre-trigger difference means that the level of phase coherence is significantly higher in the post-trigger data (during stimulation) than in the pre-trigger data (prior to stimulation). If the post-trigger and the pre-trigger data are not significantly different, the result will be close to zero. In theory, a statistically significant negative result means that the pre-trigger data was significantly more coherent at the MF.

The most important finding in this study is that the analysis of the 27 Hz ASSRs has a significantly positive result (standardized value = +0.290 SDs for the post-trigger minus pre-trigger difference; one-sided P = 0.0334) for the stimulation recordings (n = 37, pink line in Fig 2A). In other words, this means that the signal at 27 Hz is significantly more coherent during stimulation with 27 Hz ('post-trigger') than at baseline prior to stimulation ('pre-trigger'). These findings are further confirmed by the fact that additional controls (n = 37; brown line in Fig 2A) were not significantly different from zero (standardized value = +0.028 SDs; one-sided

Table 1. Distribution between gestational age groups.

	Age Group 1 (30w0d-32w6d)	Age Group 2 (33w0d-35w6d)	Age Group 3 (36w0d-38w6d)	All Recordings (30w0d-38w6d)
Total	15	16	16	47
(included/excluded)	11/4	14/2	12/4	37/10

All recordings (total, included, excluded) of this study separated by age group (w = week, d = days).

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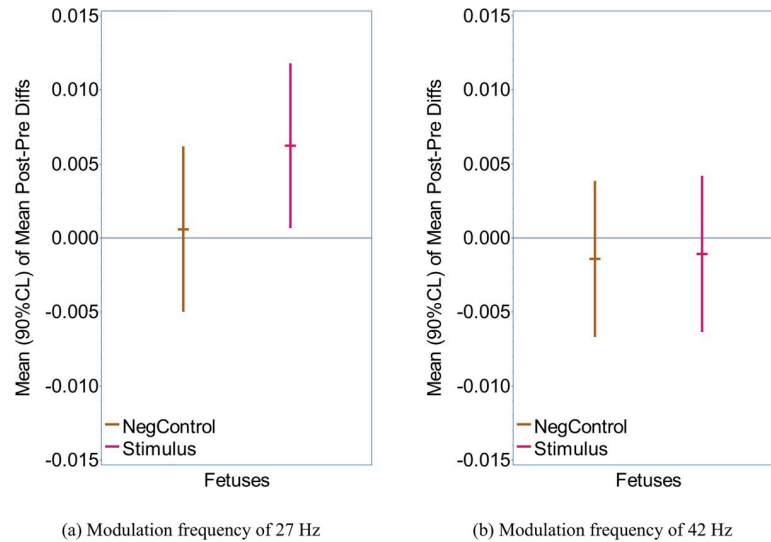(a) Modulation frequency of 27 Hz   (b) Modulation frequency of 42 Hz

Fig 2. Overall group analysis. Summary of the statistical group analysis of all recordings using a MF of a) 27 Hz, b) 42 Hz. The mean of the post-trigger minus pre-trigger differences of phase coherence is considered for the sensors within a 10-cm radius to the head coil. The error bars represent 90% confidence intervals. For each MF, stimulation recordings ('Stimulus') and control recordings ('NegControl') are displayed.

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P = 0.429). The standardized value under the Stimulus condition, though small at 0.290 SDs, was 10 times larger than the value of 0.028 SDs that it had under the control condition. In contrast, the overall analysis of the ASSR at 42 Hz did not reveal any statistically significant results. The post-trigger minus pre-trigger differences had standardized values of -0.059 SDs (one-sided P = 0.6312) for the stimulus condition, and -0.065 SDs (one-sided P = 0.6699) for the control condition (n = 37; Fig 2B).

Individual responses

Within the stimulation recordings using the MF of 27 Hz, nine out of 37 (24.3%) recordings had a statistically significant positive response, five (13.5%) had a statistically significant negative response and 23 (62.2%) had a response that was not significantly different from zero. In the control condition, six out of 37 recordings (16.2%) had a positive response, three (8.1%) had a negative response and 28 (75.7%) had responses that were not significantly different from zero. The paired-data comparison of proportions between the stimulus and control conditions yielded a test-of-symmetry chi-square of 1.79 with DF = 3 (P = 0.618).

Comparison of amplitude and phase-coherence aspect of the ASSR

The amplitude aspect of the raw data can be visualized in the power spectrum. Data from an exemplary recording is shown in Fig 3.

The background activity is higher in the lower frequencies and varies in a non-continuous manner between neighboring frequencies. A clear difference between post-trigger and pre-trigger data cannot be distinguished. However, this is not surprising given that the amplitude of the fetal ASSR may be smaller than other physiological and environmental signals by multiple orders of power. The phase aspect and the phase coherence between trials on the other hand should be less dependent on the strength or amplitude of a signal.

In the next step, we computed contour maps of the SARA sensor array in order to visualize the spatial distribution of the signals investigated. Fig 4 shows the contour maps of two
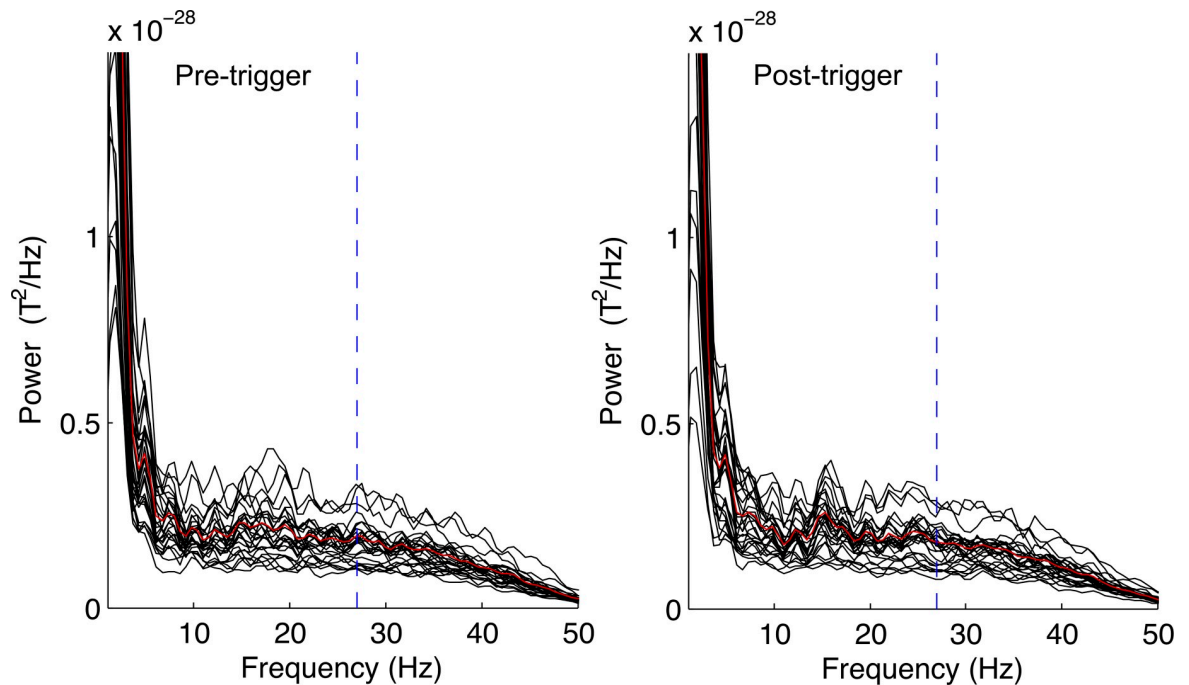
Fig 3. Power spectrum. Power spectrum of an exemplary recording at 35 weeks of gestation during stimulation ('post-trigger') and prior to stimulation ('pre-trigger'). Calculation for all sensors within 10 cm of the head coil throughout all trials using a high pass filter of 1 Hz and a low pass filter of 50 Hz. Each black line represents an individual sensor, the red lines show the average. The MF at 27 Hz is marked by a blue, dashed line.

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recordings using amplitude and phase-coherence values at a MF of 27 Hz for pre-trigger, post-trigger, and post-trigger minus pre-trigger data.

In the 27 Hz phase-coherence maps, an activation close to the head coil appeared to be present in a proportion of the post-trigger and the post-trigger minus pre-trigger (see subfigure (d), (f), (j) and (l)). Although the origin of this activation cannot be objectively determined, an ASSR would be expected to cause an increased phase coherence close to the head coil during stimulation (post-trigger) compared to baseline (pre-trigger). Therefore, it appears possible that the observed signal may represent fetal ASSR, although other causes including random events cannot be ruled out.

It is also remarkable that the pre-trigger maps in the amplitude approach (see subfigures (a) and (g)) each seem to demonstrate a specific pattern that is very similar in the corresponding post-trigger map (see subfigures (c) and (i). These patterns may represent specific physiological background activity. For example, multiple recordings exhibited an activation in the lower left and lower right sensors similar to the pattern in subfigure (a) and (c). This activation may be due to muscle activity in the maternal legs as a result of the sitting position on SARA. The activity in the center right side of subfigure (a), (c), (g) and (i) could potentially be attributed to fMCG residuals. The phase-coherence maps of the same recordings, on the other hand, show no specific common pattern in the pre-trigger data (see subfigure (b) and (h)). Instead, the baseline activity appears to be randomly distributed throughout the sensor array.
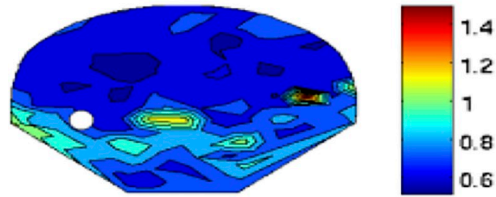
Effects of gestational age

The results of an exploratory subgroup analysis depending on fetal age groups are summarized in Fig 5. The exact statistical values are provided in S4 Table and the sample size of the subgroups can be reviewed in Table 1.
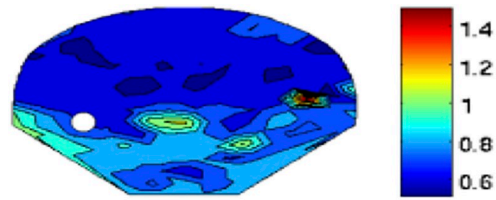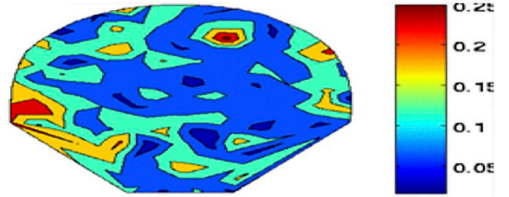
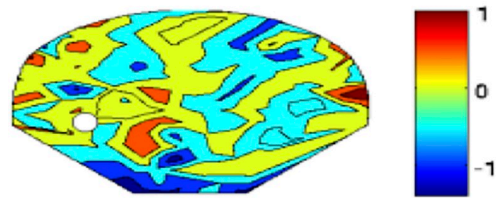(a) Rec. A 30w2d pre-trigger power spectrum
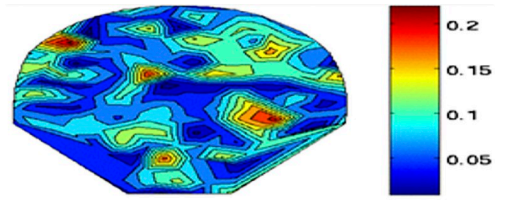
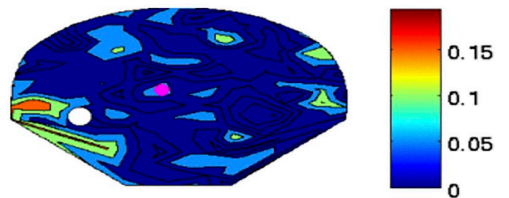(b) Rec. A 30w2d pre-trigger phase coherence

(c) Rec. A 30w2d post-trigger power spectrum

(d) Rec. A 30w2d post-trigger phase coherence

(e) Rec. A 30w2d post-trigger minus pre-trigger power spectrum

(f) Rec. A 30w2d post-trigger minus pre-trigger phase coherence

(g) Rec. B 35w0d pre-trigger power spectrum

(h) Rec. B 35w0d pre-trigger phase coherence

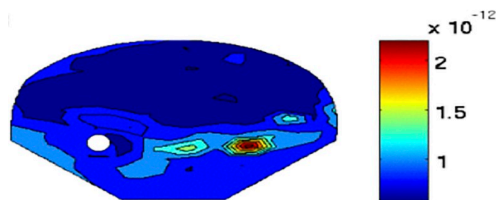(i) Rec. B 35w0d post-trigger power spectrum

(j) Rec. B 35w0d post-trigger phase coherence

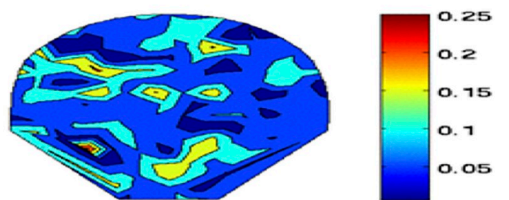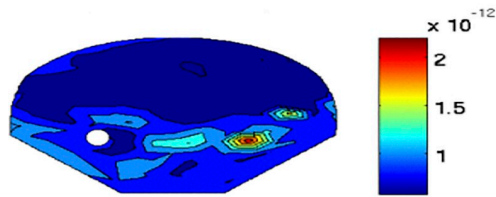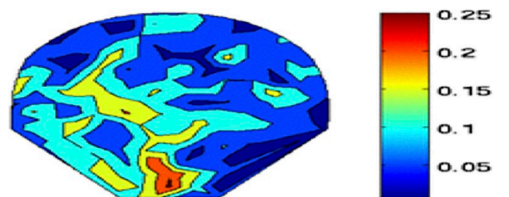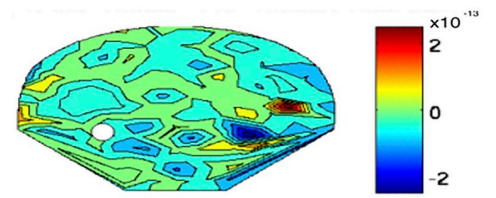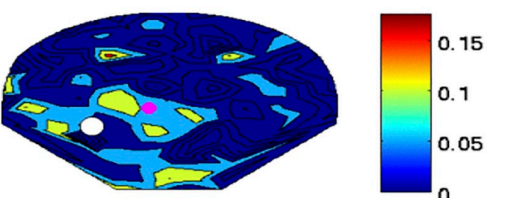(k) Rec. B 35w0d post-trigger minus pre-trigger power spectrum

(l) Rec. B 35w0d post-trigger minus pre-trigger phase coherence

Fig 4. Contour maps. Comparison between the amplitude approach (subfigures labelled 'power spectrum') and the phase coherence approach (subfigures labelled 'phase coherence') visualized on contour maps of the SARA sensor array. Subfigure (a)–(f) and subfigure (g)–(l) have been computed from two from two different subjects ('Rec. A' and 'Rec. B'). Gestational age is also displayed (w = weeks, d = days). The white circles represent the head coil, the pink circles in subfigure (f) and (l) represent the center of activity.

For the different age groups we did not observe a significant effect of the stimulus or control condition.

Effects of fetal behavioral state

The distribution of the fetal behavioral states among all recordings of this study is demonstrated in Table 2. It is important to consider that the sample size of the various behavioral state subgroups varies significantly (n = 1–15). We thus decided to perform an exploratory analysis only considering the two largest subgroups (2F with n = 15 and 1F with n = 6). All other subgroups had a very small sample size (n = 1–4) and were summarized as "others". The results are summarized in Fig 6 and the exact statistical values are provided in S5 Table.

The most important finding is that the stimulation recordings have a significantly positive result (one-sided P = 0.0358) in fetal behavioral-state group 2F, the largest subgroup (n = 15). It is also remarkable that the recordings in group 1F (n = 6) remain close to zero. These findings are further validated by the fact that none of the control groups were significantly different from zero.

Discussion

The results of our overall group analysis show a statistically significant response of the stimulation recordings using a MF of 27 Hz while recordings in control condition were not significantly different from zero (Fig 2). Out of all our statistical analyses, this group has the largest

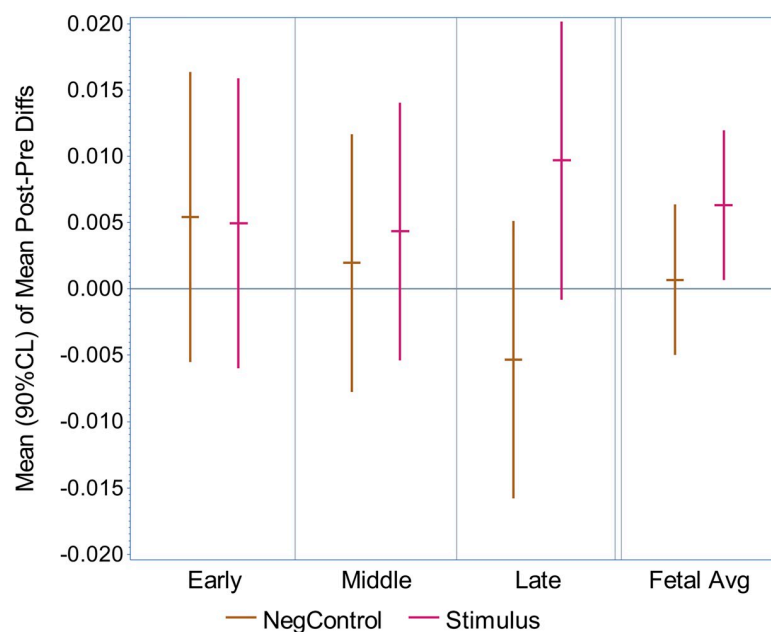**Fig 5. Subgroup analysis of gestational age.** Summary of the statistical subgroup analysis depending on gestational age groups using a MF of 27 Hz. The error bars represent the 90% confidence intervals. Gestational age group 1 ('early'), group 2 ('middle') and group 3 ('late') and their average ('Fetal Avg') are shown. Stimulation recordings ('Stimulus') and control recordings ('NegControl') are displayed.

Table 2. Distribution between behavioral states.

	Age Group 1	Age Group 2	Age Group 3	All Age Groups
Active State	4	n/a	n/a	4
Passive State	2	n/a	n/a	2
Active State → Passive State	1	n/a	n/a	1
Passive State → Active State	1	n/a	n/a	1
State 1F	1	4	1	6
State 2F	2	7	6	15
State 4F	0	0	1	1
State 1F → 2F	0	3	0	3
State 2F → 1F	0	0	3	3
State 3F → 2F	0	0	1	1
All Recordings	11	14	12	37

Distribution of the fetal behavioral states among the different gestational age groups. Recordings < 32 weeks 1 day of gestation are plotted separately in the top rows because of the different classification system of fetal behavioral states. Behavioral state groups that did not occur within this study are not displayed.

sample size and hence the largest statistical power. The fact that negative responses don't occur in the overall group analysis or in the subgroup analyses (Figs 5 and 6) further supports these findings. Of note, the AM tones used for simulation do not contain frequency contributions at the MF but only at the CF and at 'side bins' at the CF ± the MF (in our case at 500 Hz +/− 27 Hz) [53] so that artifacts from the auditory stimulation itself are highly unlikely. The data analysis was moreover completely automated so that the results are truly objective. For all those reasons, it appears likely that the recorded signal reflects a fetal ASSR.
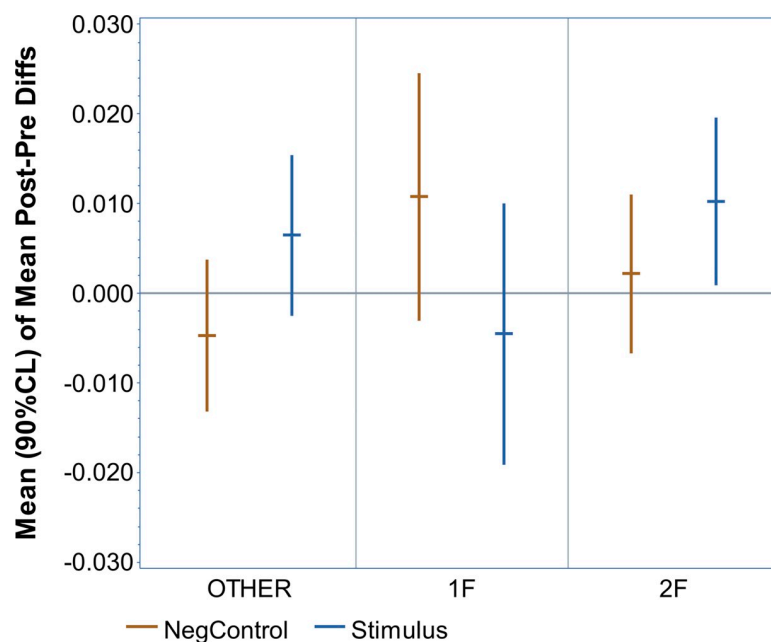
Fig 6. Subgroup analysis of fetal behavioral state. Summary of the statistical subgroup analysis depending on fetal behavioral-state groups using a MF of 27 Hz. The error bars represent the 90% confidence intervals. The most frequent fetal states '2F' (active asleep) and '1F' (quiet asleep) are displayed, all less common fetal states were summarized as 'OTHER'. Stimulation recordings ('Stimulus') and control recordings ('NegControl') are displayed.

One limitation is, that the control condition of the 27 Hz analysis was calculated during 42 Hz stimulation and vice versa for practical reasons. Future studies should consider controls with white noise, a non-modulated carrier tone or the same amplitude modulated tone used for stimulation at a volume below hearing threshold.

An important focus of the present study was to develop a method for the data analysis of ASSR testing in the fetal setting. Previous adult studies have shown that data analysis using the phase coherence aspect or the amplitude aspect of an ASSR provide similar results in terms of sensitivity and specificity [53]. However, it appears plausible that this is different when using fMEG. As demonstrated in the power spectrum and in the contour maps of the SARA sensor array, relevant physiological background activity at 27 Hz occurs in the amplitude approach but not when using phase coherence (see Figs 3 and 4). Given that the fetal brain signal is expected to be very small, the amplitude of physiological background activity at 27 Hz may be larger than the investigated ASSR by multiple orders of power. The phase coherence approach on the other hand requires the signal to be correlated to the stimulation in order to be synchronous throughout all trials. Even if environmental signals can change the phase in some trials, the overall effect will be less severe, because the recorded phase in a trial does not reflect the total strength of the signal.

Another main finding of this study is, that a MF at 27 Hz appears suitable to evoke a detectable fetal ASSR, but a MF of 42 Hz does not. A number of physiological factors can in theory explain why the signal of the ASSR is expected to be larger at 27 Hz. A large study in neonates described that although the amplitude of the neonatal ASSR was largest at 25 Hz (the lowest MF investigated) and decreases with increasing MF, this effect was offset by the longer time needed to evoke a response. Parameters such as 'time to criterion' and 'signal strength' measured by magnitude-squared coherence were more favorable in the middle and high MFs (41 Hz–88 Hz) [35]. Remarkably, this advantage was not significant for lowest CF tested at 500 Hz, because the difference in amplitude was more pronounced in the lower CFs [35]. This is particularly interesting because the present study is also using a CF of 500 Hz. In the fMEG setting it also has to be considered that the fetal brain signal is much smaller and that the strength of the fetal ASSR may be close to the detection limit. Hence, it appears possible that the higher amplitudes in the lower MFs are necessary to enable detection when using fMEG.

The enhancement of the ASSR around 40 Hz (mostly deriving from the cortex) in adult data has also not been described in studies on neonates or infants [37–39]. An early study suggests that, based on the superimposition theory for the generation of ASSRs, the increased latencies of AERRs in young children explain why an superimposition of the peaks is not possible at 40 Hz but can occur, to some extent, for MFs between 20–30 Hz. Accordant with that theory, the authors observed a small increment in the amplitude of the ASSR of young children between 20 Hz and 30 Hz [37].

The fMEG study that used a stimulation protocol similar to the one in this study to investigate AERRs to the onset of AM tones reported that the mean latencies were shortest for the MF of 27 Hz compared to an MF of 2 Hz, 4 Hz, 8 Hz, 42 Hz, 78 Hz and 91 Hz [24]. Moreover, and in line with the findings from our study, the detection rates for AERRs were highest at 86% for a MF of 27 Hz compared to 50% for a MF of 42 Hz [24] and approximately 50% in previous studies using pure tones [5]. The authors suggest, that the increased detection rates with a MF 27 may be due to a better transmission by bone conduction, the main way of fetal hearing in utero [24].

The distribution of baseline activity should also be considered. In neonatal EEG data, the background activity decreases with increasing MF [35,36]. In the fMEG setting, additional physiological and environmental signals are present (e.g. maternal MCG: 1–1.5 Hz, fMCG: 2–3 Hz, muscle contractions: 20–300 Hz [54], power line frequency in the US/EU: 60 Hz/50

Hz) and the resulting background activity varies in a noncontinuous pattern that can be subject to the local setup. For example, a relevant number of recordings in our study exhibit a visible continuous 20 Hz signal possibly connected to noise from a technical device (e.g. the camera frame rate). The first harmonic of this signal at approximately 40 Hz could in theory have distorted our 42 Hz measurements. These examples demonstrate, that each MF has to be evaluated individually in the fMEG setting.

The subgroup analysis based on fetal behavioral states seems to suggest a preferable detectability of the ASSR at 27 Hz in fetal state 2F (active asleep) compared to state 1F (quiet asleep). It has to be considered that this data are very limited due to the small sample size of the subgroups. In previous adult studies, the cortical component of the ASSR at and below 40 Hz decreases in the state of sleep [19,25,29] and even more in deeper stages of sleep [31]. In line with our observations, an early fetal study that observed changes in heart-rate pattern and fetal movement following auditory stimulation reported more frequent responses in fetal state 2F compared to fetal state 1F [55]. A previous fMEG study also reported that the majority of all fetuses with a detectable AERRs were in awake states as determined by sonography [9]. More recently, another fMEG study demonstrated that the latencies of AERRs is significantly shorter in active sleep (2F) compared to passive sleep (1F) [50]. In summary, our data are limited but the fact that our observations are in agreement with previous studies make a correlation between fetal state and fetal ASSR more likely. Future studies should therefore investigate the fetal ASSR in the context of the fetal state.

The exploratory subgroup analysis dependent on fetal gestational age showed a tendency towards a positive result in age group 3 (one sided P = 0.065) but no statistically significant responses. In theory, this trend supports the hypotheses, that the fetal auditory cortex in the majority of the fetuses in age group one and two may still be too immature to generate an ASSR to a MF of 27 Hz. However, the data are limited because the sample size of the subgroups is relatively small (n = 11–14). Thus, it is not possible to draw a final conclusion regarding potential maturational effects based on this data alone. Previous investigations of fetal AERRs to the onset of AM tones showed a change in latencies depending on gestational age for the MF of 4 Hz. In line with our study, the results were not significant when using a MF of 27 Hz but the sample size of this group was also only half the size of the 4 Hz group ($n^{(27\ Hz)} = 14$ compared to $n^{(4\ Hz)} = 28$) [24]. Further studies are needed in order to evaluate potential maturational effects of the fetal ASSR. For example, the detectability of the fetal ASSR at 27 Hz and the presence of detectable maturational changes may be dependent on fetal behavioral state. It could thus be promising to conduct another fMEG study in a larger number of fetuses selectively in fetal state 2F or even in the in less frequent the awake fetal states 3F and 4F. Another interesting direction would be to correlate our fetal findings with neonatal data. In this context, a previous fMEG/MEG study could demonstrate maturational changes of latencies of the AERR in serial studies of fetuses and neonates combined [9]. Further investigations of the neonatal ASSRs to MFs at 40 Hz and below could not only to validate our findings in the fetal setting but could also reveal other aspects of maturational changes in auditory perception.

One of the main challenges of the present study was a low signal-to-noise ratio. The ASSRs could be detected in a group analysis, but not in a significant proportion of the individual recordings. One limitation is that all sensors within a 10 cm radius around the head coil (usually approx. 25 sensors) were included in the analysis. In fetal AERR studies, the sensors with the largest amplitude (usually approx. five sensors) are determined by visual inspection and used for further analysis. Although the connection between AERRs and ASSRs is complex and ASSRs may not be produced in the exact same neurological pathways, it is likely that the ASSR is also only present in a few sensors. If we average over all sensors within a 10 cm distance to the head coil but only a few sensors contain an ASSR, the recorded average response decreases

with the number of channels without an ASSR. On the other hand, the ASSR may not be detectable if the signal is more than 10 cm away from the head coil. It would be interesting to select the sensors containing the largest AERR and to use them for an analysis of ASSRs in future studies. A number of more general adjustments to optimize the signal-to-noise ratio in ASSR testing have also been studied in adult data [27,53]. It is for example known, that a higher volume of the stimulus increases the ASSR evoked [20,25,27]. The present study is using a relatively low volume of 94 dB at the surface of the maternal abdomen. Previous studies using the SARA device have been using a volume of up to 120 dB [5,6]. The signal-to-noise ratio could also be improved by increasing the number of trials investigated. For example, the recordings of this study were only 10 minutes long but could be increased to up to 30 minutes, the maximum duration of a fMEG session on our setup. Other possibilities to increase the number of trials include decreasing the ISI between trials or also just using one MF per recording instead of two alternating MFs.

Conclusion

This work provides the first description of a method to detect fetal ASSRs using fMEG. Our results suggest that a MF of 27 Hz is suitable to evoke a recordable fetal ASSR. With regards to the data analysis, we illustrated that the phase coherence approach has notable advantages over the amplitude approach in the fMEG setting. A limitation of this study is, that the ASSR could only be objectively recorded in a group analysis. A preliminary sub-analysis of our data suggests an advantage for fetal behavioral state 2F (active asleep) compared to fetal behavioral state 1F (quiet asleep) but could not detect significant changes based on gestational age. Future studies are needed to confirm the findings from this pilot study, to improve the signal-to-noise-ratio and to increase the detection rate for individual recordings. This could be archived by technical adjustments (e.g. increased duration of the recordings, increased volume, use of only one MF per recording), systematic improvements (e.g. identification of a MF better suitable than 27 Hz, planning of recordings during a behavioral state known to be preferable) or more general changes (e.g. development of a new method for the data analysis, combination with the analysis of AERRs). A long-term goal is to establish a method for non-invasive fetal brain surveillance, to detect imminent brain damage early and to put targeted preventive or therapeutic measures into place.

Supporting information

S1 Fig. fMEG raw data. This figure shows the fMEG data of an exemplary recording called 'SS27_35w0d'. A bandpass filter was applied at 1–50 Hz, maternal and fetal cardiograms were removed, and the data was averaged over all trails. The names on the y-axis represent individual sensors, the x-axis shows time in relation to the 'Trigger A' marker. Trigger A (red line) and pre-trigger or post-trigger data (blue, dashed line) are indicated. The subfigure in the right upper corner shows all selected sensors (blue stars) within a 10 cm radius from the head coil (red square) on the SARA sensor array. Sensors that were not selected for analysis are represented by gray dots.
(TIFF)

S1 Table. Criteria for the definition of fetal behavioral states. This table summarizes the criteria used in this study to define fetal behavioral states based on a visual analysis of the fetal actocardiogram.
(DOCX)

S2 Table. Overall group analysis for the MF of 27 Hz. This table provides the exact values of the statistical analysis including the calculated standard error and p-values.
(DOCX)

S3 Table. Overall group analysis for the MF of 42 Hz. This table provides the exact values of the statistical analysis including the calculated standard error and p-values.
(DOCX)

S4 Table. Subgroup analysis according to gestational age group for the MF of 27 Hz. This table provides the exact values of the statistical analysis including the calculated standard error and p-values.
(DOCX)

S5 Table. Subgroup analysis according to fetal behavioral state group for the MF of 27 Hz. This table provides the exact values of the statistical analysis including the calculated standard error and p-values.
(DOCX)

Acknowledgments

Author Contributions

Conceptualization: Dorothea Niepel, Rossitza Draganova, Hubert Preissl.

Data curation: Hubert Preissl, Hari Eswaran.

Formal analysis: Dorothea Niepel, Bhargavi Krishna, Rathinaswamy B. Govindan.

Funding acquisition: Hubert Preissl, Hari Eswaran.

Investigation: Dorothea Niepel.

Methodology: Dorothea Niepel, Bhargavi Krishna, Eric R. Siegel, Hubert Preissl, Rathinaswamy B. Govindan, Hari Eswaran.

Project administration: Dorothea Niepel, Hari Eswaran.

Resources: Hari Eswaran.

Software: Bhargavi Krishna, Rathinaswamy B. Govindan.

Supervision: Hubert Preissl, Rathinaswamy B. Govindan, Hari Eswaran.

Visualization: Dorothea Niepel, Eric R. Siegel.

Writing – original draft: Dorothea Niepel.

Writing – review & editing: Dorothea Niepel, Bhargavi Krishna, Eric R. Siegel, Rossitza Draganova, Hubert Preissl, Rathinaswamy B. Govindan, Hari Eswaran.

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