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MODELS FOR PRETERM CORTICAL DEVELOPMENT USING NON INVASIVE CLINICAL EEG

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Abstract

The objective of this study was to evaluate the piglet and the mouse as model systems for preterm cortical development. According to the clinical context, we used non invasive EEG recordings. As a prerequisite, we developed miniaturized Ag/AgCl electrodes for full band EEG recordings in mice and verified that Urethane had no effect on EEG band power. Since mice are born with a "preterm" brain, we evaluated three age groups: P0/P1, P3/P4 and P13/P14. Our aim was to identify EEG patterns in the somatosensory cortex which are distinguishable between developmental stages and represent a physiologic brain development. In mice, we were able to find clear differences between age groups with a simple power analysis of EEG bands and also for phase locking and power spectral density. Interhemispheric coherence between corresponding regions can only be seen in two week old mice. The canolty maps for piglets as well as for mice show a clear PAC (phase amplitude coupling) pattern during development. From our data it can be concluded that analytic tools relying on network activity, as for example PAC (phase amplitude coupling) are best suited to extract basic EEG patterns of cortical development across species.

Keywords

• coherence • cortical development • full band EEG • mouse model • phase amplitude coupling • telemetry • somatosensory cortex • piglet • preterm

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Introduction

The basic question underlying our work is, whether insights from animal model systems can be used to refine medical EEG diagnostics in early preterm babies and especially to identify typical patterns in the EEG which are ubiquitously related to the status of cortical development.

Preterm birth is worldwide of growing incidence [1]. Survival rates of preterm babies are increasing due to modern health monitoring and specialized neonatal care units [2]. Unfortunately, preterm birth is associated with several risk factors during later life, including cognitive impairments [3]. One major problem is the lack of knowledge regarding a "normal" brain maturation during early development [4]. On the one hand, preterm birth per se is a non-physiologic condition. On the other hand, due to ethical constraints, brain monitoring of preterm babies is usually limited to pathophysiological indications as for example epilepsy [5].

Therefore, our aim is to evaluate the mouse and the piglet as model systems for preterm EEG patterns which are related to cortical maturation. Knowledge about an age related state of cortical maturation is the only way to distinguish between physiological development and pathophysiologic maldevelopments, because preterm EEG differs profoundly from adult EEG as well as from newborn EEG. EEG in preterm babies is discontinuous, interhemispheric coherence is weak [6] and sleep/wake stages are not as clearly established as in the EEG of adults or older children [7,8]. A discontinuous EEG is characterized by very low overall network activity and some sparse, spontaneously occurring events, like spindle bursts or delta brushes (latter also known as spontaneous activity transients [9]). It turned out, that very slow oscillations play an important role during cortical maturation [10]. Consequently we used full band EEG (fbEEG) in order to record even very slow potentials. Such waves with a duration up to several seconds are cut off with standard AC coupled EEG amplifiers [11]. A newborn mouse corresponds approximately to the 17th week of gestation in human regarding cortical maturation/EEG [12] (http://www.translatingtime.net/). 3 to 4 day old mouse pubs correspond to the 22nd week of gestation in human. Very early preterm babies in human can survive from the 23rd week of gestation on. Two week old mice correspond to a cortical maturation around the 46th week of gestation in human, which is already post term. In comparison to humans, mice are born with a preterm brain [13]. Nevertheless, typical EEG patterns like spindle and gamma bursts can also be observed in the mouse model [13-16].

The piglet is an emerging model system in the field of developmental neurobiology [17]. Therefore, knowledge is still limited but growing. Obvious advantages are the physiological as well as anatomical similarity to humans and a similar body size. A disadvantage is that piglets are precocious and therefore at least partially more mature at the date of birth in comparison to mouse pubs and human babies.

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In contrast to scientific research, the method of choice on the neonatal intensive care unit is the scalp EEG. EEGs derived from the scalp do not contain unit activity but instead exclusively global activity patterns of oscillating neural networks. Nevertheless, recent findings suggest that certain patterns in the preterm scalp EEG are predictive for the health status of former preterm children several years later in life [18]. This finding is an important step on the way to use clinical EEG as diagnostic tool for preventive treatments in the case of preterm birth. Another approach is to analyze correlations between EEG channels and/or frequency bands. Indeed, it has been shown that connectivity analysis is correlated with cortical maturation [19]. One such tool of analysis is phase amplitude coupling, also called nesting coefficient [10]. It deciphers the degree of amplitude modulation of one frequency band as a function of the phase relation to a second frequency band of the EEG, thus a special case of correlation. Delta brushes are representative examples for such nested events in the developing cortex [20].

By using the mouse and the piglet as model systems for the development of scalp EEG patterns we would like to close a methodological gap to acquire the necessary knowledge regarding early cortical maturation and related patterns in the clinical EEG. We were able to extract patterns of cortical development across both model systems with analytic tools, relying on network activity, especially phase amplitude coupling. In the future this kind of analysis in combination with telemetric full band EEG might be an interesting diagnostic tool for the neonatal intensive care unit.

Materials and Methods

Animal Experiments

All procedures were approved by the local ethics committee (#23177-07/G10-1-010/G 15-15-011), and followed the European and the German national regulations (European Communities Council Directive, 86/609/ECC; Tierschutzgesetz).

All animal procedures were performed in accordance with the [Medical Center of the Johannes Gutenberg-University Mainz] animal care committee's regulations.

mice of either sex:

For general anesthesia Urethane (1g/kg) was injected IP. Local anesthesia was achieved by lidocaine gel (Xylocain 2%, Astra Zeneca, UK).

For head fixation, a metal ring was fixed with super glue and dental cement on the skull as described in [14].

Teflon coated silver wires (advent) with a diameter of 50µm have been used for supra cranial recordings (on top of the Dura). For super cranial recordings (on top of the skull) the same wires have been deinsulated and melted at the tip. The resulting metal droplet was pressed in order to acquire a flattened round electrode tip with a diameter of approximately 1mm. The wires were initially fixed with a conductive 1:1 mixture of Kwik Cast (2-component silicone elastomer, World Precision Instruments, Sarasota, FL, USA) and NaCl containing electrode gel (signa crème, parker laboratories inc., USA) for EEG recordings. In case of age groups without known coordinates, Barrel cortex and forelimb region were pre experimentally determined by intrinsic imaging with Whisker and Forelimb stimulation by a motor driven short touch. After precise placement of electrodes with a micromanipulator, electrodes were additionally fixed with dental cement (Paladur, Heraeus, Germany and Tetric evo flow, ivoclar vivadent GmbH, Germany) and subsequently connected via molex pins to a DC multi channel systems full band EEG (fbEEG) recording setup. Finally, the recording site was confirmed by whisker and forelimb stimulation, respectively.

piglets of either sex:

We recorded EEGs from piglets directly in the pigpen. To fix the electrodes on the piglets scalp, piglets were wrapped into a piece of soft tissue to calm them down. We used no anesthesia. Disposable adhesive surface silver/silverchloride electrodes (Spes Medica S.r.l., Genova, Italy) were placed above cerebellum (between the ears) as ground, above the nose as reference and between eye and ear to record from the somatosensory cortex region. In order to assure anatomical dimension of the newborn piglets brain, we prepared brains from stillborn piglets. Whole brains were fixed for two weeks in 5% paraformaldehyde and plastinated afterwards.

Before Fixation of the electrode, the skin was cleaned with an abrasive cream (Abralyt HiCl, Easycap GmbH, Herrsching, Germany) in order to remove dead skin cells and to achieve a lower impedance. The data were recorded and send by a telemetry unit (with an AC coupled amplifier), [21]. Only sleep phases were taken into account for analysis.

Comparison of super cranial EEG recordings with dural EEG in mice

Custom made super cranial electrodes were compared with silver wire recordings on top of the skull in P3 animals. Animals were head fixed and anesthetized as described beforehand. We marked the coordinates for Barrel Cortex on the left and right brain hemisphere. 2mm apart from this position a hole was carefully scratched into the skull by the aid of a small piece of razor blade. The supracranial electrode was slowly pushed forward to the marked position, underneath the skull and afterwards fixed with dental cement. The supercranial electrode was placed on top of the skull at the marked position. With this technique we were able to record simultaneously super- and supracranial EEG at the same position.

Comparison EEG under urethane anesthesia vs. awake (local anesthesia) in mice

Newborn mice (P0 or P1) were locally anesthetized as described beforehand. Supercranial electrodes were fixed on top of the skull (forelimb and barrel cortex region of the primary somatosensory cortex). After the recording of spontaneous and evoked EEG, the same animals were anesthetized with urethane. 40 minutes thereafter EEG of spontaneous as well as evoked activity was measured again under urethane anesthesia.

Data acquisition

Initial sampling rate for mouse experiments was 10000Hz, for further analyzes, data were down sampled to 1000Hz. We used a DC coupled amplifier (multi channel systems GmbH, Germany). A Bode diagram was kindly provided by multi channel systems, showing that amplification was stable in the frequency range we were interested in (LFP from 0-250Hz).

For the piglets we used an AC coupled amplifier and a sampling rate of 500Hz.

Data Analysis

We analyzed the Data with matlab (2015a, Simulink) and with brainstorm [22]. EEG raw data were filtered with digital butterworth filters with a custom written matlab script. The filter was designed with the function butter (n=3rd order). We calculated the normalized cutoff frequency (Wn) for EEG bands delta [0-4Hz], theta [4-8Hz], alpha [8-13Hz], beta [13-30Hz], low gamma [30-80Hz] and high gamma [80-120Hz]. Wn is a number between 0 and 1, where 1 corresponds to the Nyquist frequency which is half the sampling rate (here: 500Hz for down sampled EEG data).

The numerator and denominator values (IIR filter), achieved with the function butter, were used with the matlab function filtfilt to filter the EEG data. For the delta EEG band (0-4Hz), a lowpass was used. We extracted all other EEG frequency bands with a bandpass filter design.

Phase amplitude coupling, Phase Locking Value (PLV) as well as coherence were analyzed with brainstorm software [22].

To obtain Canolty maps [23], following procedure was computed (neuroimage.usc.edu/brainstorm/Tutorials/Resting): The EEG was filtered at the low frequency of interest, using a narrow band pass filter. The amplitude troughs of the desired low frequency were detected in the signal. A time window is defined around the detected troughs in order to compute a time frequency decomposition using a set of narrow band-pass filters.

Coherence was calculated as time resolved coherence with an estimation window length of 3500ms and 50% sliding window overlap. The maximum frequency resolution was 1Hz and metric significativity $p<0.01$. Brainstorm Coherence analysis was based on the matlab function "mscohere". EEGs from all animals of a group were filtered independently with brainstorm [22]. The delta band was filtered with a low pass filter (0-4Hz). All other bands (theta:4-8Hz, alpha:8-13Hz, beta:13-30Hz, gamma:30-80Hz, hgamma:80-120Hz) were filtered with the appropriate band pass filter. Time resolved coherence was calculated for each animal and each band separately. The results for every EEG band and age group were averaged (arithmetic average, n=5 for P0,P1 and n=6 for P13,P14).The Phase Locking Value (PLV) was calculated as averaged connectivity matrix (one file per age group).

Statistics

We tested data for distribution with the Lilliefors test (matlab 2015a, Simulink). Thereafter, tests were either performed with the non-parametric Wilcoxon ranksum test or the Kruskal Wallis test and a subsequent multiple comparison test in order to achieve exact statistical relations between groups. All tests are implemented in the matlab statistics toolbox (2015, Simulink).

Results

The main question of this study is, in how far the scalp EEG during the first two weeks of life in mice and pigs does show similar maturational patterns, as for example delta brushes or a transition from discontinuous to continuous EEG, as seen in preterm babies.

In order to use mice as model system for human preterm cortical maturation, we developed miniaturized super cranial fbEEG electrodes useful for long term recordings in neonatal mice with a body weight below 1g. Furthermore, we tested the influence of different anesthesia methods (commonly used in animal experiments) on EEG band power in mice. Recording quality of our super cranial electrodes was compared with electrodes underneath the skull. Mice had an age from P0 to P14, which corresponds to cortical development from human post conceptional day 114 (17th week of gestation, very early preterm) towards post conceptional day 318 (46th week of gestation, post term) (translatingtime.net).

Effect of urethane anesthesia on EEG band power

The comparison between urethane anesthetized and awake, spontaneously active newborn mice (group P0, P1, n=6) revealed no statistically significant difference for the power of EEG frequency bands from 1Hz to 120Hz (p-values (wilcoxon ranksum) from delta to high gamma: 0.7/0.6/0.6/0.24/1/0.1) (Figure 1). Power values for urethane anesthetized mice from delta to high gamma: delta: median[4479μV²/ms], mean[4419μV²/ms] +/- 2474μV²/ms SD; theta: median[0.17μV²/

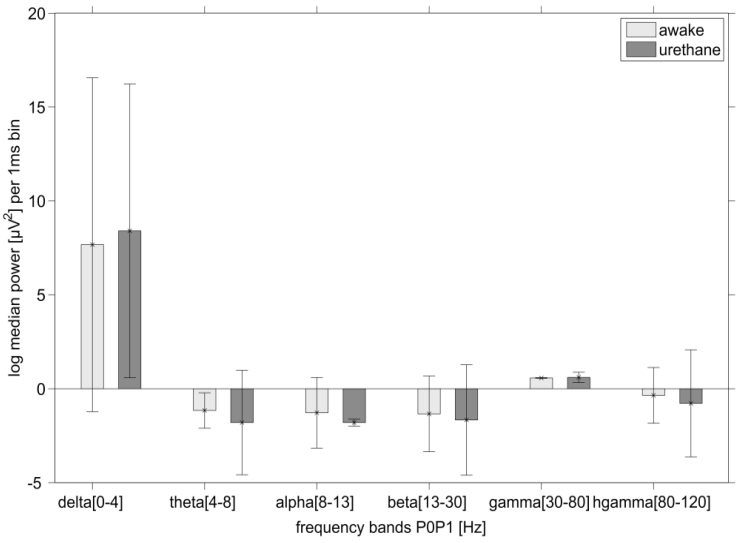

Figure1. No statistically significant effect of urethane anesthesia on full band EEG power. EEG recordings of spontaneous activity of n=6 newborn mice (P0,P1) were acquired before (awake) and after administration of light urethane anesthesia. Data were band pass filtered in order to extract EEG bands. Median band power was calculated with matlab. There is a slight tendency towards higher EEG band power under urethane anesthesia in comparison to awake animals.

ms], mean[6.76μV²/ms] +/- 16.19μV²/ms SD; alpha: median[0.17μV²/ms], mean[0.49μV²/ms] +/- 0.83 μV²/ms SD; beta: median[0.19μV²/ms], mean[0.19μV²/ms] +/- 0.05μV²/ms SD; gamma: median[1.83μV²/ms], mean[2.1μV²/ms] +/- 0.76 μV²/ms SD; high gamma: median[0.46μV²/ms], mean[0.48μV²/ms] +/- 0.06 μV²/ms SD.

Power values for awake mice (local anesthesia): delta: median[2146μV²/ms], mean[5224μV²/ms] +/- 7261μV²/ms SD; theta: median[0.31μV²/ms], mean[0.45μV²/ms] +/- 0.39μV²/ms SD; alpha: median[0.28μV²/ms], mean[0.28μV²/ms] +/- 0.15μV²/ms SD; beta: median[0.26μV²/ms], mean[0.28μV²/ms] +/- 0.13μV²/ms SD; gamma: median[1.77μV²/ms], mean[2.18μV²/ms] +/- 1.02μV²/ms SD; high gamma: median[0.7μV²/ms], mean[0.66μV²/ms] +/- 0.23 μV²/ms SD.

EEG pattern during development: from discontinuous to continuous EEG

The transition from discontinuous EEG in newborn mice (age groups P0/P1 and P3/P4, urethane anesthesia) to continuous EEG activity in two week old mice can be seen in Figure 2a-c. Due to our recording system (full band EEG) we are able to record very slow, spontaneously occurring delta waves, with superimposed faster oscillations in P1 mice (Figure 2a). Spontaneous spindles can be seen in a P3 mouse with very sparse background activity, typical for discontinuous EEG (Figure 2b). In two week old animals, the transition towards continuous EEG with ongoing activity is completed (Figure 2c). No such transition can be seen in the EEG of piglets (Figure 2 d-f) from the first day of life towards an age of 2 weeks. Recording site was the somatosensory cortex. A gamma burst can be seen in Figure 2e, recorded from a 4 day old piglet. Apart from traces 2a and 2f, which are low pass filtered (30Hz), all the other traces which are seen in Figure 2 are unfiltered raw data.

Comparison of EEG band power recorded with electrodes on top of the skull and electrodes on top of the Dura

No statistically significant difference can be observed in median EEG band power comparing supracranial electrodes (between skull and Dura)(delta: median[2097μV²/ms], mean[3048 μV²/ms] +/- 3029 μV²/ms SD; theta: median[0.68 μV²/ms], mean[2.22 μV²/ms] +/- 3.99 μV²/ms SD; alpha: median[0.37 μV²/ms], mean[0.41 μV²/ms] +/- 0.32 μV²/ms SD; beta: median[0.19 μV²/ms], mean[0.23 μV²/ms] +/-0.15 μV²/ms SD, gamma: median[1.37 μV²/ms], mean[32.38 μV²/ms] +/-75.88 μV²/ms SD) with electrodes on top of the intact skull (supercranial) (delta: median[233.22μV²/ms], mean[2022μV²/ms] +/- 4151μV²/ms SD; theta: median[0.84μV²/ms], mean[1.76μV²/ms] +/- 2.72μV²/ms SD; alpha: median[0.4μV²/ms], mean[0.38μV²/ms] +/- μV²/ms SD; beta: median[0.27μV²/ms], mean[0.26μV²/ms] +/-0.09μV²/ms SD, gamma: median[1.21μV²/ms], mean[13μV²/ms] +/-28.85μV²/ms SD) in three and four day old mice (n=6, p-values (wilcoxon ranksum) from delta to gamma: 0.4 /0.7/1/0.3/0.9) (Figure 3). Supra cranial electrodes have higher power by trend. In the high gamma range (80-120Hz), a significant difference in median EEG power between super- (median[0.39μV²/ms], mean[0.58μV²/ms] +/- 0.5μV²/ms SD) and supra cranial electrodes (median[0.28μV²/ms], mean[0.31μV²/ms] +/- 0.11 μV²/ms SD) can be observed for the 95% significance level (alpha=0.05, p-value=0.03). In this case, the EEG recorded with supra cranial electrode does show a higher power in comparison to EEGs recorded with electrodes on top of the skull.

Comparative median EEG band power and amplitudes during the first two weeks of live in mice

Median EEG delta band amplitudes are not statistically significant different between P0/P1 (median[36.35μV], mean[52.68μV]+/- 44μV standard deviation (SD)), P3/P4 (median[27.17μV], mean[37.94] +/- 34μV SD) and 2 week old animals (P13/P14,

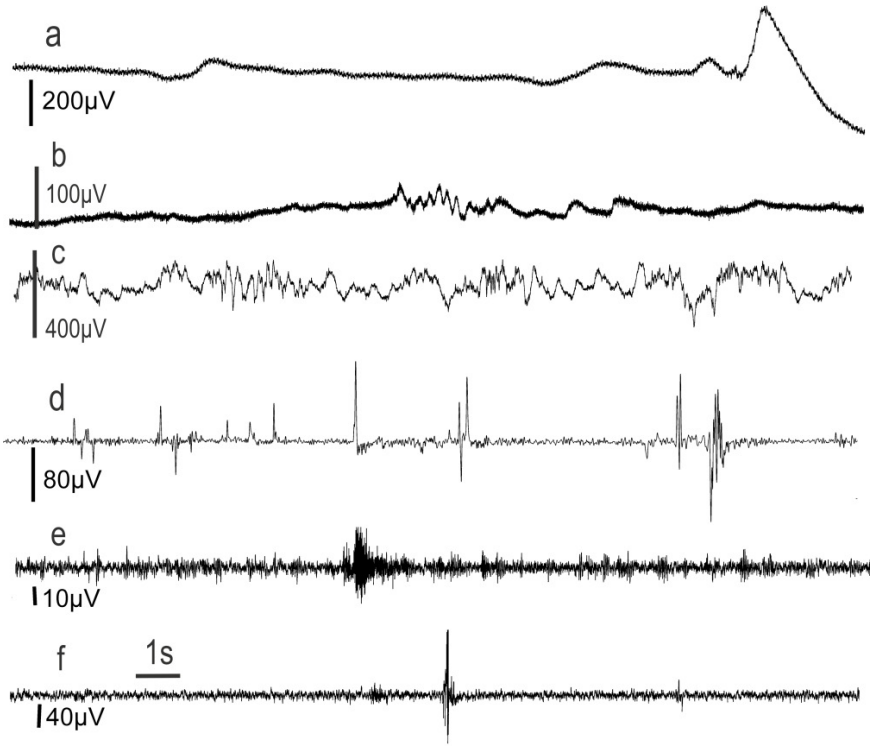

Figure 2. From discontinuous to continuous EEG during development. 20s raw EEG traces of spontaneous activity in the Barrel Cortex in a representative P1, P3 and P13 mouse and piglet, respectively. a) Discontinuous EEG recording of a one day old mouse. Slow, 6mV delta brush with superimposed faster oscillations. b) Discontinuous EEG recording of a three day old mouse. Sparse overall activity and one spontaneously occurring spindle burst. c) Continuous EEG recording of a 13 day old mouse. Comparatively high background activity, transition from discontinuous to continuous EEG completed. d,e,f) EEG raw traces for a one day old piglet (d) a 4 day old piglet (e) and a two week old piglet (f). A transition from discontinous to continous EEG is not visible. Furthermore, k-komplex like events are seen from the first day of life.

median[59.12µV], mean[67.15µV] +/- 31V SD) (Figure 4). Between the groups P0P1 and P3P4 no statistically significant difference between median amplitudes can be found for any frequency band. P0P1 mice have statistically significant lower median theta band amplitudes (median[0.48µV], mean[1.42µV] +/-2.3µV SD) in comparison to P13 P14 mice (median[9.61µV], mean[11.26µV] +/-3.3µV SD) at a significance level alpha=0.01. 3 and 4 day old mice have lower theta band amplitudes (median[0.79µV], mean[1.05µV] +/-1µV SD) in comparison to two week old animals. The same statistical relations and significance levels are true for the alpha band with the median EEG amplitudes P0P1 (median[0.48µV], mean[0.63µV] +/-0.4µV SD), P3P4 (median[0.6µV], mean[0.56µV]+/-0.2µV SD) and P13P14 (median[7.36µV], mean[7.41µV] +/-2.1µV SD). For the beta band, P0P1 median amplitudes (median[0.49µV], mean[0.48µV] +/-0.1µV SD) are statistically significant different for alpha=0.05 in comparison to two week old animals (median[5.27µV], mean[4.92µV] +/-1.4µV SD). Median beta amplitudes in P3P4 age group (median[0.51µV], mean[0.48µV] +/-0.1µV) are statistically significant different for a significance level of alpha=0.01 in comparison to two week old animals. Median gamma amplitude is not statistically significant different between P0P1 mice (median[1.24µV], mean[1.34µV] +/-0.3µV SD) and P13P14 mice (median[4.1µV], mean[3.96µV] +/-1.2µV SD) whereas P3P4 animals have statistically significant lower gamma band amplitudes (alpha=0.001, median[0.96µV], mean[0.65µV] +/-0.2µV SD) in comparison to two week old animals. Group relations and significance levels for the median high gamma band amplitudes are the same as described for the median beta band amplitudes with following median amplitudes: P0P1 (median[0.71µV], mean[0.74µV] +/-0.1µV SD), P3P4 (median[0.64µV], mean[0.65µV] +/-0.1µVµV SD) and P13P14 (median[1.34µV], mean[1.33µV] +/-0.2µV SD).

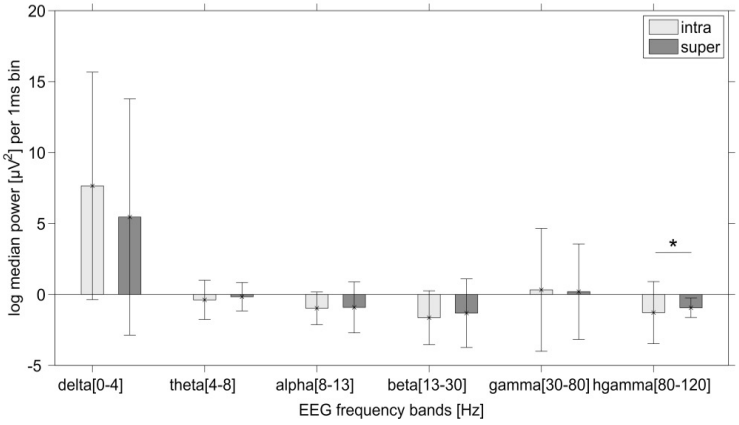

Figure 3. Verification of super cranial electrodes in comparison with supra cranial EEG electrodes in P3/P4 mice. First, the supra cranial electrode was placed. To do so, a small whole was scratched into the skull approximately 2mm away from the marked position, without bleeding. The supra cranial electrode was inserted and gently pushed forward underneath the skull until it reached the desired area. Afterwards it was fixed with dental cement towards the skull. Subsequently, the super cranial EEG electrode was fixed at the same recording site as the supra cranial electrode but on top of the skull. Median EEG band power is only statistically significant different for both electrode types in the high gamma band (80-100Hz) to a significance level of 95%.

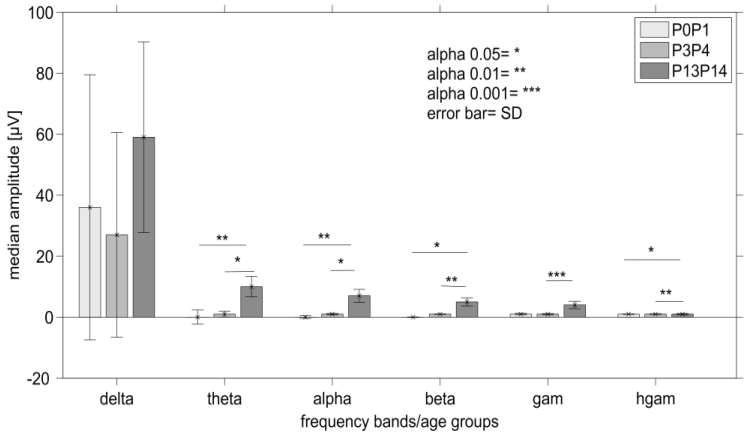

Figure 4. Median spectral full band EEG amplitudes during development in mice (n=6 per age group). Two week old mice show an exponential decay of median full band EEG amplitudes from slow oscillations in the delta range to fast oscillations in the high gamma range up to 120Hz. In contrast, 3 or 4 day old mice have relatively high delta power and more or less constant values for the full band EEG amplitudes of all faster oscillations. Newborn mice (P0/P1) deviate from both age groups, since they have a two peak distribution of full band EEG amplitudes: relatively high in the slow delta band in the faster gamma band, but statistically significant lower than two week old animals in the theta and alpha band. In contrast, median gamma band amplitudes of newborn mice are not statistically significant different from two week old animals and higher than in P3/P4 mice. Hence, we can observe a non-linear development of band amplitudes during the first two weeks of life in mice.

Similar results can be observed for normalized median power (power per ms) (Figure 5). Between the groups P0P1 and P3P4 no statistically significant difference can be found for any frequency band. Median delta band power/ms is in P0P1 (median[2036µV²/ms], mean[3118 µV²/ms]+/-3273µV²/ms SD) as well as P3P4 (median[766µV²/ms], mean[2354 µV²/ms] +/-4027µV²/ms SD) mice significantly different from two week old animals (median[3507µV²/ms], mean[5321 µV²/ms] +/-5383µV²/ms SD) at a significance level of alpha=0.05. For the age group P0P1, median theta (median[0.23µV²/ms], mean[6.82 µV²/ms] +/-16.16µV²/ms SD), alpha (median[0.23µV²/ms], mean[0.54 µV²/ms]+/-0.81µV²/ms SD) and beta power (median[0.23µV²/ms], mean[0.23 µV²/ms]+/-0.08µV²/ms SD) are significantly different from two week old animals at a level of alpha=0.01, respectively, (theta: median[92.3 µV²/ms], mean[135.75 µV²/ms]+/-81.03µV²/ms SD), (alpha: median[54.24 µV²/ms], mean[58.58 µV²/ms]+/-32.5µV²/ms SD), (beta: median[27.83 µV²/ms], mean[25.79 µV²/ms] +/-13.14µV²/ms SD). For the same frequency bands, P3P4 mice show significantly different EEG band power in comparison to two week old animals

at a significance level of alpha=0.05 (theta: median[0.63μV²/ms], mean[1.88 μV²/ms]+/-3.21μV²/ms SD), (alpha: median[0.36μV²/ms], mean[0.36 μV²/ms] +/-0.24μV²/ms SD), (beta: median[0.26μV²/ms], mean[0.24 μV²/ms] +/-0.09μV²/ms SD). Median gamma band power is not statistically significant different between P0P1(median[1.6μV²/ms], mean[1.85 μV²/ms] +/-0.76μV²/ms SD) animals and P3/P4 mice (median[16.81μV²/ms], mean[16.97 μV²/ms]+/-9.57μV²/ms SD) but there is a highly significant difference between P3P4 mice (median[0.92 μV²/ms], mean[1 μV²/ms] +/-0.41μV²/ms SD) and two week old animals at alpha=0.001. P0P1(median[0.49μV²/ms], mean[0.55 μV²/ms] +/-0.13μV²/ms SD) is statistically significant different in comparison to two week old animals(median[1.79μV²/ms], mean[1.8 μV²/ms] +/-0.57μV²/ms SD) regarding the median power of the high gamma band for a level of alpha=0.05. For the same band, the difference between median power of P3P4(median[0.41μV²/ms], mean[0.43 μV²/ms] +/-0.13μV²/ms SD) age group and two week old animals is statistically significant for alpha=0.01.

In summary, 2 week old animals show a typical exponential decline of band power from slow to fast oscillations, whereas newborn mice show a two peak distribution with relatively high delta and gamma power. The two peak distribution is already lost in 3 and 4 day old mice.

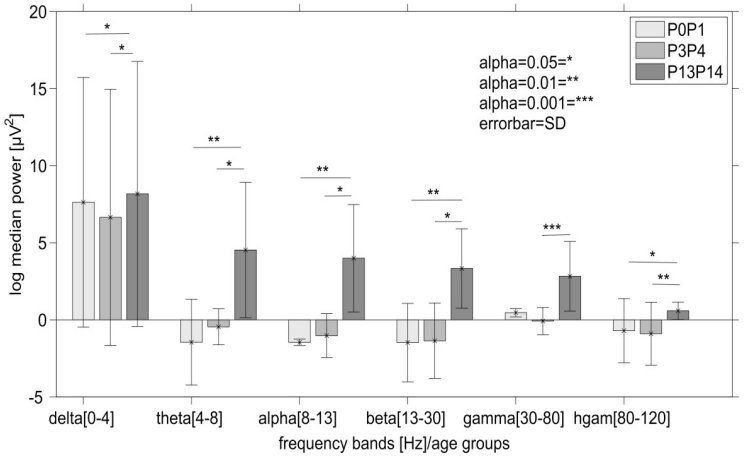

Figure 5. Age dependent development of EEG band power in mice (n=6 per age group). EEG band power is exponentially decaying for two week old mice. In contrast, newborn mice have dominant power in the delta band. P0P1 animals have a two peak distribution, with relatively high delta and gamma power. Power distribution is significantly different for newborn mice and two week old animals, despite the gamma band. There is no statistically significant difference in EEG band power between the age groups P0/P1 and P3/P4.

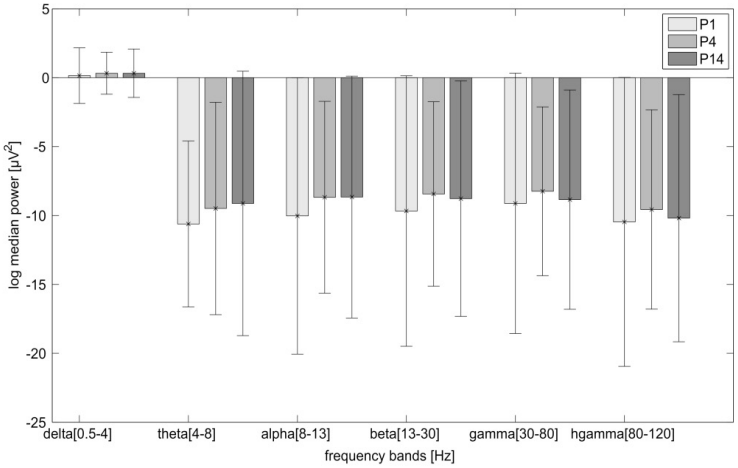

Figure 6. No significant differences can be seen for EEG band power between age groups (from 1 day old towards two weeks of age) in piglets. All piglets show a two peak distribution for spectral EEG band power, with relatively high delta as well as gamma power.

Comparative median EEG band power during the first two weeks of live in piglets

In contrast to mice, no statistically significant differences can be observed in median spectral EEG band power between the age groups P1 (one day old piglets), P4 (four day old piglets), P13/P14 (two week old) in piglets (Figure 6, Table 1). There is a tendency towards a two peak distribution for all the age groups, with relatively high delta and gamma power in comparison to the other frequency bands, especially in newborn piglets.

Major changes in Phase amplitude coupling during the first two weeks of live in mice and piglets

Amplitude modulation of a fast frequency in relation to the phase of a slower frequency gives distinguishable patterns between age groups (Figure 7) in mice. For P0/P1 and a phase frequency of 1Hz, a peak coupling in the theta, alpha and beta range can be seen (Figure 7, 1a). The 1Hz phase frequency is indicated as white sine wave. Despite a coupling between 4Hz phase frequency and a narrow band in the high gamma range as seen in the P0/P1 age group (Figure 7, 1b) there is no coupling between any of the phase frequencies tested here and the gamma band in young animals, neither in the age group P0/P1 nor in the group P3/P4. In contrast, in the age group P13/P14, coupling effects are much stronger, with a stronger phase relation and dominantly in the gamma band (Figure 7, 3a-c). Additionally, in these 2 week old animals, nearly no coupling in lower bands can be seen. Furthermore, increasing phase frequency is associated with increasing frequency of the amplitude frequency in two week old animals. For example, in Figure 7, 3b with a phase frequency of 4Hz, the amplitude modulation does take place in the gamma range

Table1: Data are not normally distributed. Therefore, we used a kruskal wallis test in combination with a multiple comparison test to compare the three age groups, n=6, respectively. There are no statistically significant differences between age groups for a significance level of alpha=0.05, as seen from confidence intervals, which do all contain zero. If the confidence interval does envelope a number range which contains zero, there is no statistically significant difference between the groups.

Freq.- band	age	Median Power [μV^2/ms]	Mean [μV^2/ms]	SD [μV^2/ms]	kruskal wallis, multcomp, alpha=0.05	
					age groups	confidence interval
delta	P1	1.16	1.17	0.13	P1/P4	[-7/6.2]
	P4	1.38	1.24	0.22	P1/P13	[-9.2/4]
	P13/P14	1.38	1.27	0.17	P4/P13	[-8.8/4.4]
theta	P1	$0.25x10^{-4}$	$0.10x10^{-4}$	$0.20x10^{-4}$	P1/P4	[-7/6.2]
	P4	$0.76x10^{-4}$	$3.03x10^{-4}$	$4.51x10^{-4}$	P1/P13	[-9.8/3.4]
	P13/P14	$1.09x10^{-4}$	$1.12x10^{-4}$	$0.68x10^{-4}$	P4/P13	[-9.4/3.8]
alpha	P1	$0.44x10^{-4}$	$0.54x10^{-4}$	$0.44x10^{-4}$	P1/P4	[-10.2/3]
	P4	$1.7x10^{-4}$	$6.5x10^{-4}$	$0.95x10^{-4}$	P1/P13	[-12/1.2]
	P13/P14	$1.73x10^{-4}$	$2.04x10^{-4}$	$1.54x10^{-4}$	P4/P13	[-8.4/4.8]
beta	P1	$0.63x10^{-4}$	$0.72x10^{-4}$	$0.55x10^{-4}$	P1/P4	[-10.4/2.8]
	P4	$2.17x10^{-4}$	$8.75x10^{-4}$	$0.10x10^{-4}$	P1/P13	[-11.8/1.4]
	P13/P14	$1.54x10^{-4}$	$2.27x10^{-4}$	$1.94x10^{-4}$	P4/P13	[-8/5.2]
gamma	P1	$1.09x10^{-4}$	$1.22x10^{-4}$	$0.79x10^{-4}$	P1/P4	[-9.4/3.8]
	P4	$2.62x10^{-4}$	$0.20x10^{-4}$	$0.20x10^{-4}$	P1/P13	[-10.4/2.8]
	P13/P14	1.43x10-4	$3.21x10^{-4}$	$3.5x10^{-4}$	P4/P13	[-7.6/5.6]
high gamma	P1	$0.28x10^{-4}$	$0.35x10^{-4}$	$0.28x10^{-4}$	P1/P4	[-8.8/4.4]
	P4	$0.7x10^{-4}$	$4.98x10^{-4}$	$7.23x10^{-4}$	P1/P13	[-9.8/3.4]
	P13/P14	$0.37x10^{-4}$	$1.01x10^{-4}$	$1.28x10^{-4}$	P4/P13	[-7.6/5.6]

from 30 to 80Hz with enhanced amplitudes in relation to the rising and falling phase of the phase frequency. With a phase frequency of 8Hz (Figure 7, 3c) the amplitude modulation does take place between 70 and 140Hz, which is already mainly the high gamma range. In this case amplitude is enhanced with peak and trough of the phase frequency. A proportional relation between phase frequency and amplitude frequency is not true for both young age groups. In P0P1 the amplitude modulation with 8Hz phase frequency does take place in the delta, theta and alpha bands (Figure 7, 1c). In the primary somatosensory cortex of P3P4 mice, the amplitude modulation does occur mainly in the delta and alpha bands with 4Hz phase frequency (Figure 7, 2b) and more pronounced with 8Hz phase frequency (Figure 7, 2c). To sum up, phase amplitude coupling changes from slower to faster amplitude frequencies during development and the coupling pattern itself gains contrast as well as strength during the first 2 weeks of life in mice.

As already seen in the mouse model, gamma band coupling can only be seen in the age groups P1 (Figure 8.1b) and P13/P14 (Figure 8.3a-c) in piglets but not in 4 day old piglets (Figure 8.2a-c). Furthermore, the coupled gamma band is shifted towards higher frequencies (here called "high gamma", up to 250Hz) values in two week old animals.

Coherence of brain hemispheres during development

Coherence in the EEG delta to beta band is much stronger in newborn mice (P0/P1, n=5) in comparison to two week old animals (P13/P14, n=6, Figure 9). Two week old mice show mainly coherence between corresponding regions: For the Barrel Cortex in the delta band and for the forelimb region of the primary somatosensory Cortex in the theta band. Similarly, newborn mice (P0/P1) do also have the strongest coherence between left and right Barrel cortex in the delta range and the strongest correlation between left and right forelimb region of the primary somatosensory Cortex in the theta range. In contrast to two week old animals, P0/P1 mice show strong intrahemispheric coherence in the delta range between ipsilateral Barrel Cortex and forelimb region of the primary somatosensory Cortex. In the gamma as well as high gamma band of newborn mice, coherence is only apparent between hemispheres. The opposite is true for two week old animals: only ipsilateral, right hemispheric gamma band coherence.

Phase locking value in the somatosensory Cortex during development

Phase locking values in the somatosensory Cortex are stronger for newborn mice (P0/P1) in comparison to two week old animals, especially in the EEG delta and gamma band (Figure 10). For newborn mice, no clear trend towards ipsi- or contralateral phase locking is visible. In the high gamma range (newborn mice, P0/P1), phase locking values are by far

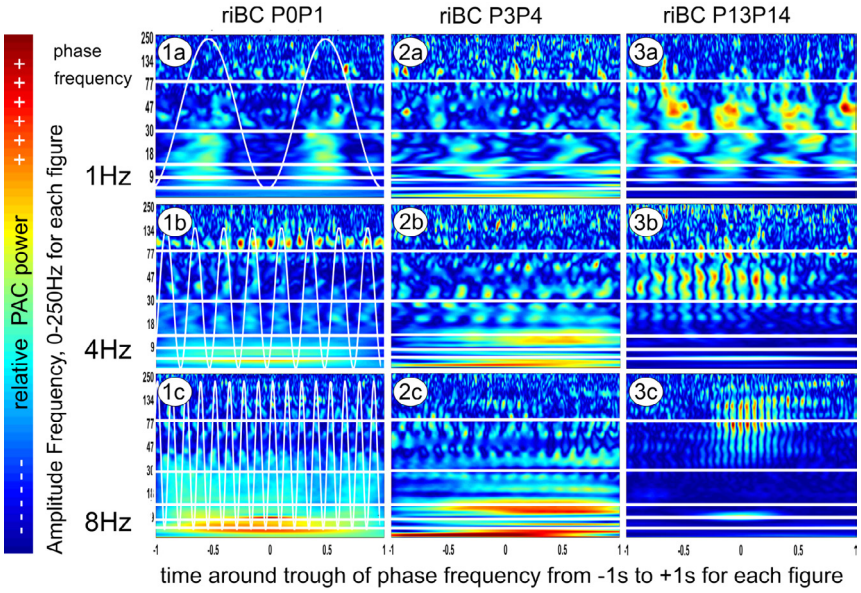

Figure 7. Visualization of cortical network maturation via phase amplitude coupling in mice (n=6 per age group). Canolty maps [23] (brainstorm: [22]) with 1, 4 and 8Hz phase frequency for modulation is changing during the first 2 weeks of life in mice. The first row (1a-3a), illustrates amplitude modulations (amplitude frequencies from 1-250Hz, y-axis) as power (color coded) relative to the phase of a 1Hz sine wave (delta range). The sine wave is shown in 1a as white line. The horizontal white lines indicate the EEG bands delta (1-4Hz), theta (4-8Hz), alpha (8-13Hz), beta (13-30Hz), gamma (30-80Hz) and high gamma (80-250Hz) range for each figure. The second row (1b-3b) illustrates amplitude modulations of EEG bands in phase relation to 4Hz phase frequency and the third row (3a-3c) does show the same for 8Hz phase frequency. Each figure depicts a time range from -1s to 1s, where the trough of the phase frequency (the pacemaker) is centered at 0s (middle of the x-axis). As a result, there are more cycles of the phase frequency per figure the shorter the wavelength is. Each figure from 1a to 3c is a group average of EEG traces of spontaneous activity in the right Barrel Cortex under light urethane anesthesia for 6 animals, respectively. In the first row (1a-3a) the amplitude modulation for EEG bands is shown relative to the phase of a 1Hz delta band wave. For newborn mice (P0P1, 1a) the amplitude of alpha and beta waves phase locked to the peak of the 1Hz phase frequency is enhanced (color coded). This may be due to nested frequencies in delta brushes. In P3/P4 (2a) the alpha band modulation is shifted from the peak towards the trough of the 1Hz phase frequency. Additionally there is amplitude modulation in the delta band. For 2 week old animals (3a) the amplitude modulation is clearly shifted towards faster frequency bands, especially beta and gamma. Furthermore, the amplitudes are enhanced during the rising and falling epochs of the phase frequency. In the second row (1b-3b) amplitude modulations in relation to a 4Hz wave are shown (4Hz sine wave is indicated in 1b). For newborn animals (1b) an enhancement at 90Hz amplitude frequency is visible, coupled to the trough of the 4Hz phase frequency. For P3 (2b) no clear pattern is visible but for 2 week old animals, an amplitude enhancement takes place in the gamma range for peak and trough of the 4Hz phase frequency. For the 8Hz phase frequency (1c-3c) amplitude enhancement does take place in the low frequency range (delta to alpha bands) in young animals (1c, 2c) whereas a clear coupling pattern with amplitude enhancement in the gamma and high gamma range can be seen in the two week old animals. Generally, delta to alpha band power enhancement is nearly not visible in 2 week old animals in contrast to the younger age groups. In P3P4 animals (second column) there is no power enhancement in the gamma range for the three phase frequencies 1, 4 and 8Hz.

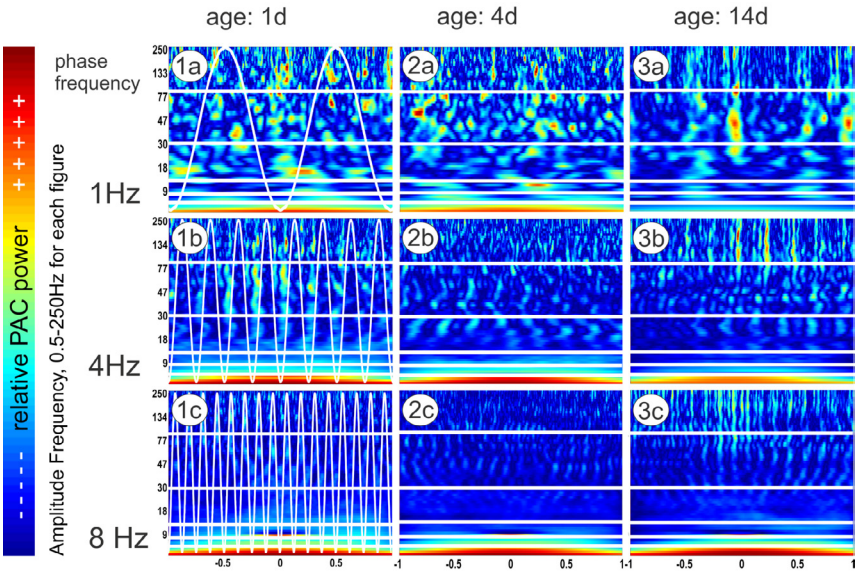

Figure 8. Phase amplitude coupling in piglets (n=6 per age group) is only apparent in 1 day old and two week old animals. Gamma as well as high gamma coupling can be seen for a phase frequency of 4 Hz in the case of 1 day old piglets. In two week old piglets clear coupling patterns can be seen for all phase frequencies from 1 to 8 Hz with the gamma and high gamma band.

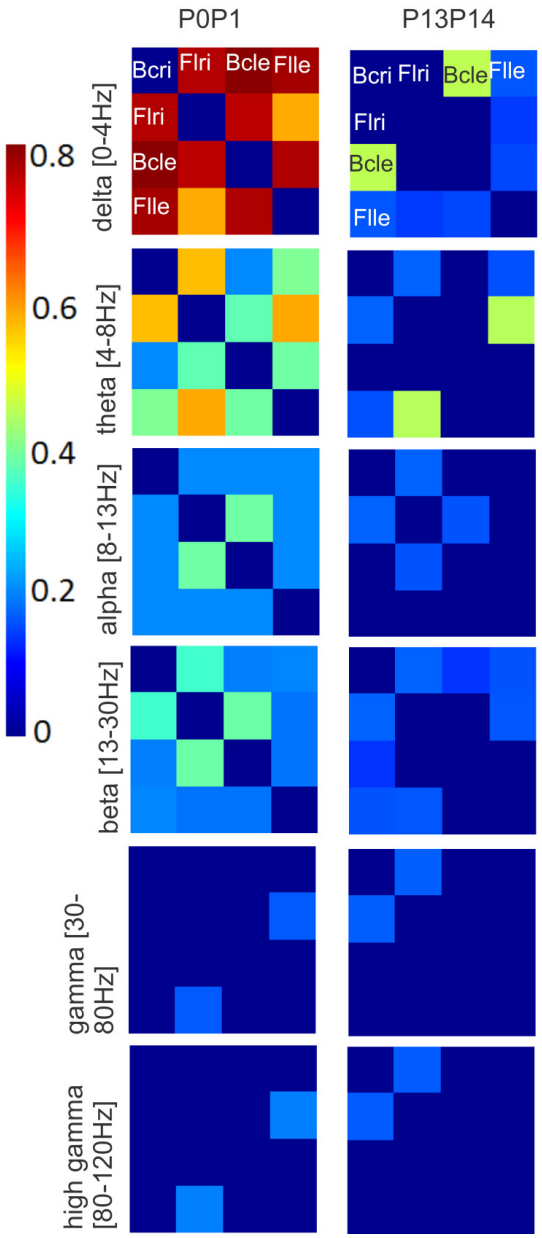

Figure 9. Development of hemisphere connectivity in mouse pubs. Coherence was calculated with the program brainstorm [22], n=5 EEG traces of spontaneous activity under light urethane anesthesia for newborn mice and n=6 for 2 week old animals were used for analyzes. Coherence (color coded with no coherence=0=blue towards full coherence=1=dark red) is shown for each frequency band separately from delta on top towards high gamma, squares right down at the bottom. Inside each square the coherence is read as coordinate system, with the order right Barrel Cortex, right forelimb S1, left Barrel Cortex, left forelimb S1 from left to right and from top to bottom. The diagonal plane is representing auto coherence and therefore color coded in dark blue. Overall Coherence values are much stronger for newborn mice in comparison with two week old animals, especially in the delta band. In two week old animals there is strong contralateral coherence between corresponding regions: for the Barrel Cortex in the delta band and for the forelimb region of the primary somatosensory Cortex in the theta band. The same is true for newborn mice, but there is additionally very strong ipsilateral coherence between adjacent regions of the primary somatosensory Cortex, especially in the delta band.

weaker than in the gamma band with strongest phase locking occurring between hemispheres. Phase locking values for two week old animals are gradually declining from EEG delta band to the high gamma band. This is in contrast to newborn mice (P0/P1), where maximal phase locking values occur in the gamma range. Similarly to newborn mice, Phase locking for the lower EEG bands in two week old mice is apparent between adjacent regions of the primary somatosensory Cortex (Barrel Cortex and forelimb region) as well as between cortical hemispheres. For the faster EEG bands (beta to high gamma), phase locking is occurring particularly between hemispheres.

Discussion

Our main finding is, that the fbEEG of neonatal mice show similarities to preterm human full band EEG patterns as for example less pronounced hemisphere coherence and a discontinuous EEG with spontaneous activity transients [6]. We were able to show fundamental changes in the EEG spectral band power and network connectivity patterns during a phase of maturation in mouse pubs, which corresponds to the critical transition period from very early preterm to full term in human infants. We observed similar results for piglets.

Why an animal model system for preterm birth at all?

In first instance, because the measurement of EEG in preterm infants is limited to cases with clinical indication. Since very early preterm birth is a life threatening status [2], other physiological parameters, as for example breathing, have priority and make EEG acquisition in the neonatal intensive care unit challenging [24]. Nevertheless, it turned out that former preterm infants have not only a statistically increased risk for cognitive impairments throughout their later lives in comparison to term born adults, they have also corresponding alterations in brain physiology and anatomy [25] [3]. Hence, there is an urgent need to distinguish "normal" developmental EEG patterns from pathophysiologic predictors of cognitive or other impairments during

later life. In a first mathematical approach, such patterns have been identified from EEG measurements [18].

Influences of analgesic treatment on EEG

A general problem are medication associated EEG alterations [26]. Preterm babies underwent very often some kind of analgesic treatment [27] [28] whereas laboratory animals most often receive anesthesia. We were able to show for newborn mice, that the spectral band power and amplitude of EEG recordings is not statistically significant altered by using a light urethane anesthesia in comparison to awake animals (fig.1). Furthermore we could show certain typical preterm EEG patterns as for example delta brushes or spindle bursts during urethane anesthesia (Figure 2). Spindle bursts under light urethane anesthesia have also been described in the EEG of rat pubs [14].

Adaptation to the clinical context: non invasive fbEEG electrodes for newborn mice with a body weight below 1g

We recorded the EEG in mice with non invasive skull electrodes in order to come closer to the recording situation on the neonatal intensive care unit. To our knowledge, this has been done for the first time in newborn mice. We validated our newly designed method by comparing the power spectral density between surface and conservative invasive electrodes on top of the Dura, as they are usually used in the scientific context for animal experiments. Despite the high gamma band, we could not find any statistically significant differences between invasive and the newly designed non-invasive electrodes (Figure 3). This might be due to the thin and soft skull in young mice and an associated very high conductivity, as has been proposed for neonates [29].

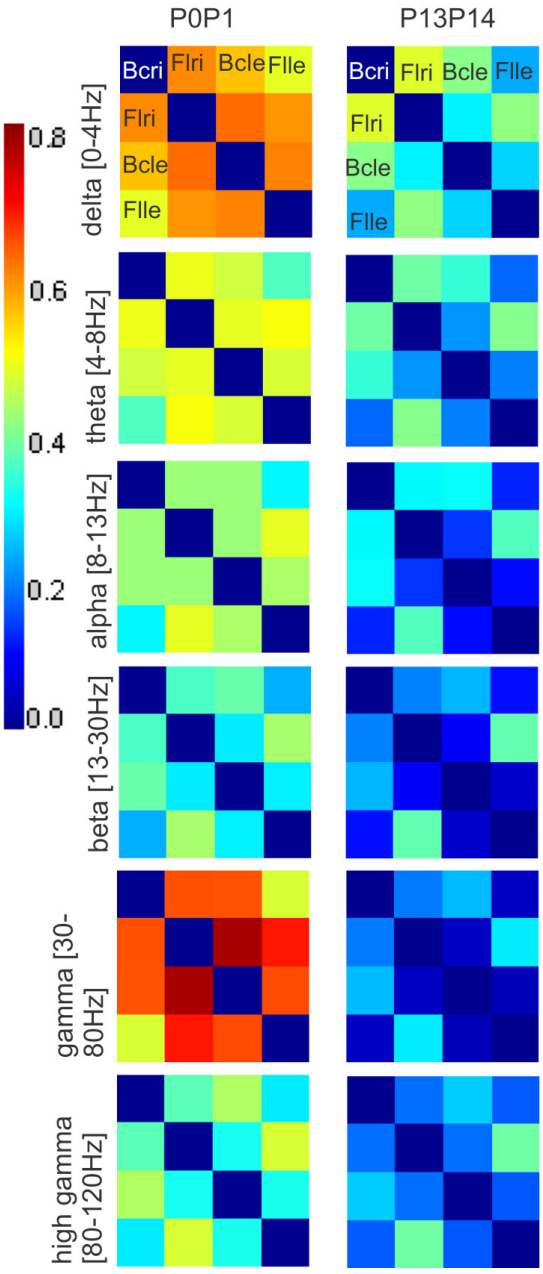

Figure10. Comparison of Phase locking values for newborn mice and two week old animals. Phase locking values are stronger for newborn mice in comparison to two week old animals. In two week old animals, phase locking values are additionally gradually declining from slow towards fast EEG bands. In contrast, newborn mice show strongest phase locking values in the gamma range and second strongest in the delta range. For newborn mice and slow frequency bands, there is no clear trend towards mainly ipsi or contralateral phase locking. For two week old animals and faster oscillations (from beta to high gamma), phase locking is mainly occurring between the right and left hemisphere.

Power spectral density as developmental marker

Very slow oscillations play an important role during early cortical development [10]. We used a full band EEG recording system to measure slow delta potentials. In fact, there is a high proportion of delta band power in the EEG of newborn mice (Figure 5). In comparison to two week old mice, which have exponentially decreasing power as well as amplitude values (Figure 4) from slow to fast oscillations, newborn mice (P0/P1) show a two peak distribution (Figure 5). They have relatively high amount of delta power as well as gamma. This pattern is already resolved in three day old mouse pubs, which have very low power for all bands but delta. One explanation

might be the shift from a subplate driven, gap junction coupled syncytium, towards a network which is mainly driven by chemical synapses [30]. It has been shown, that gap junction coupled ensembles are capable of producing oscillations of high frequencies, as for example beta and gamma [31]. Therefore, the EEG gamma band in newborn mice is most likely of different physiologic origin than the gamma band in two week old animals.

Power spectral density in newborn piglets differs somewhat from mice regarding differences between age groups. Piglets do not show significant differences between the age groups P1, P4 and P13/P14. Nevertheless, there is a similar two peak distribution with relatively high gamma as well as delta power in comparison to the other frequency bands for all the piglet age groups in contrast to mice, where this kind of spectral EEG power distribution can only be observed for the younger age groups. One reason might be that piglets do not develop as fast as mice so that it is impossible (in the given developmental window of this study) to observe the transition towards an exponential decay of power from slow to fast EEG band oscillations, as seen in two week old mice. We do not know the exact developmental correlation between age groups in mice and piglets. But one day old piglets show EEG patterns similar to K-Komplexes which are occurring in human from the third month of life onwards [32].

Does this mean, mouse pubs do not show any activity in the theta to beta band?

We used phase amplitude coupling analysis to compare the extend of nested processes between newborn mice, which correspond to human very early preterm and two week old mice corresponding to approximately the 46th week of gestation in human regarding EEG (translatingtime.net) which is already post term in human. The computed modulation index indicates, whether a faster wave changes its amplitude (power) when superimposed on top of a slower wave. With other words, whether the amplitude of the fast oscillation is dependent on the phase of a slower oscillation. This network phenomenon is also known as cross frequency coupling [33] or nesting coefficient [34].

To come back to the initial question, we observed most phase amplitude coupling events between delta and the faster EEG bands theta, alpha and beta in newborn mice (Figure 7, 1a-2c). Taking our power analysis into account it seems, as if these intermediate bands do occur mostly during nested events. This does make sense, because preterm EEG has sparse overall activity with spontaneous activity transients [34] which are most often very slow delta oscillations with nested faster oscillations, also called delta brushes [35]. Surprisingly, we found also coupling patterns in the high gamma range in newborn mice but not in three day old mouse pubs. This does fit to our spectral power analysis, where P0P1 mice have higher power in the gamma as well as fast gamma range by trend in comparison to 3 day old mice. Since the coupling pattern has no similarity with corresponding theta gamma coupling we observed in two week old mice, the fast gamma band in newborn mice might be of different physiological origin, as already discussed for the power analysis. The EEG gamma band in adult mice is associated with GABAergic network activity, especially PV interneurons [36]. Since the physiology of GABA changes drastically during early cortical development in mice [37] [38] and human [34] this assumption is very likely. And it might be a precise indicator of development or mal-development. Prior to GABA driven gamma oscillations, gap junction coupled subplate networks [30] may induce very early gamma rhythms in the cortex of newborn mice, measured here with non invasive EEG electrodes. In two week old mice, delta band is less involved in PAC (phase amplitude coupling) patterns. This is in contrast to all younger mice we recorded (P0/P1 and P3/P4) and in good agreement with data from preterm infants, published recently. It has been shown, that PAC is decreasing for nesting frequencies in the delta range and nested frequencies from 10-32Hz during the first two weeks of life in full term infants [19]. From our data it becomes clear, that two week old animals have higher nested frequencies, the higher the nesting frequency becomes. Also for 1Hz nesting frequency, the main nested frequencies lie well above 30 Hz. In contrast, in newborn mice, there is no proportional relation between nested and nesting frequency. Higher nesting frequencies result also in delta coupling as seen in the Canolty maps (Figure 7: 1c, 2c). This is not the case for two week old animals (Figure 7: 3b,3c).

We think, that these differences in the phase amplitude coupling pattern represent drastic network changes during this critical period of early cortical development. This is not only in good agreement with data from newborn infants where delta associated PAC decreases during maturation [19] but it is also in line with the occurrence of delta brushes in newborn rodents and preterm human infants [16] as well as with theta gamma PACs, seen in the EEG of adult rodents [39] and human [23]. Furthermore, we were able to show similar amplitude coupling patterns for piglets. Gamma coupling is only occurring in 1 day old (Figure 8.1b) and two week old piglets (Figure 8.3a-c) but not in 4 day old piglets (Figure 8.2a-c). Additionally, the coupled gamma band changes to higher frequencies during development (Figure 8.3a-c). Hence, it might be interesting to use phase amplitude coupling as diagnostic tool for brain maturation in the clinical context.

Developmental changes in hemispheric coherence

It is known that hemispheric coherence in preterm infants is probably brainstem driven up to the 35th week of gestation, before callosal hemisphere connections are functional [6]. Up to 28 weeks of gestational age synchronization between brain hemispheres is strong for high amplitude potentials [40]. After a desynchronized phase, synchronous activity is reactivated from a gestational age of 31 weeks onwards [40]. This might be an proximate explanation for the relatively high absolute values of interhemispheric coherence in newborn mice in comparison to two week old animals (Figure 9). Ultimately, this phenomenon might be explained by the syncytial, gap junction coupled network in younger mice in comparison to the fine tuned network, driven by chemical synapses which is already established during later stages of development

in two week old mice [30]. Another explanation could be the extended distance between electrodes in older animals, because coherence may decrease with increasing distance [41]. Nevertheless, other authors do not find volume conduction effects for coherence but an asymmetric pattern for coherence values between different anatomical locations and head axis [42]. For newborn mice, coherence is strongest for the delta band, without a clear preference towards ipsilateral or contralateral (between hemispheres) coherence. This is in strong contrast to two week old animals, where coherence is strongest for corresponding areas of the primary somatosensory cortex between hemispheres (Figure 9). Interestingly, for the Barrel Cortex in the delta band and for the forelimb region in the theta band. It has been shown that EEG coherence is occurring in topographically distinct frequency bands [41]. For comparable vigilance states, coherence might therefore be an interesting tool to tag neurodevelopment with topographical precision.

High degree of phase locking in newborn mice

We compared the phase locking values of distinct frequency bands between neonatal mice (P0/P1) and two week old animals (Figure 10). As seen for the PAC, absolute phase locking values are higher for newborn mice. Two week old animals show most phase locking in the delta band, whereas newborn mice have most phase locking in the gamma band. This is a surprising result, since the high frequency component of the EEG is most often not recorded in the clinical context. Our data indicate, that it might be advantageous to extend neonatal clinical EEG recordings to frequencies up to 100Hz. The two peak distribution in the delta and gamma band in newborn mice is hence not only a quantitative phenomenon of our power spectral density calculation (Figure 5) but also of functional relevance regarding cortical connectivity at an early stage of cortical development. Gamma driven phase locking between Hippocampus and prefrontal cortex has been shown to occur during early development in rat pubs [43].

Taken together, the mouse as well as the piglet might be promising model systems for clinical (non-invasive) preterm brain electrophysiology and development. Simple calculations as for example power spectral density as well as network analysis, like phase locking or phase amplitude coupling show stable differences between age groups, which are corresponding to very early preterm until post term in human. We established a non invasive fbEEG method for newborn mice in order to adapt to clinical methods in a better way. The raw traces of these super cranial EEG traces in developing mice nicely illustrate the transition from discontinuous to continuous EEG (Figure 2a-c). Hence, the mouse might be a powerful model system to understand early EEG patterns in a better way and especially, to distinguish between normal EEG patterns and indicators of future dysplasia in the preterm EEG. The piglet is partially also a good model system, keeping in mind that piglets, as all undulates, are precocious. We were not able to see a discontinuous EEG pattern in newborn piglets (Figure 2d-f) but instead k-komplex like EEG patterns, which are seen in human from an age of several month on [32]. This might either be due to the precocious nature of piglets or it might be due to the fact that piglets are simply much more mature during birth in comparison to mice. The last possibility would indeed mean, that piglets are very much closer to human in comparsion to mice, regarding cortical maturation. Therefore, the combination of both model systems might be ideal to study clinical patterns of cortical maturation in the scientific context.

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