High resolution Electroencephalography in Freely Moving Mice

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Abstract

Electroencephalography (EEG) is a standard tool for monitoring brain states in humans. Understanding the molecular and cellular mechanisms underlying diverse EEG rhythms can be facilitated by using mouse models under molecular, pharmacological, or electrophysiological manipulations. The small size of the mouse brain, however, poses a severe limitation in the spatial information of EEG. To overcome this limitation we devised a polyimide based microelectrode array (PBM-array) with nanofabrication technologies. The microelectrode contains 32 electrodes, weighs 150 mg, and yields noise-insensitive signals when applied on the mouse skull. The high density microelectrode allowed both global and focused mapping of high resolution EEG (HR-EEG) in the mouse brain. Mapping and dynamical analysis tools also have been developed to visualize the dynamical changes of spatially resolved mouse EEG. We demonstrated the validity and utility of mouse EEG in localization of the seizure onset in absence seizure model and phase dynamics of abnormal theta rhythm in transgenic mice. Dynamic tracking of the EEG map in genetically modified mice under freely moving conditions should allow study of the molecular and cellular mechanisms underlying the generation and dynamics of diverse EEG rhythms.
INTRODUCTION

Electroencephalography (EEG) is used to measure electrical potential differences on the brain scalp directly and has been used successfully as a widespread neuropsychological assessment tool. Changes in the regional power spectral density or connectivity strength in different brain regions have been studied with EEG, and tremendous efforts have been made to link these changes with neurological, physiological or psychological correlates. Recent developments in the fabrication of microelectrodes and noninvasive imaging modalities have been promoted to identify the cellular and physiological sources of EEG rhythms (Gonçalves SI 2006; Goncalves et al. 2006; Siapas 2009; Sirota et al. 2008; Whittingstall and Logothetis 2009). Additional ways to dissect the key elements of neural synchrony is genetic perturbation followed by electrophysiological and behavioral analysis in mice.

In recent years, the creation of genetically modified mice has allowed more comprehensive perspectives on the regulation of oscillatory dynamics, usually through investigation of neurological, behavioral, electrophysiological or anatomopathological comparisons to normal mice. These approaches have not only delivered better scientific understanding of brain oscillations but have also yielded potential drug targets for related diseases (Shin et al. 2008). Furthermore, some genes have been reported to affect spontaneous oscillations, e.g., gamma and delta (Joho et al. 1999) or cholinergic theta oscillations (Shin et al. 2005), or gamma oscillations (Llinas et al. 2007). However, most EEG recordings in mice lack spatial resolution, producing difficulties in determining the cortical origins of rhythms. Considering that field oscillations at different frequency bands have been observed in numerous cortical structures (Steriade 2000), the cortical relation to brain oscillations in conjunction with behavior remains to be described in transgenic mice. Despite its significance, lacking spatial information have always hindered the investigators to extend their data interpretation to network level. Recently, Mégevand et al. proposed a spatial mapping technique for modeling large-scale neuronal networks in mouse model using needle-type electrode cluster and mapped the event related potentials successfully in mouse model but restricted to anesthetized mice (Megevand et al. 2008).

A major technical problem in obtaining an EEG map in freely moving mice is maintaining a significant number of EEG electrodes on the mouse brain with consistent contact during a
recording period. More specifically, all the electrodes must be maintained in the same range of impedance, and mechanical stability is required to allow repeated plug-in and -out actions when the application includes longitudinal monitoring. In this article, we present a novel technique to obtain spatially resolved information of mouse cortical EEG in absence of invasive surgery to the brain. We applied soft material as the carrier substrate and fabricated polyimide-based microelectrode array (PBM-array) and produced platinum based microcircuit with lithography technique whose biocompatibility has been proven (Lago et al. 2007). Within the limited area of exposed skull, 32 electrodes were imbedded. Topographical and dynamical analysis tools also have been developed to visualize the dynamical changes of spatially resolved mouse EEG.

Firstly, we demonstrated the validity and utility of mouse EEG by localizing the cortical foci of absence seizure. Although the absence seizure is well established by spike-wave-discharges (SWD), a paroxysmal oscillation in thalamocortical network (Steriade et al. 1993), the initiation mechanism of SWD is still under debate between cortical focus theory and centrencephalic theory (Meeren et al. 2005; van Luijtelaar and Sitnikova 2006). In this article, the initiation timings of SWD were obtained throughout the cortex, and the cortical foci in parietal and frontal regions were explored. This result is expected to be used in identifying the specific contribution of neocortex and thalamus in the initiation of SWD in conjuncture with simultaneous recording of thalamus. Secondly, we confirmed the specific contribution of mouse EEG to genetic studies by characterizing irregular oscillation dynamics in cholinergic theta in phospholipase C (PLC)-β1 deficit mouse. PLC-β1 knock-out mice lacks in cholinergic theta rhythms with a reduced spectral power in urethane induced theta bands (Shin et al. 2005), however, whether the residual theta rhythm has the same origin with cholinergic theta is still unknown. Here, we analyzed the phase dynamics of cholinergic theta in wild and PLC-β1 knock-out mice and confirmed that spatial coherency of cholinergic theta is absent in the mutant, suggesting different origin of the residual theta rhythm. Our methods can be readily tested in other knock-out mouse models and expected to contribute in investigation of the generating mechanism of EEG signals in molecular and cellular levels.

MATERIALS AND METHODS

*Nanofabrication of Bifurcated and Soft Microelectrode*
The structures of PBM-array were fabricated using a well-established nanofabrication process. The electrical contacts, the connection lines, and the interconnection pads were made of 300 nm thick platinum, deposited by sputtering on a spin-coated polyimide substrate (Pyralin 2611, HD Microsystems, Bad Homburg, Germany) of 5 µm thickness. After patterning of the metal layers using a photolithography process, a second layer of polyimide of the same thickness was spin-coated on top of the structure. The electrical contacts and the interconnection pads were then opened through selective reactive ion etching of the polyimide layer. Two connectors with 16 pins each (Omnetics Connector Corporation, Minn., USA) were attached to the interconnection pads using a conductive glue, to provide an interface to the recording equipment. In order to improve the electrical properties of the electrode, the contacts were platinized by electroplating.

**Characterization of Microelectrode**

Before and after platinization, the electrodes were electrochemically characterized by means of impedance spectroscopy (measurement amplitude: 50 mV, frequency range: 10 Hz - $10^5$ Hz). The measurements were performed using a three-electrode set-up with a platinum counter electrode (PT 1800, Schott Instruments, Mainz, Germany) and a silver/silver chloride reference electrode (B 2920, Schott Instruments, Mainz, Germany). The set-up was connected to an electrochemical interface (1287, Solartron Analytical, Farnborough, UK) and a frequency response analyzer (1255, Solartron Analytical, Farnborough, UK). The measurements were performed at room temperature in physiological (0.9%) saline solution. Prior to impedance spectroscopy, the electrode was cycled between -0.6 V and +0.9 V (scan rate: 0.1 V/s), in order to remove organic residues on the electrode surface and thus to stabilize the impedance measurements (Franks et al. 2005).

**Animal Preparation and Surgery**

All surgical, handling, and experimental procedures were conducted in accordance with the guidelines for the Institutional Animal Care and Use Committee, following Act 1992 of the Korea Lab Animal Care Regulations and associated guidelines. The experiments on signal performance and pharmacologically driven seizure were performed with 7-8 weeks female C57BL/6J-129S4/SvJae hybrid mice (19 g -23 g). The experiments on urethane-induced theta were performed on 10 week old F1 homozygous male mice and wild-type littermates obtained by cross C57BL/6J(N8)PLC-β1+/− and 129S4/SvJae(N8)PLC-β1+/− mice, whose
genotypes were determined by polymerase chain reaction (PCR) analysis. For PBM-array implantation, the animal was anesthetized with an intraperitoneal dose of Avertin (2%, 20 μl/g), and then head-fixed in a stereotaxic apparatus (David Kopf Instruments, Model 902, Tujunga, CA). One inch incision was made at the midpoint of the scalp and exposed by micro clamps. Prior to placing the PBM-array, the skull was wiped with a saline-soaked cotton ball to remove any tissue debris as well as to give moisture to the skull for better adherence to the PBM-array. A wet condition helps the branches of PBM-array be pulled to the skull by Van der Waals forces. The PBM-array was carefully aligned so that the vertical midline met the midline of the skull and the bregma met the vertical midline and the upper edge of the 3rd branch. An important aspect of the securing process is to ensure that the branches of PBM-array are adhered to the skull prior to the administration of glass isonomer. For electromyogram (EMG) recording, a Teflon-coated tungsten electrode was inserted into the dorsal neck and grounded with the PBM-array ground electrode. After positioning electrodes, self-curing glass isonomer (Vivaglass CEM, Ivoclar Vivadent, Germany) was carefully coated over the PBM-array to cement the PBM-array and the bone. After curing, the incised skin was sutured. The animal was returned to a cage with no littermates under a thermal lamp for recovery.

**EEG Recording**

For initial experiment signal performance, a Grass 8-16C amplifier (Grass Technologies, West Warwick, RI) was applied with band-pass filtering from 0.3 Hz to 70 Hz with a 60 Hz notch. The analog signals were digitized by a 16-bit Digidata 1440A (Molecular Device, USA) at a sampling frequency rate of 1 kHz. For experiments on seizure and theta mappings, EEGs were recorded with a SynAmps amplifier (Neuroscan Inc. El Paso, TX). The low impedance electrode on the left was used as a reference electrode and the other low impedance electrode on the right was used as a ground electrode. Signals were recorded by Scan 4.3 (Neuroscan Inc. El Paso, TX) at a sampling rate of 1000 Hz with bandpass of 0.1 Hz – 100 Hz. The dynamic range of mouse EEG recordings was within 100 μV and the impedance of skull-electrode measured by Scan 4.3 was below 100 kΩ at test frequency of 30 Hz.

**Data Analysis**
All the off-line analysis was carried out on a PC by means of in-house programs developed in the Matlab (Mathworks, Natick, MA) programming platform.

**Statistical Analysis**

Descriptive statistical analysis of behavioral parameters before and after PBM-array implantation was performed by the Paired t-test. As for the conventional parameters of EEG (e.g., frequency, power values, phase delay, coherence, and phase synchronization index), the Kruskal-Wallis one-way analysis of variance tests were applied. Each frequency band was filtered by Butterworth zero-phase symmetric using Filter Design Toolbox in Matlab. All differences were regarded as significant if $p < 0.05$.

**Detection of Spike-Wave-Discharge (SWD)**

The characteristic patterns of SWD in rodent EEG are 3-5Hz repetitive high-voltage negative spikes (20–50 msec of spike width, positive in case of depth-EEG) followed by larger high-voltage positive wave (~200 msec of wave width, negative in case of depth-EEG) lasting longer than 1 sec (van Luijtelaar and Coenen 1986). SWD extraction procedures is following:

(i) the Daubechies 5 wavelet analysis was performed and the filtered signals were reconstructed from decomposed signals at the 5th, 6th, 7th, and 8th levels, corresponding to 15.6 - 31.3, 7.8 - 15.6, 3.9 - 7.8, and 2.0 - 3.9 Hz, respectively in case of 1 kHz sampling rate.

(ii) The positive peaks larger than predetermined threshold values were detected. In this study, the threshold values were set to be $5 \times \text{standard deviation of baseline}$. (iii) The inter-peak distance in msec was calculated. If the difference in neighboring inter-peak distance is smaller than 20 msec and the values of inter-peak distance are in the range of 180 - 250 msec, we defined the moment as equidistance intervals. (iv) The standard deviation of EMG was calculated at each equidistance interval and only quiescent moments were selected. (v) The rising and falling slopes to each peak within the equidistance interval were calculated. When rising is faster than falling repetitively, we considered the interval as SWD moment. The onset of SWD was defined at the moment of the first peak in the equidistance interval.

**Topographic Mapping**

Prior to interpolation, each EEG channels was divided by normalization factor, which was defined by the average power of the range, 130 Hz to 170 Hz within quiescent moment of the animal. Ellipsoidal boundary was arbitrarily drawn based on the electrode coordinates and
imaginary points were generated in a linearly spaced way between electrode positions. The potential value of the imaginary points were calculated from cubic spline interpolation method. For the boundary values, we adopted the 10 percentiles of the dataset. The topographic map was displayed by drawing contour of the matrix.

**Function Connectivity**

In case of epileptic signals, phase synchronization index was used to scale the synchrony level. The instantaneous phase, \( \phi(t) \) of the measured EEG signal was obtained by Hilbert transform (Piersol 1986)

\[
\Delta \phi_{n,m}(t) = \phi_n(t) - \phi_m(t),
\]

and bra-ket notes an ensemble average, and \(|...|\) means absolute values of the complex number (Rosenblum et al. 1996). Please note that the index ranges from 0 (independent fluctuations, i.e., \( \Delta \phi_{n,m} \) is randomly or uniformly distributed) to 1 (perfect phase synchronization, i.e., \( \Delta \phi_{n,m} \) through the observation time).

In case of oscillation signals, coherence is used as a measure of synchrony between EEG signals, calculated between pairs of signals as a function of frequency. The coherence \( C_{xy}(f) \) for two continuous time series \( x(t) \) and \( y(t) \) is defined as the absolute square of the cross-spectrum \( P_{xy}(f) \) normalized by the auto spectra \( P_{xx}(f) \) and \( P_{yy}(f) \), having values in the interval from 0 to 1 (Bendat and Piersol 1986). A significance threshold for the coherence is then determined by \( 1-0.05^{1/(T-1)} \), where \( T \) is the number of EEG segments, as an estimate of the upper 95% confidence limit under the hypothesis of independence (Halliday et al. 1995).

**Phase Dynamics of Oscillation**

The phase of the Hilbert transform of EEG signals is often used in determining the phase of the signals in the case of a narrow-band oscillation. A narrow-band oscillation can be expressed as

\[
x(t) = A(t)e^{i\phi(t)},
\]

which resembles a sine wave with slowly varying frequency and amplitude or a superposition of sine waves with similar frequency. The amplitude \( A(t) \) and the instantaneous phase \( \phi(t) \) of the signal are uniquely determined by Hilbert transformation. The phase relationship between two EEG signals has been widely applied to EEG studies, including the characterization of synchrony of human epileptic or noisy signals.
(Freeman et al. 2003; Lai et al. 2007), investigation of underlying control mechanisms of
certain oscillations such as spindle or alpha/beta (Nikulin and Brismar 2006; Varela et al.
2001), and definition of large-scale integration among cortical areas (Engel et al. 1991;
Roelfsema et al. 1997).

RESULTS

Development of Bifurcated and Soft Electrode for Mouse Skull

Standard Coordinates for Mouse EEG

The electrode arrangement for mouse EEG tomography needed to cover the exposed skull
and electrodes needed to be evenly distributed so that an accurate scalp potential map could
be obtained for estimating the current sources inside the brain. The area of electrode coverage
was collected from C57BL/J6 mice (weight range 25 g – 30 g), which were chosen due to the
availability of neuro-anatomical atlases (Paxinos and Franklin 2008). The shape and size of
the active electrodes were determined for a circle with a diameter of 500 μm, at which the
maximal EEG signal-to-noise ratio was measured (Figure 1A). Reference and ground
electrodes were designed to be on both sides of the cerebellum. The null space between
electrodes was cut off to enhance flexibility. The electrode array has a bifurcated structure
with six branches on each side (Christmas tree shape), and each branch has two or three
electrical contact points, covering the exposed skull in an evenly distributed manner (Figure
1B). The inter-distance between two neighboring channels was 1.42±0.31 mm in Av±Std. The
coordinates of the active electrode were decided to be in the middle of one cortical area based
on the mouse brain atlas (Paxinos and Franklin 2008): ±0.5/2.3 (in mm, anteroposterior/lateral, secondary motor cortex, M2), ±1.5/2.3 (M2), ±0.55/1.04 (M2), ±1.93/1.04 (primary motor cortex, M1), ±2.12/-0.48 (primary somatosensory cortex, fore/hind limb regions, S1FL/S1HL), ±3.5/-0.48 (primary somatosensory cortex, barrel field, S1BF), ±1.12/-1.96 (medial parietal association cortex, MPtA), ±2.88/-1.96 (S1BF), ±4.05/-
1.96 (secondary auditory cortex, AuV), ±1.13/-3.04 (secondary visual cortex, V2), ±2.88/-
3.04 (primary visual cortex, binocular area, V1B), ±4.13/-3.04 (primary auditory cortex, Au1), ±1.0/-4.14 (V2), ±2.24/-4.14 (primary visual cortex, monocular area, V1M), ±4.05/-4.14
(temporal association cortex, TeA), under the condition that the vertical midline and the
upper edge of the 3rd branch lays exactly on the bregma point (origin of coordinate axes).
Figure 1C shows a photograph of the complete electrode structure, including the connector. The PBM-array was made thin in order to sit on the mouse skull naturally with no introduction of additional force. For the electrode substrate, we used polyimide. Polyimide films with various thicknesses from 5 μm to 40 μm were tested for adherence to the mouse skull. We found that 10 μm is a critical thickness for quick placement of the electrodes. After wiping the skull with a saline soaked cotton ball, a 10 μm thickness film easily adhered to the skull with no additional force or glue and remained for hours, whereas a 15 μm thickness film was easily detached from the skull due to the tension of the film.

The nanofabricated microelectrode had an impedance range of 2 kΩ to 3.5 kΩ in the frequency range of 10 Hz – 10^4 Hz. As an example, Figure 1D shows the absolute values of impedance and Figure 1E shows phase shifts of the platinized contacts showing the electric performance of the sample. All the electrodes exhibited an exponential decrease in impedance with respect to frequency, which is a characteristic behavior for a general electrode-electrolyte interface.

Each electrical contact was aligned to the interconnection pad, which was arranged to fit two double-row microconnectors (NSD-16-VV, Omnestics Connector Corporation, Minneapolis, MN). The microconnectors were attached to the electrode pads with the aid of conductive glue. An instant adhesive was applied to encapsulate the edges of the connector to enhance the mechanical stability. The integrity of the electrode and connector connection eased implantation dramatically and also made the study subjects accessible to the recording machine on demand.

**Screw-Type Electrode Vs. PBM-array**

We compared the signal quality between the PBM-array and the conventional screw electrode by simultaneous measurement of both types during epilepsy simulation. In this application, one mouse (25 g, 10 weeks) was anesthetized with Avertin (2%, at a dose of 20 ml/kg bodyweight) and mounted in a stereotaxic apparatus (Lab Standard, Stoelting Co, IL, USA). The film electrode was carefully cut to cover only left hemisphere. One screw electrode was placed on the right lobe (AP: -2.5 mm, L: +2.5 mm) and the contact of the film electrode was placed in a symmetrical position to the screw electrode with respect to the midline lobe (AP: -
The reference electrode was implanted on the center of frontal lobe and bifurcated into two connectors to be used as a common reference. The ground electrode was placed over the occipital lobe 2 mm dorsal to the lambda point. We applied γ-Butyrolactone (GBL, Sigma, MO, USA) to the animal with a dosage of 75 mg/kg to induce bilaterally synchronous spike-wave-density (Snead et al. 1999). We observed that the spike-wave-discharge (SWD) occurred approximately 30 min after the administration of the GBL. In our study, the frequency of SWD was about 9 Hz-13 Hz. Figure 1F shows a sample time series showing SWD collected from both film and screw electrodes. The signal level of the film electrode was 10 times less than that of the screw electrode under the same amplification settings, but waveform and frequency information was successfully delivered with the film electrode.

The implantation of the film microelectrodes took less effort and time compared to the conventional screw electrodes. Figure 1H shows the dorsal view of the mouse with electrodes on its skull. After applying dental cement for fixation, we sutured the incised scalp. The microconnector enabled us to connect the recording wires in a convenient manner and the multichannel EEG acquisition can be performed in a freely moving condition (Figure 1I). The detailed procedure is described in the method section.

**EEG Recordings in Freely Moving Mice**

After implantation and fixation of the film microelectrode array on mice skulls using dental cement, no severe disorder, such as limpness or withering, was observed throughout the study.

**Long-Term Stability of EEG Recordings for Longitudinal Study**

The levels of signal-to-ratio (SNR) were evaluated in longitudinal recording over a month of six freely behaving animals to explore the long-term stability of the EEG signals. With EEG, the definitions of signal and noise are unclear due to the complex properties of EEG recordings. We selected theta rhythms as signals in the frequency range from 4 Hz to 8 Hz. For the background noise, the frequency range of 190 Hz to 230 Hz was chosen to avoid the harmonics of 60 Hz line noise and/or any other physiological rhythms. Figure 2 shows the average and standard error mean of temporal development of SNR from 8 days to 41 days after surgery divided by the SNR on the 8 days after surgery. We observed that the signal
quality was maintained for about one month after surgery and dramatically deteriorated after this period.

Movement artifacts

The recording duration and the quality of the data are highly dependent on mechanical stability versus movement of the subject. A co-registration of video, motion sensor, and EEG recordings assured no significant changes occurred during mild movements such as sniffing, drinking water or licking paws. On inspecting the raw EEG results, protruding artifacts sometimes appeared; e.g., when the animal bumped into a wall (Figure 3A) or jumped with a 180 degree turn (Figure 3B). On the other hand, scratching the ear, which is a very perturbing moment in terms of interface noise, did not evoke either a transient or lasting artifact (Figure 3C). The example in Figure 3D shows the power of EEG rhythms while an animal jogged on a treadmill with three different speeds (5 cm/s, 10 cm/s, 15 cm/s). The activity level is the vector magnitude of three dimensional accelerometer signals. Some rhythms showed increased power compared to the resting state, but no correlation to activity levels was found. Our successive recordings suggested that the condition of the connector jack or insulation of the wire lead were crucial in barricading any unwanted noise.

Functional Brain Mapping of Mouse EEG

Spatio-temporal mapping of multichannel EEG signals to the mouse brain is similar to other EEG topographic mapping techniques in human EEG. However, in mouse EEG, the cortex region covered by the electrode is limited to the frontal, central, parietal and upper temporal regions, which restricts the precise identification of a zone of an EEG event in terms of depth. While the potential mapping delivers surface information, nevertheless noninvasive cortical localization of EEG in functioning mouse can image the generation or dynamics of certain rhythms or epileptic signals or event-related signals in gene modified animals

Characterization of Seizure Onset: A Case of Spike-Wave-Discharge

Absence seizure were recorded on mice skulls with PBM-array by systemic administration of gamma-butyrolactone (GBL, 50 mg/kg) (Snead and Bearden 1980). GBL is known to generate spike-wave-discharge in thalamocortical networks (Ishige et al. 1996). Sample traces of EEG and EMG with SWD episode are exhibited in Figure 4A and the SWDs robustly occurred in GBL model. The electromyogram (EMG) was simultaneously recorded
in the dorsal neck and only motionless SWD episodes were exclusively extracted. The absence seizure globally was observed in all channels, and it was noticeable that strong signals were detected favorably in the parietal and frontal cortex. The power was evaluated both for non-epileptic and SWD intervals and the spatial distribution of ensemble average of power ratio during SWD with respect to the baseline was mapped in Fig. 4B (N=10, one mouse). The same length of time interval at 2 sec after cessation of SWD was picked as baseline. Compared to the baseline, power of the signal increased significantly particularly in somatosensory area including barrel, limb area, whereas the power of SWD in posterior parietal or occipital lobe did not increase significantly during SWD. We found that the power pattern of SWD did not hold the hemispheric symmetry. In our ten SWD samples, only two samples had SWD dominance in right hemisphere, and the other SWD had preference to left hemisphere. The cross-channel connectivity was mapped by evaluating phase synchronization index for every pair of channel and only the pairs of increased connectivity with statistical significance were overlaid in Fig. 4B. It is noticeable that the cross-channel connectivity increased during absence seizure mostly in the ipsilateral way, except the connection between prefrontal and occipital cortex. The SWD onset was identified by detecting the first peak of the SWD and the ensemble averages of the onset time, $\tau$ in msec and the peak strength in $\mu$V were depicted in Figs. C and D, respectively. $\tau$ was defined by the time delay of the first peak at the corresponding channel to the firstly appeared SWD within the whole channels. It is noticeable that left parietal cortex dominantly leaded the absence seizure within the cortex. Although there are a heterogeneity in onset patterns, for example, frontal onset was observed in some cases, the probability of onset area shows that left parietal corresponding to primary somatosensory cortex leads the SWD in the cortex as shown in Fig. 4E.

Characterization of Rhythm Phenotype: A Case of Irregular Cholinergic Theta Rhythms in PLC $\beta_1$ Knock-Out Mouse

The advantage of studies using transgenic mice is in the availability of valid behavioral mouse models. We applied our EEG mapping technique to map a deficiency of hippocampus theta rhythms in the phospholipase C (PLC)-$\beta_1$ -/- mouse by i.p. injection of urethane (0.8 g/kg). PLC-$\beta_1$ -/- mice have been shown have a lack of cholinergic, type II, hippocampal theta rhythms (Shin et al. 2005). As expected, the EEG map of PLC-$\beta_1$ +/- mouse shows robustly synchronized theta oscillations (Figure 5A). Over the entire cortex, the theta
oscillations occur dominantly with almost zero time lags. On the other hand, as characterized
by previous hippocampus local field recordings, PLC-β1 -/- mice showed a deficit of
cholinergic theta oscillation. In our 30 channel EEG measurements, rhythms were not
completely abolished in all the channels, however. Average power spectra for all the channels
re-affirmed that PLC-β1 -/- mouse had diminished theta rhythms with frequencies lower than
wild type littermates (Figure 5E). Topographic maps of theta amplitude plot the regions of
the brain where theta activity are the most prominent (Figures 5C and 5D). Additionally, the
long-range connectivity of neuronal activity has also been derived from the coherence of
theta at two different sites and is depicted as connecting lines on the topographic maps. The
coherence map shows that the cholinergic theta in PLC-β1 +/+ mouse maintains coherence
over the brain, and additionally shows that the frontal area has dynamics independent of theta.
On the other hand, theta exists in PLC-β1 -/- mice, but in a spatially local and incoherent way,
implying that the network generating theta is partially broken down, nonexistent or modified.

From a temporal point of view, these power changes in theta bands are associated with
impairments in the ability of distributed networks to establish precise synchronization of
neuronal assemblies oscillating at theta frequencies. The distributions of phase differences
between theta rhythms at two different sites were evaluated for all combinations and plotted
in Figure 5F. The distribution of sharp peaks around zero was found in case of PLC-β1 +/+ 
mice theta oscillations, whereas a broad distribution of phase differences were seen in
association with PLC-β1 -/- mice rhythm. The theta oscillations in PLC-β1 +/+ mice were
formulated in a synchronous manner over the brain, and the theta oscillations in PLC-β1 -/- 
mice occurred in an asynchronous manner. Considering that urethane-induced theta rhythms
are synchronous (Buzsáki 2002; Buzsáki et al. 1986; Fox et al. 1986), the irregular
propagation of theta rhythms in PLC-β1 -/- mice suggests that the theta power observed in
PLC-β1 -/- mice might have different origins from urethane-triggered resonances shown in
the wild type.

**DISCUSSION**

Here we report on a new method for high-resolution EEG mapping in freely behaving mice
using a nanofabricated microelectrode array and neuroimaging techniques. This method
solves the problem of limitations in acquiring spatial information using conventional mouse
EEG by allowing EEG using multiple electrodes distributed over the skull. To our knowledge, there is no comparable method for EEG mapping of freely moving mice that can be used to obtain functional brain imaging phenotypes in mutant mice.

Use of nanofabricated PBM-array was the key element in achieving a high-density electrode configuration in our method. PBM-array have been used for human electrocorticograms (ECoG) (Mercanzini et al. 2007; Rubehn et al. 2009) and rat ECoG (Yeager et al. 2008), but have not been applied to mouse EEG to the best of our knowledge. We chose this material because of the light-weight and flexible characteristics, the biocompatibility that has been proven in cell viability tests (Sun et al. 2008), and results from morphological studies after chronic implantation (Lago et al. 2007). The impedance spectrum of PBM-array is relatively wide and tunable mostly by adjusting the size of electrical contact. In chronic recordings, PBM-array has been proved to have great signal fidelity in recording cell-level activities (Rousche et al. 2001) and cortical potentials in addition to demonstrating long endurance in nerve (Stieglitz et al. 2000) or cortex (Rubehn et al. 2009; Yeager et al. 2008) stimulation. In our study, determining the correct impedance range for the electrodes was an important issue. The signal-to-noise ratio for different sizes of electrical contacts (Figure 1A) narrowed down the impedance range and pilot recordings with different pitch sizes lead to the setting of the current design parameters. In the future, more rigorous studies must be carried out in regard to the impedance between brain and electrode interface, which we expect will be a function of the impedance of the electrode, frequency of the rhythm, and conductivity of the tissue.

**Advantages of spatially resolved mouse EEG**

During the absence seizure in both hemispheres using one electrode anchored by a microscrew and PBM-array on the other hemisphere, the bilaterally synchronized spike-wave-discharges were recorded producing almost identical waveforms with neither frequency nor phase distortion (Figure 1F). This suggests that PBM-array will successfully replace microscrew electrodes for skull EEG recording, especially when spatial information is desired. From the perspective of measurement capabilities, a flexible, ultra-thin, film-type, high-density EEG electrode offers a number of advantages compared to existing screw type electrodes. First, film-type electrodes can reduce surgery times dramatically with no need for invasive surgery to the skull or brain. This not only produces less stress in the animal but also shortens the recovery time after surgery. Our experience with PBM-array informed that
recording immediately after cement cured is possible. Secondly, PBM-array do not require an anchoring process on the skull hence, do not induce hemorrhaging in the brain during surgery. Blood clot under the skull has been the main source of artifacts unrelated to brain activation. Thirdly, the flexibility of fabrication with polyimide enables us to make null spaces between electrodes, and secondary electrode or needle can be applied for multi-modal recording or simultaneous microinjection. In sum-up, application of PBM-array reduces the stress not only in the animal but also in the experimenter by delivering efficient and more successful way of implantation.

Physically, the spatial distribution of the electric source is estimated by a potential map in space. The electromagnetic model of the synchronized synaptic action in neuronal groups is a current dipole (Nunez and Srinivasan 2006) and the location and size of a neuronal circuit generating the synchronized behavior can be gauged from the potential map. Therefore, multichannel recording is required for the characterization of synchronization. Figures 5C-D show the potential maps of theta rhythms in wild and transgenic mice, respectively. This topographic information discerns the cortical regions involved in certain rhythms or patterns and cine-modes can be generated to visualize the generation, propagation, and termination of the waves. Furthermore, higher-level cognitive mechanisms are associated with functional relationships between different brain regions and one channel EEG is not adaptable to the study of multiple-range connectivity of the brain during cognitive processes. The spectral coherence between different regions of brain visualized the cortical connectivity of the mouse brain. In summary, recruiting more electrodes presents not only spatio-temporal dynamics of neuronal synchrony but also the functional connectivity of different cortical regions.

**Localization of absence seizure onset in mice**

The investigation of generating mechanisms of SWDs during absence seizure has been one of the most popular topics in neuroscience and specific contribution of brain regions or cells or neurons or molecules are still under investigation. A systemic diagram incorporating molecular to systemic level needs to be built. Our demonstration showing the SWD onset from intact brain of behaviorally unrestricted mice is one trial to understand the molecular backgrounds of SWDs in system level. We observed that the initiation of cortical SWD happened in a focal manner. Mostly parietal and frontal cortex initiated SWD and by scoring the probability of seizure onset, left primary somatosensory cortex was the dominant leader
of SWD. This result is in line with previous finding with a rat model with spontaneous
absence seizure showing that SWDs are initiated in the facial region of the somatosensory
cortex (Meeren et al. 2002). Recently, genetic study have contributed to the understanding of
generation of absence seizure in the molecular level, for instance, $\alpha_{1}$G subunit of low-
voltage-activated T-type Ca2+ channels in thalamocortical relay neurons are involved in the
genesis of absence seizure (Kim et al. 2001), on the other hand, phospholipase C $\beta$4 in
thalamic reticular neuron conduces absence seizure (Cheong et al. 2009). However, it is true
that these genes have a role as modulator rather than exterminator or source for SWDs. A
dynamic mapping of SWD in these genetic models is expected to visualize in which moment
or which circuit these genes are involved in generation of SWD.

Phase synchrony and dispersion with EEG in mice

Some opinions hold that the process of integration is based on the interplay between phase
locking and phase dispersion across different bands and at different moments in time (Varela
et al. 2001) and EEG phase synchrony reflects that exact timing of communication between
distant but functionally related neural populations (Sauseng and Klimesch 2008). One of the
primary issues in high density mouse EEG is how precisely the locking and dispersion of
phase can be tractable in terms of temporal and spatial resolution. Recently published work
on hippocampal theta oscillations beautifully demonstrated the propagation of theta along the
septotemporal (vertical) axis of the hippocampus using multichannel silicon probes (Lubenov
and Siapas 2009). An equiphase contour was observed within layers in their study, and
therefore the travelling wave nature was not observable with our horizontally arranged PBM.
However, the topography of theta phase offsets across the brain was demonstrated (Figures
5A and 5B). Theta oscillations occurring in PLC $\beta$1 +/-, wild type, mice were synchronized
with zero delay (Figure 5A), whereas theta oscillations in the PLC $\beta$1/-/- mice were locked to
each other both in- and out-of-phase (Figure 5B). Over the entire period of time, the phase
differences between two sites in PLC $\beta$1 +/- mice remained small, whereas those values in
PLC $\beta$1/-/- mice had a broad distribution (Figure 5H). This evidence clearly shows that PLC
$\beta$1/-/- mice do not have a global clock. This information is useful especially when some
residual rhythms remain in the power spectrum (Fig. 5C) but decisions need to be made
whether their origin is the same as for wild type mice.
The expected contribution of transgenic mice to the understanding of mechanisms underlying EEG rhythms

Recordings from EEG are now taken very seriously as important signals from the brain. As noted in the introduction, EEG in transgenic mice has been used to dissect brain rhythms related to genetic deficiency, which are directly linked to signaling via related receptors or ion channels, eventually disturbing signal transmission in the network. The most intriguing questions arise when the altered rhythm observed in mutant mice is correlated with cognitive or behavioral dysfunctions. It may not be the most significant example, but PLCβ1 knock-out mice, lacking cholinergic theta waves (Shin et al. 2005), showed locomotor hyperactivity and sensorimotor gating deficits as well as cognitive impairments (Koh et al. 2008; McOmish et al. 2008). The historical data of Lennox (Lennox and Davis 1950) and related works on human EEG in twins (Pelliccioli and Garioni 1955) suggested that there exists syndrome-specific genotypes. Apart from linkage to a specific pathological gene, integration of electrophysiological, pharmacopathological or neuroanatomical studies with recordings from mouse brains will allow identification of molecular, neuronal or circuitry targets as potential causes of pathology. The in vivo study of brain wave mechanics over the entire brain will add complementary knowledge to the current neuroscience database in an effective way.

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**Figure Legends**

**Figure 1.** Mouse skull EEG electrodes: Polyimide Based Microelectrodes (PBM). (A) The signal-to-noise ratio (SNR) for various sizes of electrical contacts in PBM. EEG bipolar and monopolar recordings were obtained on the mouse skull. SNR was calculated for delta, $\delta$ (1 Hz - 4 Hz) and theta, $\theta$ (5 Hz - 8 Hz) rhythms. The SNR (dB) was obtained by calculating $10\log\left(\frac{P_{\text{signal}}}{P_{\text{noise}}}\right)$, where $P_{\text{signal}}$ is the mean power spectral density for corresponding frequency band and $P_{\text{noise}}$ is the mean power spectral density between 190 and 230 Hz. The SNR curves show that the range 400 mm – 500 mm has a relatively large SNR. (B) Arrangement of high density mouse EEG electrodes. (C) Photo of fabricated PBM. Approximate dimensions of PBM-array are noted. The layered structure of the PBM-array is shown in the inside of the box. (D) The absolute impedance ($|Z|$) in Ohm of the PBM-array plotted with respect to the measurement frequencies. Standard deviations are marked as error bars. (E) Phase shifts in degrees plotted with respect to the measurement frequencies. Standard deviations are marked as error bars. (F) EEG recordings from a mouse after administration of GBL. Both electrodes were located symmetrically around the midline. The EEG signal of the film electrode was multiplied by 10. In both EEG figures, SWD epileptic patterns were observed. The horizontal bars indicate the SWD epileptic events. (G) The signal-to-noise ratios (SNR) of film and screw type electrodes for delta (1 Hz - 4 Hz), theta (5 Hz - 8 Hz), alpha (9 Hz - 12 Hz), beta (12 Hz - 30 Hz), gamma (30 Hz - 60 Hz). The bars represent the average of SNR from five mice and the error bars are the standard error mean. The SNR of film and screw electrodes did not show any statistical significance (one way ANOVA test for significance level, p-value of 0.05). The SNR values were calculated as defined in Fig. 1(A). (H) Mouse with exposed skull after placement of PBM. Any membrane layers on the skull were removed and a small amount of saline was applied over the skull for adhesion of the electrode to the skull. (I) A freely moving mouse in one of our EEG recording sessions.

**Figure 2** Long-term EEG recording in PBM-array implanted mice. The vertical axis is the SNR normalized by the SNR on 8th day after implantation averaged over 6 mice to normalize the individual difference in SNR scale. The error bars indicates the standard error means. SNR values were calculated as defined in Fig. 1(A). In each day, one hour of recording has
been carried out and 10 minutes of awake period were used for calculating the power spectral density.

**Figure 3.** EEG recordings are stable with mouse head motion. (A) Raw traces of co-registered one channel EEG (top) and accelerometer (bottom) of the mouse in a freely moving condition, around the time that the mouse knocked its head against the cage wall. Note that EEG continues after the brief transient. (B) Raw traces of co-registered one channel EEG (top) and accelerometer (bottom) of the mouse in a freely moving condition, around the time that the mouse jumped with a 180 degree turn. Similar actions sometimes resulted in artifacts in EEG. (C) Raw traces of co-registered one channel EEG (top) and accelerometer (bottom) of the mouse in a freely moving condition, around the time that the mouse started to scratch its ear. Note that the head motion appeared as large amplitude fast oscillation in the accelerometer but did not evoke either transient or lasting artifacts in EEG. (D) Treadmill test while the mouse jogs on the treadmill with three different speeds (5 cm/s, 10 cm/s, and 15 cm/s). Each test stage lasted for five minutes and resting periods were recorded before and after exercise for five minutes (noted as 0 cm/s). The activity level is the vector magnitude of the 3-axis accelerometer signals (black dots, solid line). The powers of rhythms were calculated every 10 seconds and their average and standard deviations were drawn in bar graphs with error bars. Some rhythms showed increased power compared to the resting state, but no correlation to the activity level was found.

**Figure 4.** Localization of cortical foci of absence seizure in mouse brain. (A) A sample trace of EEG experiencing absence seizure induced by i.p. injection of GBL. The number follows the channel montage defined in Fig. 1B. The horizontal and vertical axes indicate time and voltage, respectively. The scales are denoted in the left bottom of the figure. The displayed signals were filtered by zero-phase Butterworth bandpass filter (0.5 Hz - 100 Hz). During absence episode, EMG was relatively quiescent. (B) Topography of ensemble average of power of SWD normalized to the baseline periods were mapped (N=10). Power means an integration of spectral power over in the frequency range of 2.0 - 31.3 Hz. The value in the colorbar represents the ratio of power during SWD to power during baseline. The lines represent the functional connectivity produced by SWD. Phase synchronization index during SWD and baseline period was evaluated for every pair of channels. Kruskalwallis test, a nonparametric one-way ANOVA test was performed and the pairs with p-value smaller than
0.05 were marked by black lines. Not a single pair showed decreased connectivity in a statistically significant way, hence all the pairs linked by lines increased connectivity during SWD in a statistically significant way. (C) Topography of ensemble average (N=10) of time delay, $\tau$ in msec of the first peak of SWD at each channel with respect to the earliest peak throughout all the channel. (D) Topography of ensemble average (N=10) of peak strength, $V$ in $\mu$V at the first SWD. (E) The average probability of seizure onset at each location. The probability of seizure onset was found by a scale function $\sim 1/\tau$ satisfying $\int c/\tau d\tau = 1$. It is noticeable that the somatosensory cortex is the dominant leader in SWD generation.

**Figure 5.** EEG recordings of theta rhythm in the mouse brain. Simultaneous cortical recordings from 30 channel EEG under urethane anesthesia in (A) PLC-$\beta_1$ +/+ and (B) PLC-$\beta_1$ -/- mice. Raw traces are ordered in the left panel and the amplitude of theta rhythm filtered by zero-phase butterworth filter with passband from the 4 Hz – 8 Hz bands are color-coded in the right panel. Up and down phases of theta rhythms have red and blue colors, respectively. Black dots mark the positive peak of theta waves. Lack of theta coherence between channels appeared in a PLC-$\beta_1$ -/- mouse. Topography and the coherence map of theta rhythms in (C) PLC-$\beta_1$ +/+ and (D) PLC-$\beta_1$ -/- . The colorbar indicates the values of power spectral density (mV$^2$/Hz) in theta band (4 Hz - 8 Hz). Black dots indicate the channel positions. Lines were drawn between two channels, whose coherence exceeded the significant threshold, which was 0.79 to visualize the connectivity. The power and coherence maps of PLC-$\beta_1$ +/+ show that the cholinergic theta are globally synchronized. In PLC-$\beta_1$ -/-, theta oscillation locally present and coupling between oscillations are spatially segregated. (E) Average power spectrum of 30 EEG channels. Here the $x$ axis and $y$ axis represent frequency and power spectral density, respectively. Error bars represent the standard deviations across the channels. The plots show peaks at 5.3 Hz and 3.9 Hz in PLC-$\beta_1$ +/+ and PLC-$\beta_1$ -/- mice, respectively. Note that theta rhythm in PLC-$\beta_1$ -/- mouse was not totally abolished. (H) The distribution of phase differences between two different sites for theta rhythms in PLC-$\beta_1$ +/+ (black) and PLC-$\beta_1$ -/- (red). Sharp peak around zero phase difference in PLC-$\beta_1$ +/+ indicates the temporal delays of theta oscillation in different cortical regions are small and oscillations are locked to each other. On the other hand, the presence of broad distribution in PLC-$\beta_1$ -/- indicates that the oscillations are not dynamically coupled to each other.