Limitations of ex vivo measurements for in vivo neuroscience

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A long history of postmortem studies has provided significant insight into human brain structure and organization. Cadavers have also proven instrumental for the measurement of artifacts and nonneural effects in functional imaging, and more recently, the study of biophysical properties critical to brain stimulation. However, death produces significant changes in the biophysical properties of brain tissues, making an ex vivo to in vivo comparison complex, and even questionable. This study directly compares biophysical properties of electric fields arising from transcranial electric stimulation (TES) in a nonhuman primate brain pre- and postmortem. We show that pre- vs. postmortem, TES-induced intracranial electric fields differ significantly in both strength and frequency response dynamics, even while controlling for confounding factors such as body temperature. Our results clearly indicate that ex vivo cadaver and in vivo measurements are not easily equitable. In vivo examinations remain essential to establishing an adequate understanding of even basic biophysical phenomena in vivo.

biophysical | electric field | nonhuman primate

The use of cadavers to study human anatomy has a long history in medicine and science. For the neurosciences, centuries of postmortem examination have provided a fundamental understanding of human brain structure and organization, as well as the effect of a range of disease processes (1). With the advent of powerful in vivo imaging methods (2), the study of cadavers has become less important in modern-day neuroscience research. However, cadavers are still regularly used to examine possible methodological artifacts in brain imaging studies because of the complete absence of neuronal activity with largely preserved anatomical structures (3, 4). Numerous studies have relied on cadavers to establish the conductivity of differing tissue types, with notable discrepancies compared with in vivo measurements (5–8). Similarly, in developing noninvasive transcranial electric stimulation (TES) procedures, some have suggested the use of cadavers for measuring electric fields generated in the brain during stimulation. Such knowledge is crucial to efforts aiming to optimize brain stimulation, whether focusing on the spatial accuracy of stimulation, the magnitude of a dose actually delivered to an individual, or the possible variations in delivery across individuals. The most recent of these gained widespread attention because of its conclusion that TES-induced currents have poor penetration through the scalp and skull (9). These findings seem to reinforce concerns about the effectiveness of current dosing levels (10); however, to understand their implications, it is first important to determine how well cadaver-based conductivity measurements approximate in vivo conditions.

Death initiates a cascade of biochemical processes affecting the biophysical properties of body and brain tissues, which can make the generalization from ex vivo results to the in vivo case problematic. However, it is not clear how in vivo and ex vivo measures would differ in magnitude and direction. Here, we examine the effect of changes in biophysical properties before and after death on electric fields in the brain induced by TES in a nonhuman primate model. We implanted a measurement array consisting of four linear array probes, each with 10 or more 2-mm ring electrode contacts at 5-mm intervals along the shaft. The contacts spanned the anterior–posterior extent of the brain along four vectors targeting the temporal pole, anterior thalamus, and lateral and medial prefrontal cortices. We demonstrate large pre- to postmortem changes in electric field strength induced by TES, as well as altered frequency dependencies of electric fields. In addition, we show a consistent effect of body temperature on electric field strength, underscoring a significant confounding factor in cadaver studies.

Results

In vivo electric field strengths were found to be highest near occipital contacts close to the posterior stimulation electrode (Fig. 1). Across all contacts (mean over 14 contacts), a 25% increase in field strength [$t(13) = 10.08$; $P < 0.001$] was found in the ex vivo condition immediately after death (Fig. 2*A*, *Left*). On repeat measurement at 6 and 7 d later (temperature, 4 °C), we noted a stable ∼200% increase in field strength relative to the in vivo measurement [$F(2,26) = 77.69$; $P < 0.001$]. Although the relative spatial electric field distribution was stable across measurements (Fig. S1), with spatial correlations $r(12) > 0.97$ ($P < 0.001$), the increases occurred across all contacts. Finally, in the control experiment conducted 14 d later, a temperature-dependent linear decrease in electric field strength was observed with a difference of 38% between 4 °C and 37 °C [$F(6,78) = 80.26$; $P < 0.001$; Fig. 2*A*, *Right*]. Again, the relative field distribution remained largely unaffected by temperature [spatial correlations $r(12) > 0.88$; $P < 0.001$; Fig. S2],

Significance

Understanding the physiology of noninvasive brain stimulation requires precise knowledge of biophysical properties of brain tissue (e.g., conductivities). Numerous researchers have tried to measure these properties using in vitro, in vivo, and ex vivo preparations in nonhuman animals or human tissue, though findings have tended to vary across studies. We measured electric fields in the nonhuman primate brain during transcranial electric stimulation both in vivo and ex vivo. We found large changes in electric fields between in vivo and ex vivo measurements that increased with postmortem time, along with a significant effect of body temperature on electric field strength. Our findings underscore the necessity of nonhuman animal models for systematic study of brain stimulation effects under biophysically realistic conditions.

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Conflict of interest statement: A.O. is an inventor on patents and patent applications describing methods and devices for noninvasive brain stimulation.

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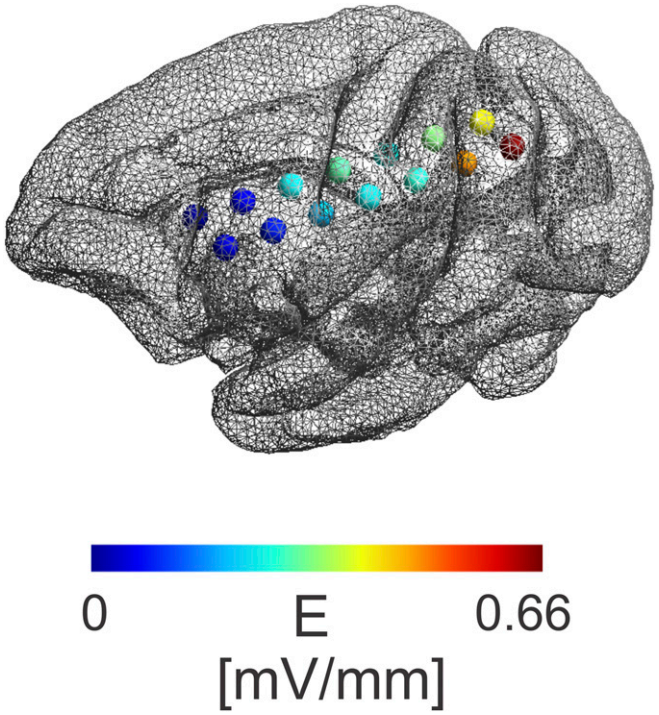

Fig. 1. Measured electric field strength in millivolts/millimeter for a scaled stimulation intensity of 1 mV/mm across contacts (in vivo measurement). Higher electric field strength was found for contacts closer to the occipital cortex.

and changes in electric field strength occurred linearly across all contacts [correlation between temperature and mean electric field strength r(5), −0.99; $P < 0.001$; Fig. 2*A*, *Right* and Fig. S3]. However, the electric field strength found at body temperature in the cadaver was still considerably greater than in the in vivo case, with the mean electric field enhanced by 29% [0.73 vs. 0.52 mV/mm; $t(13) = 5.26$; $P < 0.001$]. Interestingly, the electric field measured in the brain did not decrease with temperature to the same extent as the one measured on the scalp, such that the brain/scalp ratio of the mean electric field increased with temperature [$r(5) = 0.98$; $P < 0.001$; Fig. S4]. Along with overall electric field strength, the frequency dependence of electric fields was significantly altered between measurements [$F(3,60) = 21.45$; $P < 0.001$; Fig. 2*B*]. A slight dampening of electric fields over higher stimulation frequencies was observed for the in vivo case, as previously reported (10), which was basically absent when measured 6 or 7 d later [$t(20) = -4.3042$; $P < 0.001$]. The electric field strength for individual contacts is shown in Fig. S5.

Discussion

The present work suggests that accurate evaluation of the biophysical properties of transcranial stimulation methodologies remains critically dependent on in vivo measurement. Specifically, our results reveal significant differences in electric field strength between in vivo and ex vivo measurements, which increased over time, as the cadaver was stored at a low temperature for several days. We were able to directly relate increases in electric field strength during storage to body temperature by warming the cadaver and demonstrating temperature-dependent decreases in electric field strength. A biophysical explanation for this observation is that with increasing temperature, tissue conductivity increases. In simple terms, electrical conductivity is related to ion mobility; in a warmer medium, ions have a greater mobility, which results in an increased conductivity. For current controlled electrical stimulation, an increased conductivity means that a lower voltage difference (and thus lower electric field) needs to be applied to pass a fixed current through the volume conductor. Even matching the body temperature to the in vivo temperature (37 °C) did not help to equate the field strength measured postmortem to that observed in vivo. This suggests lasting changes in the electric properties of the head/brain tissues occur after death and appear to be compounded with time. One caveat is that the temperature distribution might be less uniform in the ex vivo measurements because of the absence of blood circulation. This could lead to slight changes in the conductivity ratios compared with the in vivo case. Differences between the in vivo and ex vivo states for measurement of stimulation effects were further corroborated by the altered frequency response of electric fields over different stimulation frequencies between in vivo and ex vivo

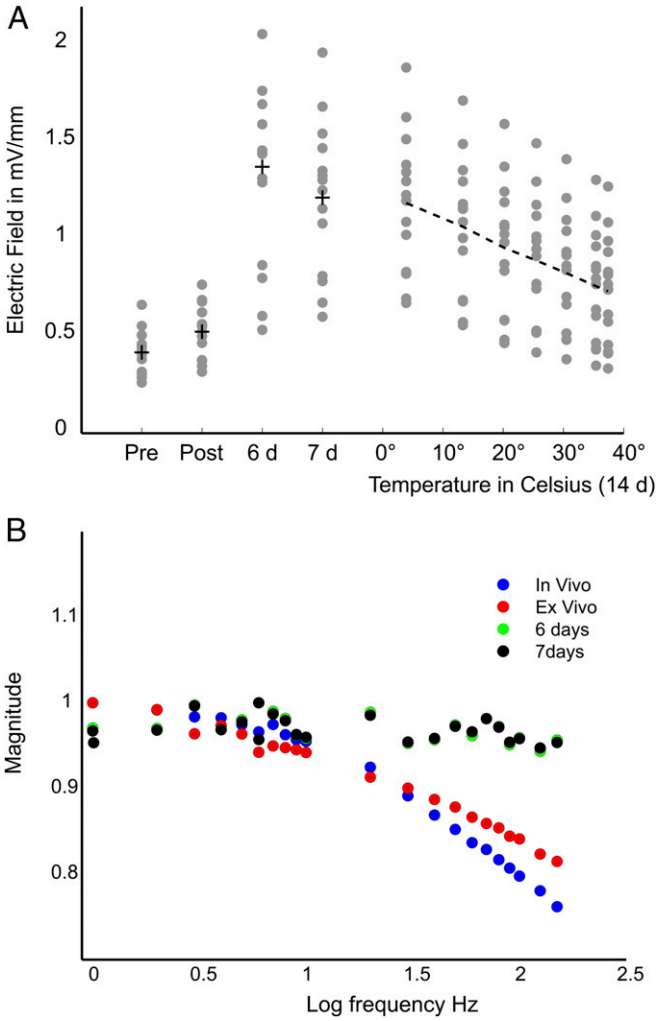

Fig. 2. (*A*) Electric field strength (in millivolts/millimeter for a scaled stimulation intensity of 1 mA for all individual contacts (each dot) across different measurements (+, mean across contacts). A slight increase in field strength was found from pre- to postmortem within the same session (*Left*). Measurements after 6 or 7 d showed strongly enhanced field strength (*Middle*). Electric field strength showed an inverse relationship with temperature (*Right*, measured after 14 d), with highest fields measured for 4 °C and lowest fields for 37 °C (although still higher compared with the in vivo measurement). (*B*) Frequency response of measured voltages. Shown is the Fourier magnitude of measured voltages (mean across all contacts) in dependence of the applied stimulation frequency (from 1 to 150 Hz in log units). For the in vivo case (blue), a decrease in magnitude was found with increasing frequency, which was reduced in the ex vivo case (red) in the same session. Any frequency dependency was largely absent for the ex vivo case measured after 6 or 7 d (green and black).

measurements. A previously suggested mechanism for the relatively small low-pass filter effect observed in vivo is the polarization of cell membranes (11). With the disintegration of cell membranes after death, it is conceivable that the filtering effect is further diminished, leading to the flattened frequency response. Overall, our results show large differences between in vivo and ex vivo measurements related to the biophysical changes of brain and head tissues that accompany death.

Our findings do not negate concerns about the appropriateness of current TES dosing levels. Independent of the in vivo vs. ex vivo question, research in both monkeys and humans had already raised concerns about the magnitude of current dosing levels, as well as the presence of substantial differences among individuals with respect to the electric field strengths generated (10). However, the minimum effective electric field strength needed to induce robust physiological effects remains a topic of ongoing research and cannot be addressed from a cadaver study. As highlighted by a recent paper (12), even in vivo, the challenges of accurately measuring the response of the brain during stimulation are substantial as a result of several sources of artifact in measurement. In addition, it is important to note that most studies claiming a meaningful behavioral response to TES actually make use of extended periods of stimulation, or multiple sessions, suggesting that changes are gradual and accrue over time. Finally, it is worth noting that recent work has suggested the contributions of inadvertent cranial nerve stimulation to outcomes, further complicating an effort to definitively rule out the utility of TES (13). None of the studies to date can appropriately address the implications of these findings regarding the utility of TES.

Physiological effects of weak electric fields have been found in in vitro slice preparations at 0.5 mV/mm (14, 15), and even at 0.2 mV/mm (16). A minimum threshold of 1 mV/mm has been suggested on the basis of in vivo measurements in rodents (17). Electric fields measured in vivo in humans fall within this range; however, rather at the lower end. Importantly, these effects are subthreshold and only lead to a modulation of ongoing activity. For suprathreshold effects, much higher electric fields are needed. Typical fields for transcranial magnetic stimulation that cause membrane depolarization are in the range of 100 mV/mm. Strategies for safely generating higher-dose electric fields in the brain are under active development (18). Other approaches using multiple stimulation electrodes could also help overcome some of the limitations of current stimulation approaches by inducing more focal fields in specific brain regions (19, 20). The measured ratio of scalp to brain electric fields of around one does not imply that equal amounts of currents are flowing on the scalp compared with the brain. This is because the needle electrodes were placed at a position that exhibited weaker fields compared with locations close to the stimulation electrodes. The amount of current entering the brain is determined by the ratio of the conductivities of scalp, skull, cerebrospinal fluid, and brain. It is conceivable that the conductivity ratio changed with death, making it more favorable for currents to pass through the brain, creating higher electric fields.

In sum, our findings strongly reinforce logical cautions against directly translating from ex vivo results to in vivo cases, even for the examination of artifacts or biophysical consequences of brain and head anatomy; the cessation of brain activity at death appears to be but one of the relevant factors. As a consequence, our results highlight the necessity of in vivo translational models even for the most basic biophysical measurements, if results are to be relevant to in vivo human applications.

Methods

Experimental protocols were approved by the Institutional Animal Care and Use Committee of the Nathan Kline Institute for Psychiatric Research (NKI), and recordings were conducted according to approved guidelines. Details of implantation and experimental procedures can be found in ref. 10. One female Cebus monkey (11 y, 2.9 kg) was implanted with a MRI-compatible headpost (Cilux) and four linear array depth electrodes (Adtech), inserted through a small craniotomy over the left occipital cortex; this was subsequently sealed with nonconducting bone cement. The four electrodes with a total of 42 contacts (5 mm spacing) were oriented along a rostrocaudal axis terminating in medial prefrontal cortex, frontal eye field, anterior hippocampus, and anterior lateral thalamus. Electrode locations were identified on a postimplantation MR image.

Stereo-EEG was recorded in four sessions, three of which were ex vivo. The same BrainAmp MR amplifier [Input Impedance 10 MOhm; common-mode rejection, >90 db; high-pass frequency, 0.016 Hz; low-pass frequency, 250 Hz (Butterworth filter with a slope of 30 db/octave); measuring range, ±16.384 mV; resolution, 0.5 muV/bit; sampling rate, 5 kHz; Brain Products] was used in all sessions. Experiments were conducted ∼1.5 y after electrode implantation. Ground and reference electrodes were placed on the scalp over the left and right temporal region, respectively.

In the first recording session, the monkey was anesthetized with ketamine 10 mg/kg, atropine 0.045 mg/kg, and diazepam 1 mg/kg, followed by 2% isoflurane in 100% oxygen. Measurements were performed under anesthesia to keep any stresses on the monkey low, as well as to keep the monkey still over an extended measurement period. Anesthesia reduces intrinsic brain activity, which could have an influence (albeit small) on the electric field measurements (signal during TES is at least 100 times stronger than intrinsic activity). During the whole session, the monkey was placed on a heating pad to ensure an approximately constant body temperature. Two small round stimulation electrodes [3.14 cm^2, Ag/AgCl with conductive gel (SigmaGel)] were used in all sessions, and TES was applied using the Starstim system (Neuroelectrics, current controlled stimulation). Stimulation electrodes were attached over the left occipital cortex and middle forehead, which results in electric fields aligned approximately in anterior–posterior direction. First, we measured the electric field strength in the brain by applying a 10-Hz oscillating current with 200 μA for 30 s (5 s ramp up/down). Signals were bandpass filtered between 9 and 11 Hz, and electric fields were calculated as the first spatial derivative of the voltage profile. Electric field measurements were performed for the medial prefrontal cortex and frontal eye field electrodes. Electrode contacts that exhibited clipping artifacts or excessive 60 Hz noise were excluded from the analysis. Clipping artifacts and excessive 60 Hz noise were identified by visual inspection. We excluded eight contacts in the analysis, resulting in a total of 14 contacts included in the analysis. We calculated uncertainty bounds on the induced electric fields based on the SD over the course of 10 stimulation cycles (Dataset S1). Deviations in the field strength were generally small (<1%). In a second measurement, we evaluated the frequency response of electric fields over a range of stimulation frequencies. We applied 21 different frequencies between 1 and 150 Hz in randomized order with other stimulation parameters kept identical to the first measurement. The specific set of frequencies used included from 1 to 10 Hz in 1-Hz steps, from 10 to 100 Hz in 10-Hz steps, and 125 and 150 Hz. We computed the magnitude of recorded potentials using the fast Fourier transform over a time window of 13.1 s of data for each channel.

After these in vivo measurements during the first session, the monkey was killed with an i.v. injection of Euthasol (pentobarbital sodium, 100 mg/kg; phenytoin sodium, 12.5 mg/kg). Thirty minutes after death, the electric field and frequency-response measurements were repeated in the same manner as premortem. At the end of the experiment, the cadaver was placed in a sealed container in a refrigerator at 4 °C.

In a second experimental session, 6 d after the first session, the cadaver was taken out of the refrigerator and the experiment was repeated. As a result of greatly increased signal amplitudes, which we noted at the outset of the session, electric field measurements and frequency responses were recorded with a stimulation intensity of 100 μA to stay within the dynamic range of the recording system. To test for the reproducibility of the initial results, the same recordings were repeated in a third experimental session 1 d later. A stimulation intensity of 50 μA was used, as one channel was clipping at 100 μA. During the third session, the temperature of the head was measured with an orally inserted electronic temperature probe at 4.6 °C.

To test for possible effects of temperature on electric field strength in the corpse, another control experiment was conducted in a fourth experimental session 14 d after the first session. For that purpose, the corpse was placed over a water bath mattress, and the temperature was slowly increased from an initial 3.9 °C to approximately body temperature at 37.4 °C over the course of 2.5 h. Temperature was monitored with the same orally inserted probe. We measured electric field strength at 10 Hz with a 50 μA intensity (30 s duration, 5 s ramp up/down) at seven different temperatures. In this session, we also measured the electric field on the scalp with needle electrodes over the left temporal cortex. Five needle electrodes were attached on the scalp over the left temporal cortex (∼1 cm spacing) with an anterior–posterior arrangement (similar to the orientation of the depth electrodes). This arrangement allows for the estimation of anterior–posterior-oriented electric fields. The location of needle electrodes was

about halfway between the occipital and frontal stimulation electrodes. This setup was chosen to estimate the effects of body temperature on scalp electric fields and to maintain the distance between stimulation and the needle electrodes, which is essential to avoid excessively large fields and the resulting amplifier saturation. To compare results across different measured intensities, recorded voltages were scaled to an effective intensity of 1 mA (which is typically used in TES studies) according to Ohm's law. This will only scale electric field strength, while keeping the relative aspects of the electric field distribution (e.g., spread) identical.

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