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Highlights

- Minipigs can be implanted safely over motor cortex before frontal sinuses develop
- Production of grunts activates the motor/premotor region around vocal onset
- This vocal-evoked potential lasted for the duration of the vocalization

Abstract

Background: Investigating brain dynamics underlying vocal production in animals is a powerful way to inform on the neural bases of human speech. In particular, brain networks underlying vocal production in non-human primates show striking similarities with the human speech production network. However, despite increasing findings also in birds and more recently in rodents, the extent to which the primate vocal cortical network model generalizes to other non-primate mammals remains unclear. Especially, no domestic species has yet been proposed to investigate vocal brain activity using electrophysiological approaches. **New method**: In the present study, we introduce a novel experimental paradigm to identify the cortical dynamics underlying vocal production in behaving minipigs. A key problem to chronically implant cortical probes in pigs is the presence and growth of frontal sinuses extending caudally to the parietal bone and preventing safe access to neural structures with conventional craniotomy in adult animals. **Results**: Here we first show that implantations of soft ECoG grids can be done safely using conventional craniotomy in minipigs younger than 5 months, a period when sinuses are not yet well developed. Using wireless recordings in behaving animals, we further show activation of the motor and premotor cortex around the onset of vocal production of grunts, the most common vocalization of pigs. **Conclusion**: These results suggest that minipigs, which are very loquacious and social animals, can be a good experimental large animal model to study the cortical bases of vocal production.

Keywords

Vocal production, pig, porcine model, motor cortex, neural implant, ECoG

Introduction

Animals communicate through multiple types of acoustic signaling (Chen and Wiens, 2020), and many vertebrates, including birds and marine and terrestrial mammals, have the ability to produce vocalizations that often differ depending on the type of information they want to communicate (Brudzynski, 2010). In primates, two types of vocal behaviors can be distinguished that have been proposed to stem from two different neural pathways (Jürgens, 2002; Hage and Nieder, 2016; Hage, 2018; Mooney, 2020): Innate affective and learned volitionally controlled vocalizations. Innate vocalizations are determined genetically and are typically produced automatically by cingulate-subcortical networks in reaction to a stimulus or a situation (such as fear, pain, hunger, or surprise). By contrast, learned vocalizations, characterized by single or sequences of stereotyped calls or more complex modulated sounds, are acquired by experience and volitionally controlled by cortical networks involving motor, premotor and inferior frontal areas (Coudé et al., 2011; Hage et al., 2013; Fukushima et al., 2014; Okubo et al., 2015; Roy et al., 2016; Gavrilov et al., 2017; Loh et al., 2017). Cortical networks have also been shown to be important for vocal learning and temporal patterning in birds and mice (Aronov et al., 2008; Long and Fee, 2008; Okobi et al., 2019; Tschida et al., 2019; Mooney, 2020). Although noticeable variations of anatomical pathways exist between humans and non-human primates (NHPs) that may explain at least in part the unique ability of humans to speak (Rilling et al., 2008), the brain networks underlying vocal production show strong anatomical similarities between humans and NHPs (Petrides et al., 2005; Hage, 2018) and to some extent birds (Chakraborty and Jarvis, 2015). However, until now, very few neurophysiological data highlighting the cortical dynamics underlying vocal production have been reported in animal models other than NHPs, rodents and birds. Especially, no domestic species has yet been proposed to investigate vocal brain activity using electrophysiological approaches.

The domestic pig (*sus domesticus*) is a highly social species that produces a large variety of vocalizations in different situations. Several decades ago, (Kiley, 1972) described ungulates vocalizations and their causations. She proposed a classification of adult pig vocalizations and highlighted four principal types of calls (grunts, barks, screams and squeals) and their situations of occurrence. She also described many variations and subcategories, giving an overview of the potential richness of the pig's vocal repertoire. Further ethological studies have later reported the existence of vocal call subcategories such as long grunts (Marchant et al., 2001) or even intermediate call types such as grunt-squeals (Appleby et al., 1999), suggesting that the vocal repertoire of pigs might be more continuous. Recent studies classified vocalizations in ungulate species (Tallet et al., 2013; Garcia et al., 2016) and concluded that a discrete system was adapted, even if there are evidences of gradation between acoustic categories. Beyond these works, a number of studies have been conducted in situations relevant for commercial farming to assess and improve the welfare of pigs (Fraser, 1975; Jensen and Algers, 1984; Jensen and Redbo, 1987; Xin et al., 1989; Weary and Fraser, 1995; Manteuffel et al., 2004; Schön et al., 2004; Whittemore and Kyriazakis, 2006; Moura et al., 2008; da Silva Cordeiro et al., 2013; Illmann et al., 2013). These works have thus improved our understanding of pig vocalizations and how they reflect their mental or physical state. Moreover, pigs are domestic animals that are easier to care for compared to wild species such as NHPs, and have been increasingly attracting attention in the field of neurotechnology and neuroscience (Félix et al., 1999; Jelsing et al., 2006; Lind et al., 2007; Saikali et al., 2010; Van Gompel et al., 2011; Knösche et al., 2015; Paek et al., 2015; Benavides et al., 2017; Bech et al., 2018; Ulyanova et al., 2018; Ernst et al., 2018; Simchick et al., 2019; Vrselja et al., 2019; Cho et al., 2020; Slopsema et al., 2021; Ritter et al., 2021) including cognition (Gieling et al., 2011). In particular, minipigs have become a purpose bred for research. They are smaller yet physiologically in all other ways similar to agricultural pigs. For research lasting longer than 3 weeks, minipigs are thus preferable both for handling ease and welfare considerations. Furthermore, they have a convenient body size for surgical procedures and given their anatomical similarities to humans, they are often used as preclinical models (Selek et al., 2014; Khoshnevis et al., 2017, 2020; Schiavone et al., 2018; Fallegger et al., 2021).

In the present study, we introduce a novel experimental paradigm to identify the cortical dynamics underlying vocal production in behaving minipigs. A key problem to chronically implant neural probes in a pig's brain is the presence and development of frontal sinuses preventing safe access to neural structures with conventional craniotomy in adult animals (Gierthmuehlen et al., 2011; Torres-Martinez et al., 2019). These cavities indeed extend far caudally within the skull of adult animals from the frontal to the parietal bones. Performing a craniotomy in such condition leads to cross the sinuses and thus to make a connection between the implanted zone and the nasal cavity, which ineluctably eventually leads to postoperative infections. Here we first show that implantations of soft ECoG grids can be done using conventional craniotomy in minipigs younger than 5 months, a period when frontal sinuses are not yet well developed. Using wireless recordings in behaving animals, we further show activation of the motor and premotor cortex around the onset of vocal production of grunts, the most common vocalization of pigs.

Materials and Methods

Animals

This study was carried out in Aachener (Carfil, Oud-Turnhout, Belgium) and Göttingen (Ellegaard, Dalmose, Denmark) minipigs. Eight animals were imaged to determine a surgical procedure allowing chronic implantation of cortical probes, and two animals were implanted with soft implants as described below (one of which broke its implant connector before recordings could be conducted). Experiments were conducted in compliance with European (2010-63-EU) and French (decree 2013-118 of rural code articles R214-87 to R214-126) regulations on animal experiments, following the approval of the local Grenoble ethical committee ComEth C2EA-12 and the French Ministry of Research (APAFIS#5221-2016042816336236.V3). Animals were typically housed in groups with weekly renewed enrichment materials and ad libitum water. Implanted minipigs were also housed most of the time with other congeners. Regular examinations were performed by a veterinarian and animals were socialized to ease human-animal interactions. At the end of this study, the implanted minipig in which we recorded cortical activity was rehabilitated in a pedagogic farm as a retirement.

Imaging (CT-scan and MRI)

In order to overcome the problem of frontal sinuses during the craniotomy, CT-scans were acquired at different ages between 3 and 12 months to assess the development of frontal sinuses. CT images were acquired at the Grenoble Hospital using a GE LightSpeed RT 16 CT Scanner (Helicoïdal acquisition, 1.25 mm slice thickness and step) except for the 2 animals aged 5.5 and 12 months, for which they were provided by Ellegaard. CT-scans were used to define the craniotomy area over the skull as proposed previously for NHPs (Chen et al., 2017). For logistical reasons, no CT-scan was performed for one of the implanted animals (CH596), so the CT acquisition of the other implanted animal of similar age and same strain (BA638) was used in this case. For CT imaging, each animal was pre-medicated with an IM injection of 1mg/kg Azaperone (Stresnil®, Elanco, Suresnes, France). After 15-20 minutes, an anesthesia was induced by intramuscular (IM) injection of 5mg/kg Tiletamine-Zolazepam (Zoletil® 100, Virbac, Carros, France). For the two animals that were implanted, a pre-surgical T1-weighted MRI (T1-MDEFT, 1-mm slice thickness, 0.43x0.43 mm^2 pixels, TE: 4 ms, TR: 2000 ms, Flip angle: 20°) was performed under a general anesthesia induced as for CT-scans and then further maintained under Isoflurane (Isoflo®, 2-2.5%).

Surgery preparation using 3D modeling and printing

Chronically implanted devices in large animals require protection components to secure fragile electronics and connectors from hits, bites or even breaking. To do so, customizable pieces adapted

to the animal anatomy were developed using 3D modeling following a procedure inspired by a previously proposed method for NHPs (Chen et al., 2017). The CT images were imported in the InVesalius software (2007-2017, Center for Information Technology Renato Archer) and segmented to obtain a 3D model of the skull. The MR images were processed with 3D Slicer software (http://www.slicer.org) (Fedorov et al., 2012) to segment the brain and visualize the cortical gyrus and sulcus anatomy before implantation in order to make accurate anatomic assessment of the motor cortex for surgical planning. The 3D models obtained by CT-scan and MRI were then combined using the Blender software (https://www.blender.org/) to model the full minipig's head. Based on this reconstruction, the coordinates of the craniotomy were planned according to the motor cortex location to access the implantation area (Figure 1a).

A custom metallic oval chamber (60x50mm) was further designed to be screwed on the skull during the implantation surgery (Figure 1b, top). This chamber was adjusted to the minipig anatomy to closely fit its skull surface. Its role was to cover and protect the craniotomy area and house the connector of the implant. To ensure good resistance against possible damages, this chamber was 3D printed in biocompatible TA6V titanium (X3D, Lyon France). To close this chamber, a thick hood was 3D printed in transparent resin using a Form 2 printer (Formlabs, Sommerville, USA) (Figure 1b, bottom). This piece could be easily removed by the experimenter for each recording session to connect the recording devices.

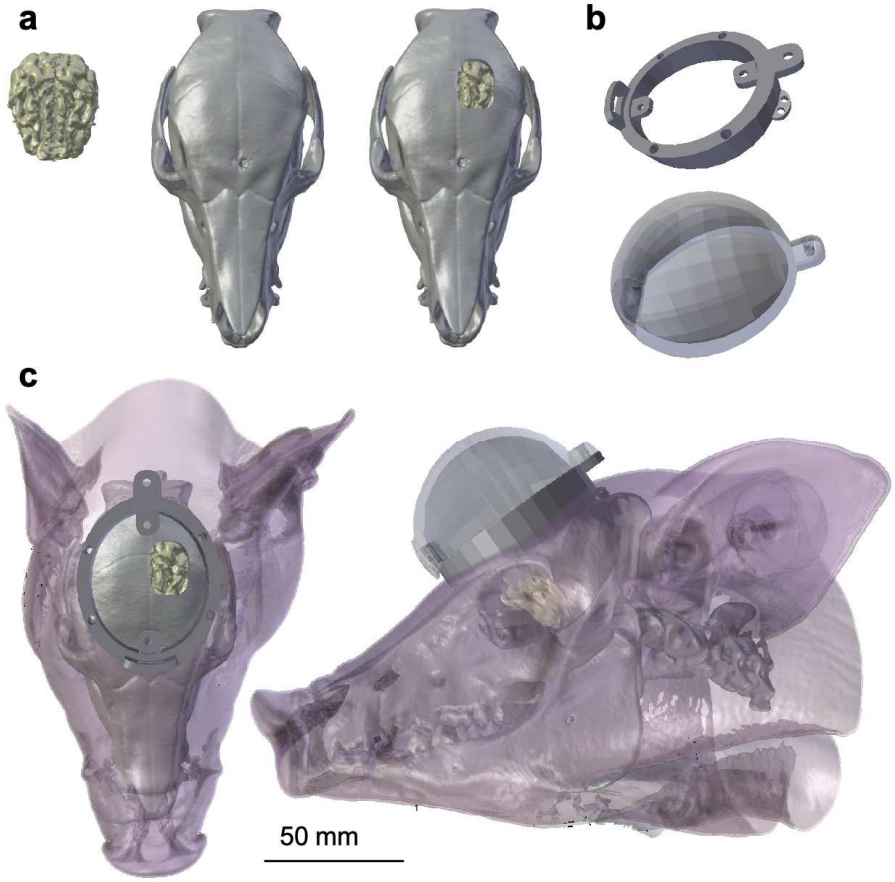

Figure 1. Surgery planning using 3D modeling. *(a) 3D modeling of a minipig's brain based on T1 MR images, and skull based on a CT-Scan. Right: The craniotomy area could be planned before the surgery to target the motor cortex. (b) Titanium chamber to be screwed on the skull (top) and plastic protective cap (bottom). (c) Full head reconstruction where the skin is also represented together with the chamber and the protective hood closing the chamber. Scale in (c) also applies to (a).*

Implantation surgery

In the present study, two Aachener female minipigs (BA638 aged 4 months and CH596 aged 4.5 months) were chronically implanted with a soft electrocorticographic (EcoG) array made of a silicone (polydimethylsiloxane, PDMS) substrate and housing 32 electrode contacts arranged in a 4x8 layout covering 5x15 mm^2 and routed to a 36-pin Omnetics connector (Figure 2a) together with a reference and a ground wires (PFA coated gold wires, Science Products). This implant was designed to fit the left motor cortex of the animal (Figure 2b) based on previous anatomical studies (Sauleau et al., 2009; Saikali et al., 2010; Benavides et al., 2017). The detailed properties of this implant as well as preliminary results have been reported elsewhere (Fallegger et al., 2021). Animal BA638 broke his cap and the implant tail before recordings could be performed. The geometry of the cap was then modified to avoid this problem with animal CH596 for which chronic recordings could be obtained and reported below. One recording of this animal corresponds to the one reported in (Fallegger et al., 2021).

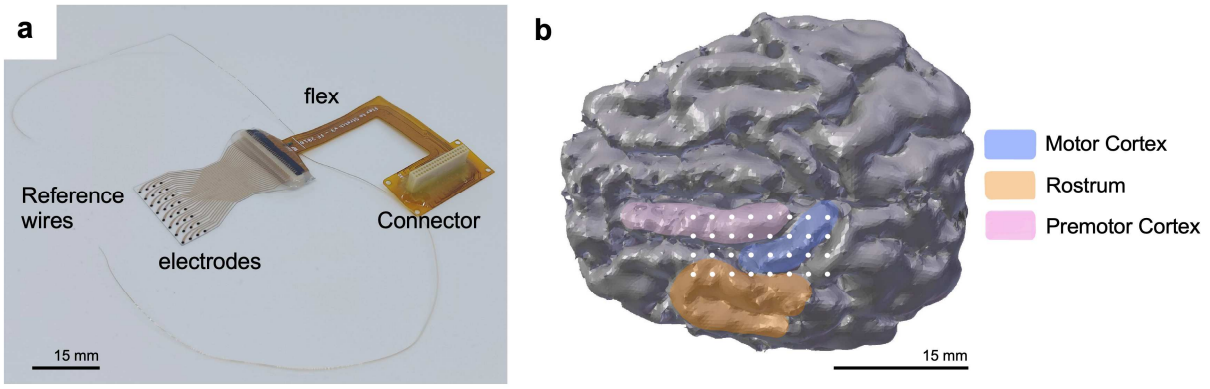

Figure 2. Soft cortical implant used in this study. *(a) General view of the soft implant housing 32 electrodes routed to an Omnetics® connector* (Fallegger et al., 2021). *(b) Reconstruction of the cortical surface of the implanted animal CH596 showing the eventual position of the electrodes with respect to the motor and premotor areas (Sauleau et al., 2009; Saikali et al., 2010; Benavides et al., 2017).*

For the surgical implantation, the animal was initially premedicated and anesthetized using IM Stresnil and Zoletil as for CT and MRI imaging (see above). A trimmer was then used to shave the hair over the head from the eyes up to the back of the ear's roots. Then, the pig was placed in a stereotaxic frame (David KOPF Instrument, USA) over a warming blanket to prevent the peri-operative hypothermia. The pig's head was typically fixed by the ear bars with a slight extension of the head. To continue the general anesthesia, sedation was maintained by a continuous inhalation of isoflurane 2% (Selek et al., 2014; Khoshnevis et al., 2017, 2020; Torres-Martinez et al., 2019). Ketoprofen (3 mg/kg, Ketofen® CEVA) was administered IM to better maintain the anesthesia and improve postoperative analgesia. Before beginning the surgery, the skin incision site and extensive area surrounding it were disinfected by wiping skin with betadine scrub 4% and dermal 10% immediately prior to draping. Cardio-respiratory functions were monitored throughout the surgical procedure. Local analgesia was provided by subcutaneous (SC) injections of lidocaine (xylocaine adrenaline) before incising the skin according to the later position of titanium chamber on the skull.

An oval incision was done to remove a part of the scalp (Figure 3a). Using a periosteal elevator, the periosteum was retracted gently to the edge of the skull. The entire exposed area of the skull was well dried and cleaned. The craniotomy area was drawn over the left hemisphere of the skull based on the skull anatomical cues such as bregma, occipital crest, temporal crest, and central tubercle of nuchal crest and according to the position of the motor cortex relative to bregma. In practice, the craniotomy extended roughly from 5 mm posterior to 20 mm anterior to bregma and from 2 mm to 18 mm lateral of the midline. The skull was drilled gently with electric surgical drill

(Medtronic). When the full thickness craniotomy has been reached, the bone flap was lifted away from the skull (Figure 3b). After exposing the dura mater, its surface was dried and cleaned to ensure no further bleeding. Then the dura mater was cut with micro-dissecting scissors to reach the implantation area on the brain surface (Figure 3c).

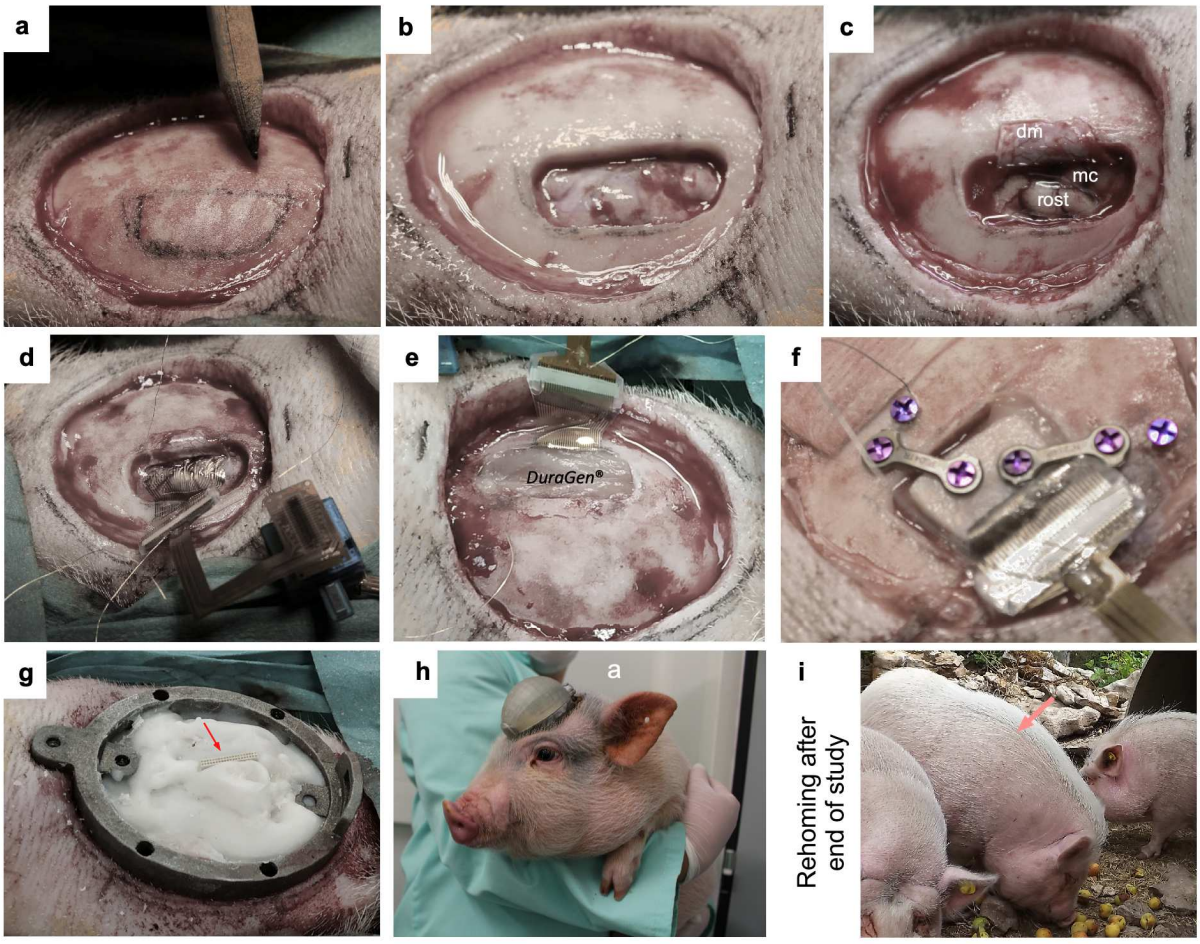

Figure 3. Chronic implantation of a soft ECoG implant. *(a) Oval opening of the skin and drawing of the craniotomy based on CT-scan. (b) Craniotomy after removal of portion of the skull (bone flap). (c) Craniotomy with opened dura matter (dm) over motor cortex (mc) and rostrum (rost). (d) Positioning of the soft implant. (e) duraplasty by Duragen®. (f) closure of the craniotomy with bone flap. (g) Titanium chamber screwed on the skull and cemented connector (arrow). (h) Implanted animal several days after surgery with protective cap closing the chamber. (i) Explanted animal CH596 (middle pointed by arrow) rehomed with congeners in a farm after ending its investigation. All panels refer to animal CH596 except panel f corresponding to animal BA638 (the same approach was used for CH596 but no picture of this step was taken during the surgery).*

The soft implant was positioned to cover the premotor and motor cortices (Figure 3d), slightly extending caudally to the postcentral gyrus. After the implantation, unilateral duraplasty was performed using an onlay, suture-free, 3-dimensional-collagen matrix graft (*DuraGen®*, Integra LifeSciences) (Figure 3e). To close the craniotomy, the bone flap was slightly thinned and placed back on the Duragen, and then fixed on the surrounding skull using titanium strips and screws. The titanium chamber was then fixed on the skull using self-drilling titanium screws. The reference wires were rolled around and laced on supplementary bone screws inserted next to the craniotomy area (Figure 3f). Then, the interior space of the chamber was filled with bone cement (CMW1 + Gentamycin®, DEPUY) to seal the craniotomy and surrounding area and to fix the Omnetics connector (Figure 3g, arrow). At the end of the surgery, the chamber was closed by the 3D printed

hood to protect the implant connector when the animal was replaced in the housing pen (Figure 3h). After surgery, the animal was monitored in a recovery room, pain relief was anticipated with an injection of Ketoprofen (3mg/kg). Prophylactic antibiotics (Kesium® CEVA) and anti-inflammatory agent (Metacam® Boehringer Ingelheim) were administered for 7 days postoperative. At the end of the study, the explanted animal was rehomed in a farm through the GRAAL society (https://www.graal-defenseanimale.org/) (Figure 3i). Two subsequent animals (one Göttingen and one Aachener) were implanted following a similar procedure with other arrays, for which we only report thereafter the anatomical evolution of their skull after explantation (Figure 6).

Simultaneous electrophysiological, audio and video recordings

Neural activity was recorded using the wireless W2100 system (MultichannelSystems, Reutlingen, Germany). A 2m x 2m x 2m recording pen was built and sound-attenuated using several layers of acoustic foam (Figure 4).

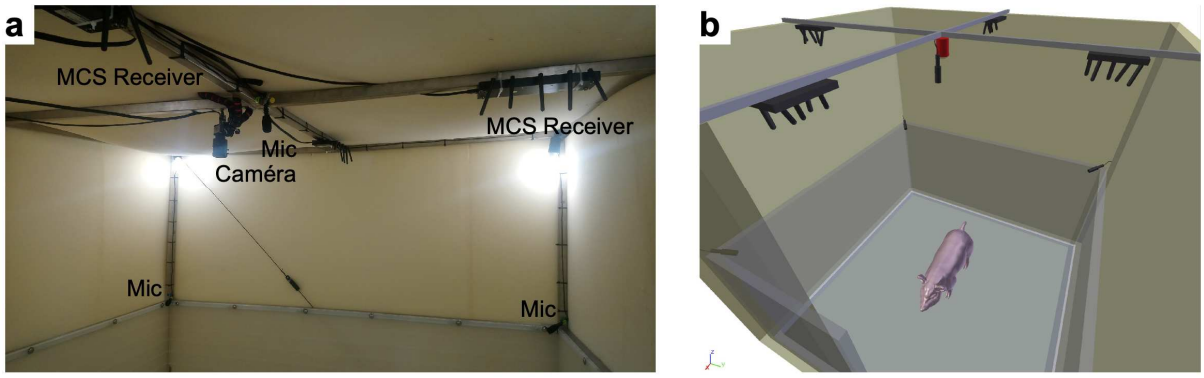

Figure 4. Experimental setup for synchronous recording of vocal and cortical data in minipgs. *(a) Roof of the recording pen with the recording setup. (b) 3D schematics of the recording pen with a behaving animal.*

Four wireless receivers were fixed on the roof of the pen and connected to 2 recording interface boards located outside the pen. Five microphones (Pro45, Audio-Technica Inc, USA) were positioned in the pen, one at each corner 1m from the floor and one on the ceiling in the center of the pen, where a camera (UI-3140CP-C-HQ Rev.2, IDS Imaging, Obersulm, Germany) was also positioned to monitor the animal behavior. During a typical experimental session, the protecting hood was detached and a wireless HS32 headstage (MultichannelSystems, Reutlingen, Germany) was connected to the implant. Then, the animal could be let to move and behave freely in the pen under the supervision of an experimenter. However, in order to avoid that the animal damages the headstage against the floor or the walls, an experimenter placed her on her laps or in her arms. This physical contact was also a way to arouse more vocalizations. Synchronous cortical, audio and video data acquisitions were performed with the MCS experimenter software. Neural data was acquired at a 20-kHz sampling rate after 1-5000 Hz bandpass filtering and 16-bits AD conversion at the headstage level. Audio data was acquired at 20kHz after amplification by a sound card (OctoMic II, RME-Audio, Haimhausen, Germany). Video was acquired at 50Hz, synchronized with the neural and audio data.

Neural data processing

Cortical data was band-pass filtered between 1 and 10 Hz and evoked potentials were computed by averaging single trials locked to the onset of vocalizations (detected by thresholding the audio signal and classified manually) and correcting the baseline with respect to the [-3s, -2 s] interval preceding the vocalization onset. We then evaluated the reproducibility of the averaged evoked potential by building a distribution of bootstrap averages (Yvert et al., 2002). If N vocalizations were recorded, N trials were drawn with replacement from the original set of N trials, and averaged and baseline-corrected. This procedure was repeated 1000 times. To further assess the statistical significance of vocalization-induced cortical activations, we used the following approach. A 60-second rest period

void of vocal production was considered and used to select N resting trials, which were in turn averaged and baseline corrected. The standard deviations corresponding to both the vocalization and the resting averages were also computed and a Welch t-test was performed to compare both distributions at every time point of the average potential. A threshold was set at 0.05 and individual electrode values at all time points for which the p-value of the Welch test was below this threshold were considered to correspond to statistically significant activity. This procedure was thus further retained to build spatiotemporal maps of cortical activity using our previously developed NeuroMap software (Abdoun et al., 2011) (freely available at https://sites.google.com/site/neuromapsoftware/).

Results

Normal development of frontal sinuses

From the several CT acquisitions made at different ages of control Aachener and Göttingen minipigs, it was observed that before 5 months of age, the sinuses have not yet extended too caudally and the motor cortex could be accessible through a conventional craniotomy (Figure 5). Thanks to this acquired information, the minipigs were implanted at 4-4.5 months of age using a craniotomy exposing the motor cortex and part of the rostrum and prefrontal cortex (see Figure 3).

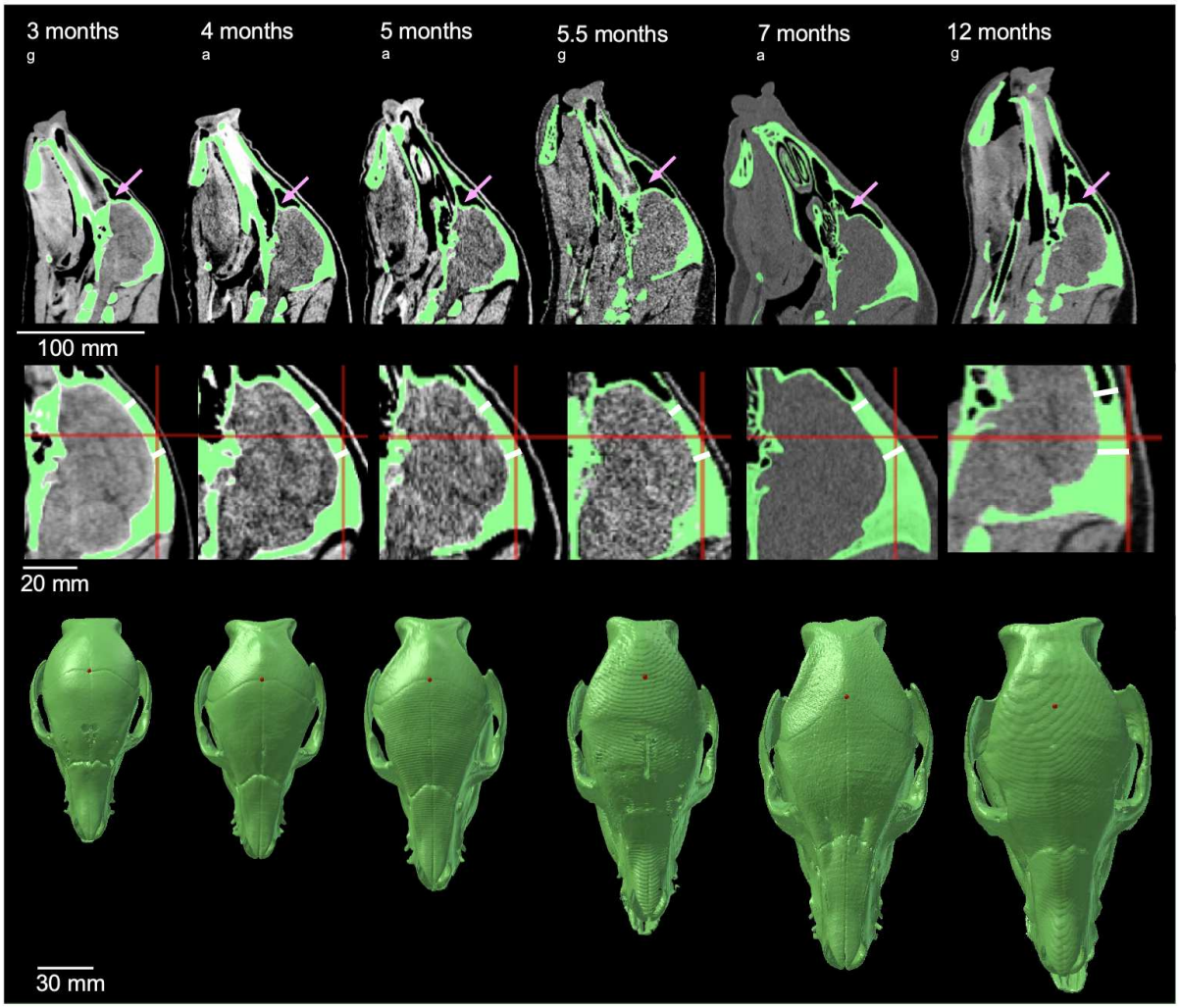

Figure 5. Development of frontal sinuses in minipigs between 3 and 12 months of age. *Top row: Mid-sagittal CT images of the whole head at various ages. The skull in colored in green and the frontal sinus is indicated by*

the pink arrow. Middle row: close-up view of the brain area, with a representation of a typical target craniotomy over the motor cortex (white marks). The red cross indicates Bregma. Bottom row: Top view of the skull with Bregma indicated by a red dot. a=Aachener minipigs, g=Göttingen minipigs.

Development of frontal sinuses in case of chronic implantation

We then analyzed the volumes of the sinuses of two other animals that had been implanted with other types of implants but the same chamber and craniotomy and for which CT-scans were obtained after their implants had been removed, and compared them to that of a control non-implanted animal. As illustrated in Figure 6, we found that in the implanted animals, the sinuses did evolve caudally but around the zone of the skull that was exposed to position the chamber. In particular, the sinuses did not develop in the zone of the craniotomy, so that no infection could be induced by their evolution after the implantation.

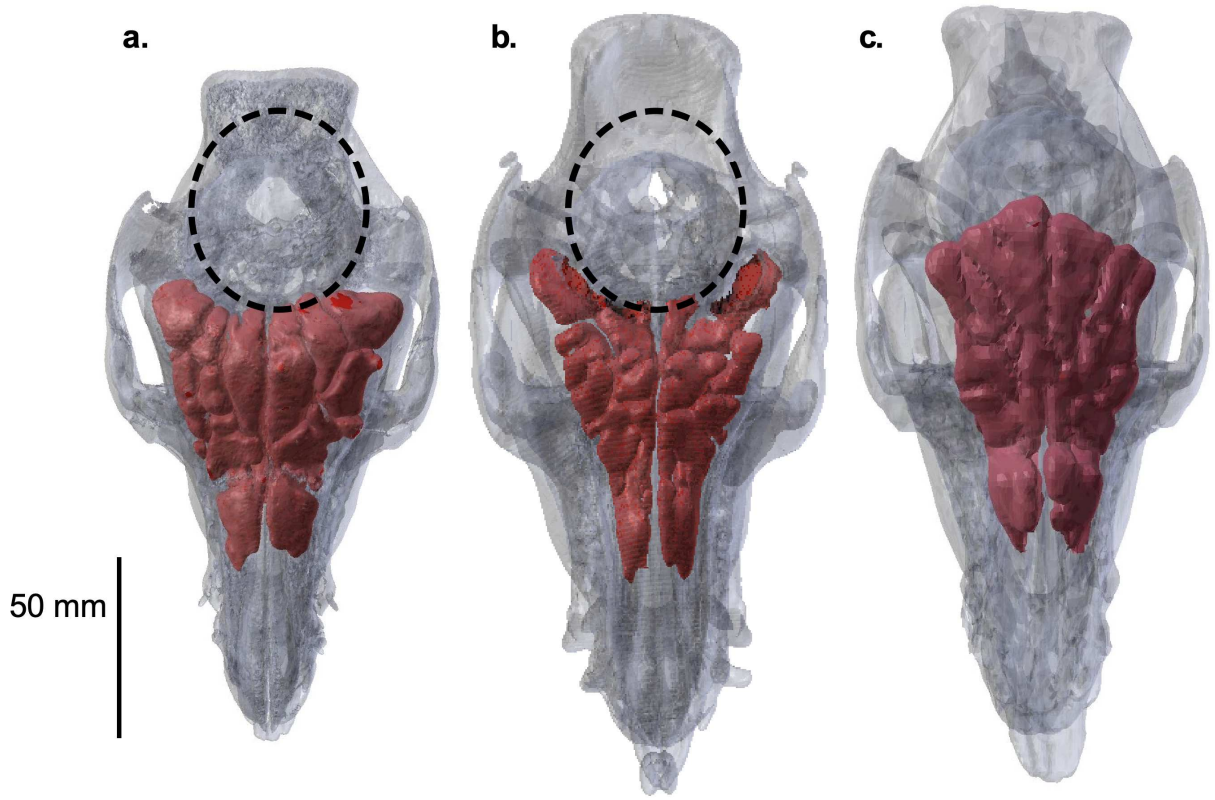

Figure 6. Development of frontal sinuses in minipigs following an implantation. *The volume of the frontal sinuses is reconstructed in red from CT-scans acquired in two implanted animals after they have been explanted and in one control animal. The position of the chamber is indicated by a dashed line for the implanted animals. a) 7.5-month-old Göttingen minipig 3 months after implantation. b) 12-month-old Aachener minipig 7 month after implantation. c) Control not implanted 12-month-old Göttingen minipig.*

Vocal production activates premotor and motor areas

Two recordings were performed 2 and 4 weeks after implantation, respectively, an example of raw data being shown in Figure 7a. We observed a decrease of the baseline noise level of the recording between both sessions (1-500 Hz: $p=0.9*10^{-5}$; 1-10 Hz: $p=1.2*10^{-5}$, Wilcoxon rank sum tests) (Figure 7b&c). Minipigs produced different types of vocalizations, most of which were grunts. We thus focused on this particular vocalization (Figure 8a). In the first session, we observed statistically significant cortical activity on several electrodes of the array (Figure 8b&c), with an initial anterior activity of -6 µV peaking on average 41 ms before vocal onset over the premotor cortex followed by a more caudal activity over the motor cortex peaking on average 120 ms after vocal onset with an amplitude of 11 µV. The overall spatiotemporal dynamics of this vocal production related activity is

illustrated in Figure 7d on the cortical anatomy. In the second session, no premotor activity was observed but we found statistically significant cortical activity over the motor cortex consistent with that observed in the first session. This activity peaked on average 157 ms after vocal onset with an amplitude of 5.4 µV. A comparison of this motor activity between both sessions is presented in Figure 9. The cortical responses over the motor region were visible at the single trial level, especially in the second session (Figure 9a). These motor potentials lasted for the duration of the vocalization (Figure 9b), which were on average longer in the second session compared to the first (385 ms versus 210 ms). In particular longer grunts elicited longer evoked potentials (Figure 9a). Across both sessions, the spatial extent of this activity was very similar (Figure 9c), and tended to follow more closely the cruciate gyrus housing the primary motor cortex in session 2 compared to session 1, possibly indicating that the implant tended to better match the cortical folding over time post-implantation.

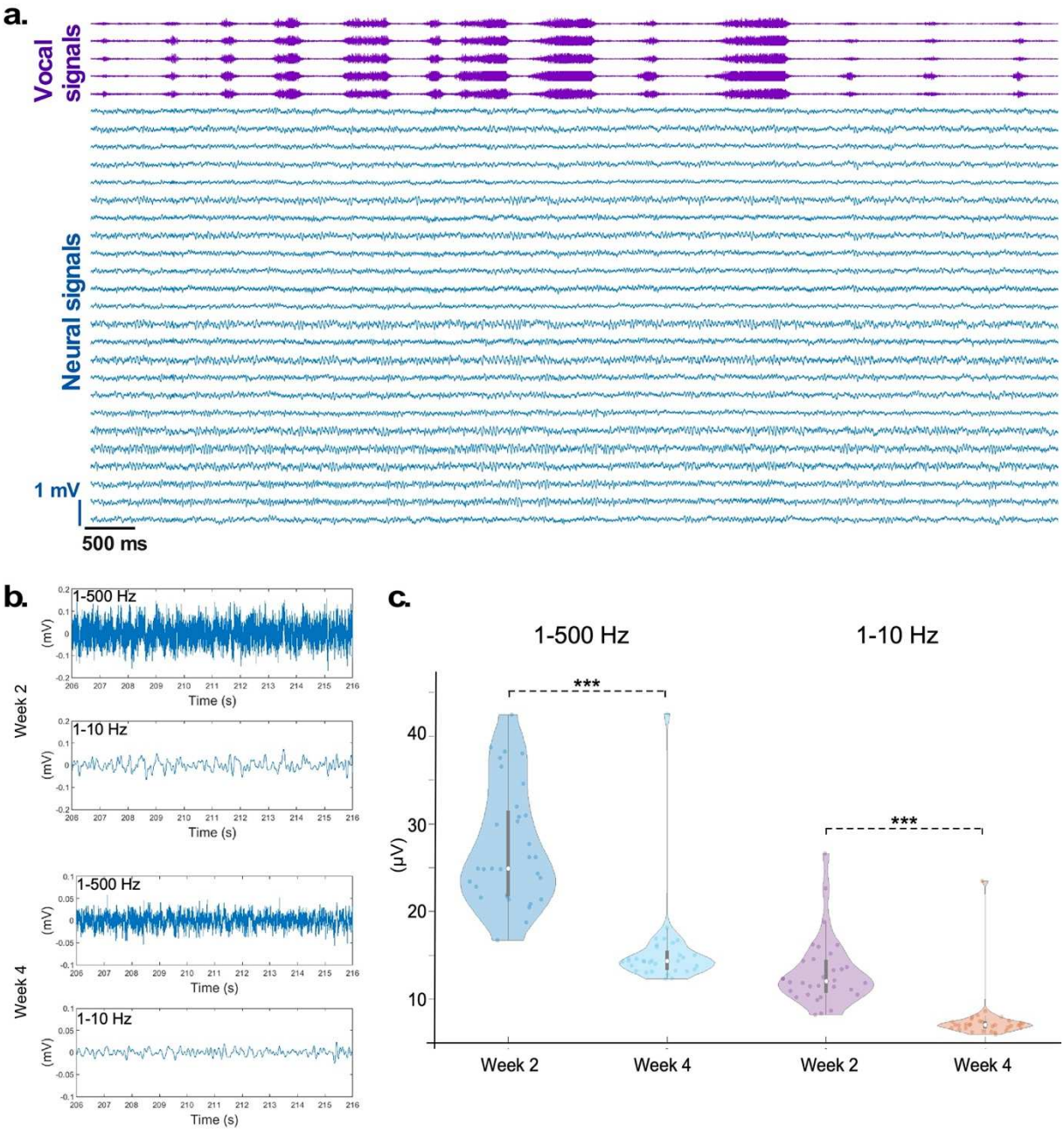

Figure 7. Evolution of baseline noise amplitude between 2 and 4 weeks after implantation. *(a) Example of 10 seconds of simultaneous audio and raw neural data recorded in animal CH596. (b) Example of raw signal for one electrode in the 1-500 Hz and 1-10 Hz frequency bands recorded at 2 and 4 weeks post implantation. (c)*

*quantification of baseline noise (**computed as the standard deviation of the signal over 60 seconds**) for all the electrodes. These signals were obtained while the animal was awake and correspond to the baseline signal used to threshold the activity maps in Figure 8 and 9 (see methods).*

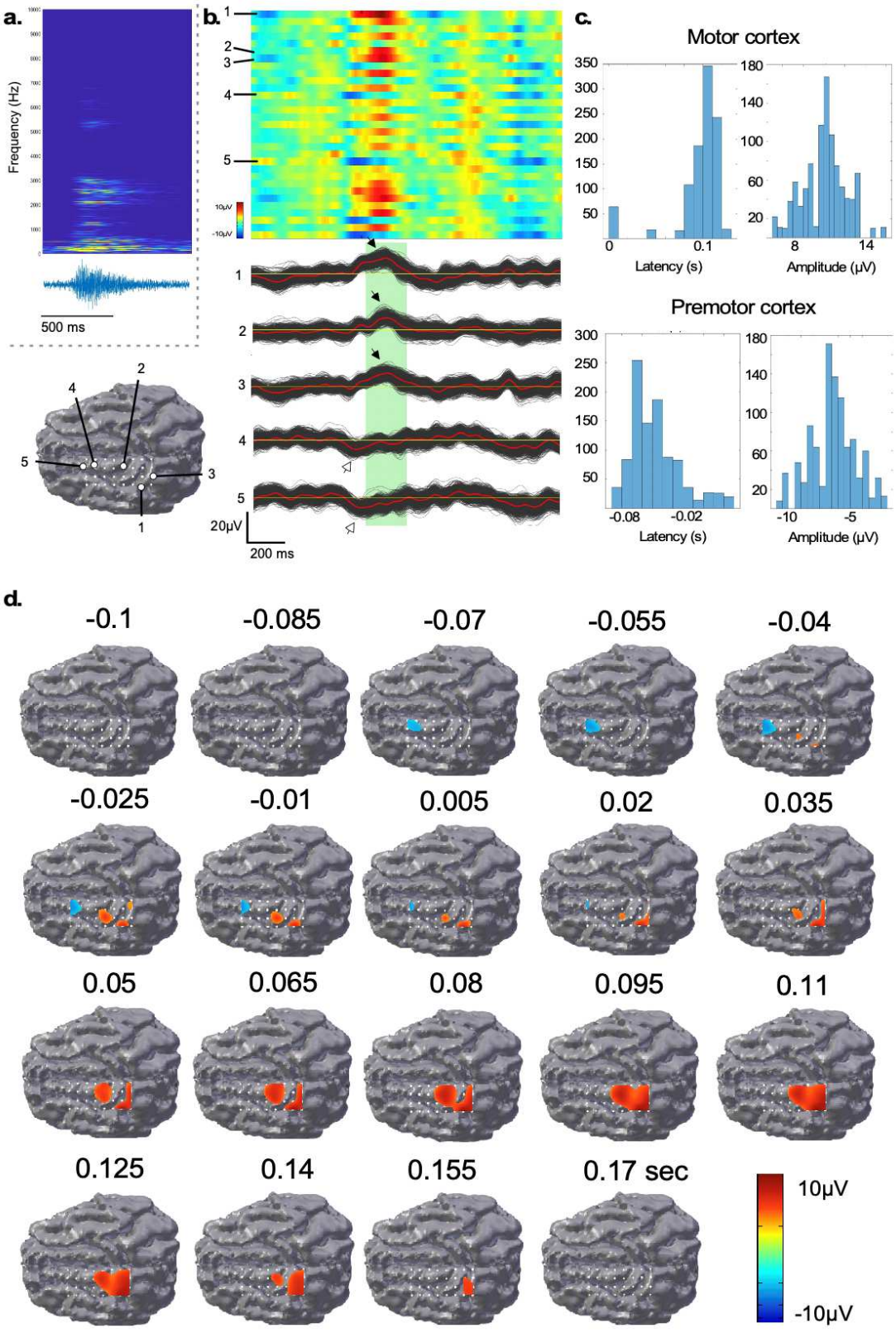

Figure 8. Cortical responses to vocal production of grunts in minipig CH596 over the motor/premotor region. *(a) Example of spectrogram and raw audio signal of a grunt vocalization. (b) Averaged evoked potentials (n = 75 grunts) color scaled across the array and for 5 electrodes of interest. Red curve= original average; black*

curves: bootstrap averages. The average vocal duration is represented by the green interval, the horizontal yellow line represents 0 µV. (c) Distributions of the latencies and amplitudes of the motor (black arrows in b) and premotor (white arrows in b) responses obtained from the bootstrap averages. (d) Time-varying spatial maps of statistically significant vocal-triggered cortical potential over the brain anatomy.

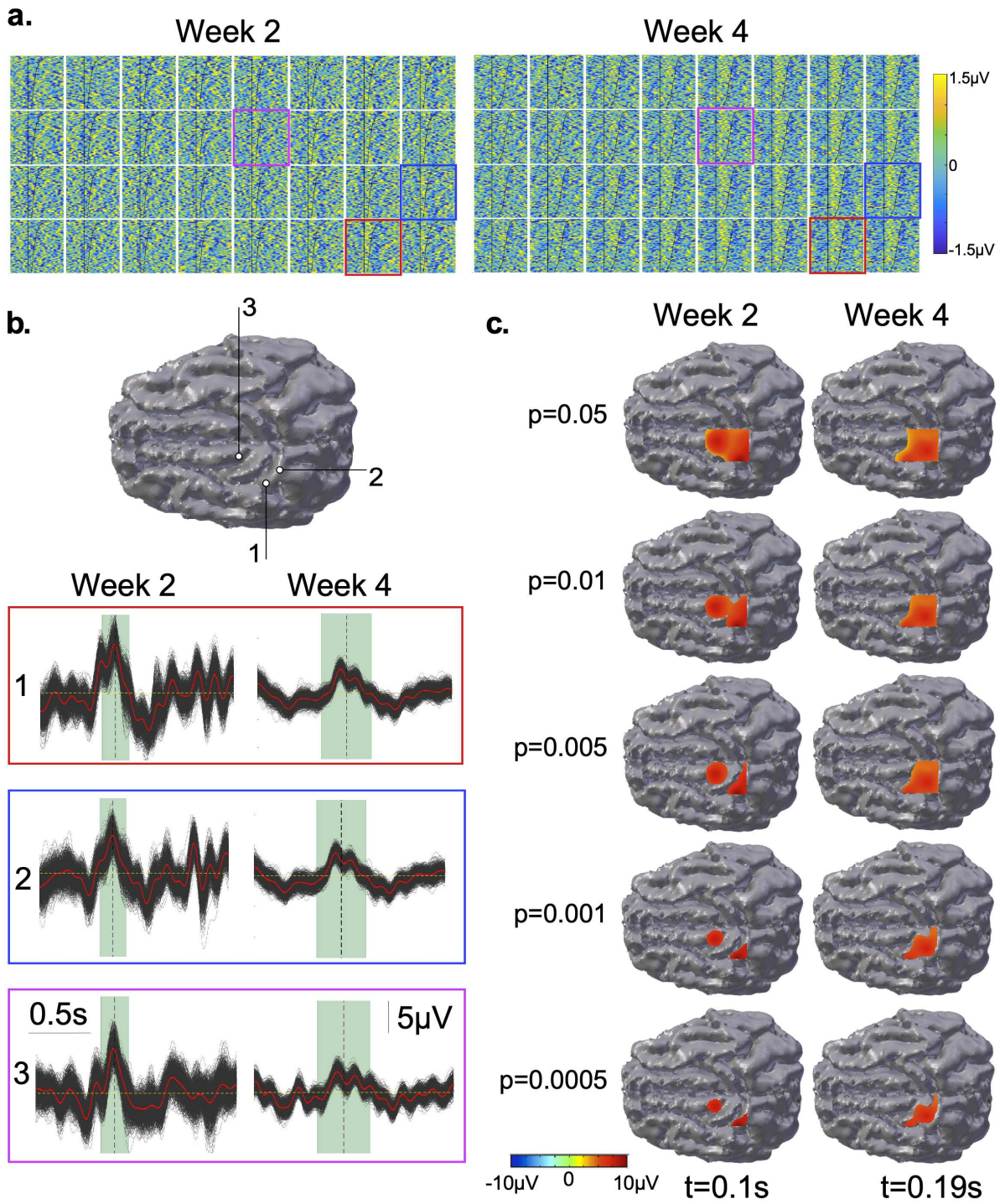

Figure 9. Comparison of cortical responses to vocal production of grunts between two recording sessions 2 and 4 weeks after implantation, respectively. *(a) Color-coded vocal-evoked potentials for all vocal occurrences (1 line per vocalization) and all electrodes displayed according to the 4x8 grid as in Figure 2b. The vertical black line indicates vocal onsets and the black curve on its right indicates the vocal offsets. Trials are sorted by vocal durations, the shortest corresponding to the lowest line of each color plot. (b) Bootstrap (black) and original (red) averages for 3 electrodes located over the motor region (framed in a). The green area indicates the mean vocal duration for each session. The yellow dashed line indicates 0 µV. (c) spatial extent of the motor cortical activity assessed at different statistical levels of the Welch test (see Methods) for each session. These*

maps correspond to the activity at the time of the middle of the vocalization as indicated by the vertical dashed lines in panel b.

Discussion

The primary goal of this paper was to describe an experimental approach enabling chronic recordings of cortical activity underlying vocal production in behaving minipigs. Planning implantation of cortical electrode arrays in young animals was found to be key to make possible a craniotomy over the motor cortex and even more frontal areas, without the usual risk of infection encountered when such surgery is performed in adult animals with well-developed frontal sinuses. Based on CT images, we found that sinuses cover the anterior part of the brain at around 5 months of age, so that performing implantation by this age avoids crossing the meninges cavities and thus opening a direct route of infection from the nasal cavity to the brain. Interestingly, no such route further develops over the months following the implantation. As shown in Figure 6, the proposed surgical procedure indeed modifies the course of development of the sinuses so that they do not invade the part of the skull bone over which the skin and the periosteum have been removed. The sinuses continue to grow caudally after the implantation but skirt around this area (Figure 6b). As a result, no infection developed at the level of the craniotomy and the brain tissue over the months after surgery.

Working with minipigs with chronic cortical implants requires practical precautions to ensure the sustainability of chronic recordings. In particular, these animals like to explore and interact with their environment with their head. This implies a reliable protection of the zone of extracutaneous connectors necessary for external connections with the recording system. The suitable solution we found was the fixation of a titanium chamber over the skull closed by a solid removable plastic hood (Figure 1 & 3). It was important that the hood did not present any poking asperities that the animal could use against its pen environment to apply a lever force that would break its attachment to the chamber and expose the implant connector. Because the scalp was removed over the area of the chamber, regular care was needed to maintain the external ridge of the chamber clean and avoid local infections. Importantly, a recovery of full skull coverage by the skin was observed after the chamber was removed when explanting the animal. This regrowth process took 1-2 months depending on the individual, and ensured that the animal could then be rehomed in a natural farming environment (Figure 3i).

The paradigm proposed in this paper opens the way to use minipigs as a new chronic experimental model for neuroscience research and particularly to study the cortical bases of vocalization in a large non-primate mammal species. Minipigs indeed represent interesting subjects for this field, because they are very loquacious animals, producing many vocalizations spontaneously, especially when in groups or when interacting with humans. Moreover, they are domestic animals with advanced cognitive capabilities (Broom et al., 2009; Gieling et al., 2011) that can be handled easily by humans without the need of coercive relationships, which is another asset for investigating behavioral paradigms.

Previous findings in NHPs, birds, and mice, have been increasingly describing the involvement of cortical structures in the production of vocalizations, especially when volitionally produced. In primates, this cortical network involves the primary motor, premotor SMA, and inferior frontal regions (Gemba et al., 1995, 1997, 1999; Romanski et al., 2005; Coudé et al., 2011; Hage and Nieder, 2013; Plakke et al., 2013; Roy et al., 2016; Gavrilov et al., 2017), and its neuroanatomical organization shares large similarities between NHPs and humans (Loh et al., 2017; Nieder and Mooney, 2020). Yet, the extent to which it can generalize to other mammals remains unknown. Here, in the animal presented in this study to illustrate the proposed paradigm, we found that the production of grunts, the most common vocalization of pigs, elicited an activation of the motor and premotor region,

starting about 40 ms before vocal onset over the premotor cortex and then involving the motor cortex. The activity over the motor cortex was consistent across both recording sessions and its duration was found to follow the duration of the vocalization (Figure 9a, right). This observation thus suggests that the motor cortex is engaged during vocal production in minipigs. Here, we did not train the animal in any particular way to volitionally produce vocalizations. Vocalizations were rather produced spontaneously while interacting with the experimenter. The fact that a cortical activity was observed could be due to the fact that the animal was on the laps or in the arms of the experimenter, and that vocalizations could thus have been produced volitionally. Future work will be required to highlight this aspect in more details in freely moving animals and also to identify more extensively the cortical network underlying vocal production in pigs and in particular to determine whether it may share similarities with the frontal network previously highlighted in primates.

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Conflict of Interest

FF. and S.P.L. hold various patents in relation with the present work and are cofounders of Neurosoft Bioelectronics SA.

Author contributions

Marie Palma: Methodology, Software, Validation, Formal analysis, Investigation (surgeries, in vivo recordings), Writing original draft, Visualization; **Mehrdad Koshnevis:** Methodology, Validation, Investigation (surgeries), Writing original draft; **Marie Lion:** Methodology, Software, Validation, Formal analysis; **Cyril Zenga:** Investigation (surgeries, in vivo recordings); **Samy Kefs:** Investigation (CT-Scans); **Florian Fallegger:** Methodology and Resources (soft implants), Manuscript review and editing; **Giuseppe Schiavone:** Methodology and Resources (soft implants); **Isabelle Gabelle Flandin:** Supervision and Investigation (CT-Scans); **Stéphanie Lacour:** Supervision and funding acquisition (soft implants); **Blaise Yvert:** Conceptualization, Methodology, Software, Validation, Investigation (surgeries, in vivo recordings), Writing original draft, Visualization, Supervision, Project administration, Funding acquisition.

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