● *Original Contribution*

TRANSCRANIAL FUNCTIONAL ULTRASOUND IMAGING IN FREELY MOVING AWAKE MICE AND ANESTHETIZED YOUNG RATS WITHOUT CONTRAST AGENT

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Abstract—Functional ultrasound (fUS) imaging by ultrasensitive Doppler detection of blood volume was previously reported to measure adult rat brain activation and functional connectivity with unmatched spatiotemporal sampling (100 μm, 1 ms), but skull-induced attenuation of ultrasonic waves imposed skull surgery or contrast agent use. Also, fUS feasibility remains to be validated in mice, a major pre-clinical model organism. In the study described here, we performed full-depth ultrasensitive Doppler imaging and 3-D Doppler tomography of the entire mouse brain under anesthesia, non-invasively through the intact skull and skin, without contrast agents. Similar results were obtained in anesthetized young rats up to postnatal day 35, thus enabling longitudinal studies on postnatal brain development. Using a newly developed ultralight ultrasonic probe and an optimized ultrasonic sequence, we also performed minimally invasive full-transcranial fUS imaging of brain vasculature and whisker stimulation-induced barrel cortex activation in awake and freely moving mice, validating transcranial fUS for brain imaging, without anesthesia-induced bias, for behavioral studies. (E-mail: mickael.tanter@espci.fr) © 2017 The Authors. Published by Elsevier Inc. on behalf of World Federation for Ultrasound in Medicine & Biology. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

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INTRODUCTION

A wide range of genetic models established mice as the most important model for pre-clinical studies in neuroscience, but to date, the use of whole-brain neuroimaging modalities, such as functional magnetic resonance imaging (fMRI), is limited in this species. Indeed, the number of articles reporting fMRI studies in mice is much lower than that in rats: an analysis of the bibliography on PubMed (September 2016) using the key words "fMRI AND mice" returns 31 articles, whereas "fMRI AND rats" returns 158 articles; that is, more than five times more studies were performed in rats. This disparity may be explained by the difficulties encountered in performing high-quality fMRI imaging in mice compared with rats,

typically lower detection sensitivity, image distortions and signal losses (Nasrallah et al. 2014). Another strong limitation of fMRI studies in rodents is that rat or mouse brain imaging typically requires anesthesia to prevent animal movements. However, the use of anesthesia excludes behavioral and cognitive experiments and raises questions about modification of neuronal metabolism and cerebral blood flow during anesthesia (Lahti et al. 1999). Several protocols have therefore been proposed to adapt fMRI experiments to awake rodents. Rats can be constrained to prevent movements during awake brain imaging by using restraining methods combined with specific habituation procedures to minimize stress (King et al. 2005; Lahti et al. 1998), but this technique is rarely applied to fMRI acquisitions in awake mice (Desai et al. 2011). Another approach to keep the animals conscious during the experiment is to use muscle relaxants to paralyze the body (Peeters et al. 2001). Consequently, although fMRI is a powerful non-invasive imaging method with relatively

high spatial and temporal resolution, the need for immobilization strongly limits awake experiments. Other techniques such as electrophysiology, optical or photoacoustic imaging enable the recording of neuronal activity or imaging of blood vessels in mobile animals (Packer et al. 2015; Yu et al. 2015), but with a very limited field of view (Tang et al. 2016) and typically through invasive procedures. Because of these limitations, the development of novel highly resolved brain imaging technologies, optimized to non-invasive imaging of awake and freely moving mice, is an important scientific objective.

We recently reported that functional ultrasound (fUS) imaging (Macé et al. 2011) is possible in awake and mobile rats using a small ultrasonic probe fixed on the rat head (Sieu et al. 2015). The possibility of imaging the brain in mobile rats confers a huge advantage to fUS over fMRI, in addition to its other advantages such as high spatial and temporal resolution, lower acquisition and operating costs, no maintenance and a portable scanner. However, the skull remains an obstacle in fUS imaging for ultrasonic waves. Indeed, direct transcranial Doppler imaging of the adult rat brain is not possible because of skull-induced attenuation and aberrations of ultrasonic waves. Consequently, currently the use of an injected contrast agent (Errico et al. 2016) or a surgical procedure to produce a craniotomy (Sieu et al. 2015) or a thinned-skull window (Osmanski et al. 2014) is required for Doppler and functional ultrasound imaging of the adult rat brain. The thinning procedure is less invasive and less complex than a complete craniotomy and allows longitudinal studies to be performed, but the image quality rapidly degrades over time (Urban et al. 2014) because of bone regeneration. An improvement of this technique is described in Sieu et al. (2015) in which a polymer prosthesis, transparent to ultrasound, is sealed in place of the removed skull to protect the brain. This approach offers a large field of view and enables longer longitudinal studies as the imaging conditions remain stable over 1 to 2 mo in the rat. However, the requirement to use these invasive approaches had not yet been investigated in small rodents such as mice and young rats. Importantly, in these animals, the skull is rather thin, suggesting the yet to be proven possibility of direct non-invasive functional imaging, provided that suitable specific hardware and software tools are developed.

In this study, we first determined that transcranial ultrasensitive Doppler images can be obtained non-invasively in anesthetized mice of different ages, through the intact skull and skin. In addition, we also performed non-invasive transcranial ultrasensitive Doppler imaging in young rats at different ages. In anesthetized adult mice, we found that 3-D tomographic acquisition and representation of the whole-brain vasculature is possible though intact skull and intact skin. These results pave the way for longitudinal functional studies of the developing brain. Finally, we designed a novel ultrasound setup and adapted sequence developed for high-quality transcranial fUS imaging in awake and freely moving mice.

METHODS

Animals

Animals were housed four per cage in a controlled environment (22 ± 2°C, 50% relative humidity, 12/12 dark/light cycle) and were provided with food and water *ad libitum.* To minimize stress during the experimental procedure, mice were given a 7-d acclimation period after their arrival. All animals received human care in compliance with the European Communities Council Directive of 2010. This study was approved by the local committee for animal care (Comité d'éthique en matière d'expérimentation animale No. 59, C2 EA-59, "Paris Centre et Sud") under Agreement No. APAFIS#3323-2015122411279178_v3-3.

The study of age-dependent changes in imaging quality was carried out on five C57Bl/6 mouse pups, and five Sprague-Dawley rat pups, respectively from the same litter (undetermined sex, Janvier Labs, Le Genest St. Isle, France). Whole-brain ultrafast Doppler tomography acquisition was carried out on an 8-wk-old C57Bl/6 male mouse (Janvier Labs). Freely moving experiments were carried out in three C57Bl/6 male mice (Janvier Labs), 8 wk old at the beginning of the experiments.

Ultrasonic probes and scanner

Two different high-frequency ultrasonic probes were used with an ultrafast research ultrasound scanner (Aixplorer, SuperSonic Imagine, Aix-en-Provence, France) running MATLAB (The MathWorks, Natick, MA, USA). In young rats the acquisitions were performed using a 15-MHz ultrasonic probe (128 elements, 0.08-mm pitch, Vermon, Tours, France). In mice, a new ultralight probe prototype (15 MHz central frequency, 128 elements, 0.110-mm pitch, 8-mm-elevation focal distance and 400-μm-elevation focal width) was used both in anesthetized and in awake freely moving mice. This new ultralight ultrasonic probe has been specifically designed for awake rodents. Compared with our previous design used for mobile rat experiments in Sieu et al. (2015), its thickness has been reduced by a factor of 5 (from 1.85 to 0.4 cm) and its weight by a factor of 3 (from 12 to 4 g), and its cable has been made more flexible (Fig. 1a). The probe was designed in-house and manufactured by Vermon according to these specifications.

Ultrasound sequence for transcranial ultrafast Doppler acquisition

Vascular images were obtained using the ultrafast compound Doppler imaging technique (Bercoff et al. 2011).
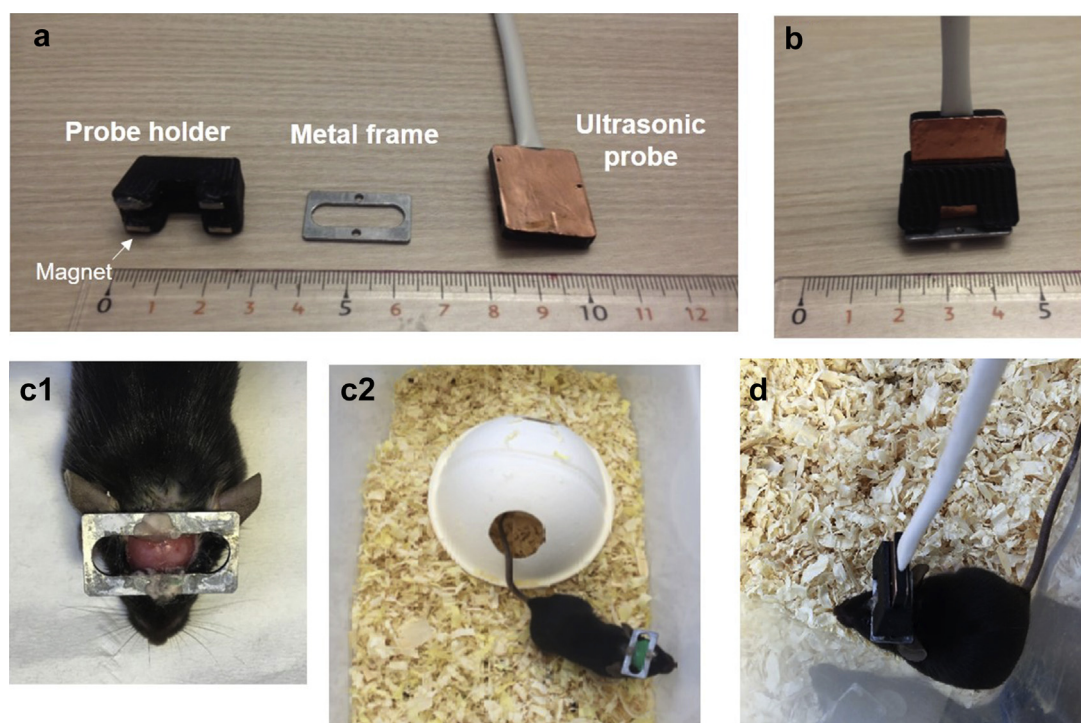
Fig. 1. Experimental setup for transcranial functional ultrasound (fUS) acquisitions in freely moving animals. (a) The setup for clip-on probe fixation for experiments in freely moving mice comprises three parts: the probe, the magnetic probe holder and a light metal frame chronically fixed over the intact skull. (b) Assembled setup for freely moving mice. (c1,c2) Mouse with the chronically fixed metal frame. The metal frame is the only part of the setup preserved on the mouse head between experiments. The intact skull (c1) of the mouse is protected by a Kwik-Cast plastic layer between experiments (green part in c2). (d) Mouse with the full setup on its head during an experiment.

Each frame was a compound plane wave frame (Montaldo et al. 2009) resulting from the coherent summation of backscattered echoes obtained after successive tilted plane wave emissions (typically 11 compounded tilted plane waves separated by $2°$). A stack of hundreds of such compounded frames was acquired with very high frame rate (typically 200–400 frames at 500-Hz frame rate, recording over several cardiac cycles). Then, the blood flow signal was extracted from the tissue signal by filtering the image stacks with a dedicated spatiotemporal filter using singular value decomposition (SVD) (Demene et al. 2015). Each transcranial Doppler image in developing young rats and mice was obtained from 400 compounded frames acquired at 500-Hz frame rate, using 11 tilted plane waves separated by $2°$ ($-10°, -8°, -6°, -4°, -2°, 0°, 2°, 4°, 6°, 8°, 10°$) acquired at a 5500-Hz pulse repetition frequency (PRF).

Ultrasound sequence for the 3-D transcranial Doppler scan in mouse

For the fully non-invasive transcranial 3-D scan in mouse, 200 compounded frames were acquired at 500-Hz frame rate, with the same tilted plane wave configuration ($-10°, -8°, -6°, -4°, -2°, 0°, 2°, 4°, 6°, 8°, 10°$) to form one ultrasensitive Doppler image. This sequence was repeated for different scan orientations as described below.

Ultrasound sequences for freely moving functional ultrasound experiments in mice

For fUS acquisitions, ultrasensitive Doppler images were acquired every second over 5 min. Each ultrasensitive Doppler image was obtained from 200 compounded frames acquired at 500-Hz frame rate, using five ultrasonic tilted plane waves ($-4°, -2°, 0°, 2°, 4°$), acquired at a 2500-Hz PRF.

An optimization of this ultrasonic acquisition sequence was required for experiments in freely moving animals. A two-way transmit and receive apodization (Cobbold 2006) was used to minimize unwanted ultrasound signals transmitted to and backscattered from muscles. This apodization corresponds to the introduction of independent weighting gains on the different channels based on a hamming window. Because of this apodization, 64 lateral elements over the 128 elements of the probe are used at less than 50% maximal amplitude, effectively blinding both sides of the image on approximately 3.5 mm.

Motorized linear four-axis stage for probe positioning and 3-D scanning in anesthetized rat pups and mice

The positioning of the probes above the head of the animal was performed with a motor system setup enabling three degrees of translation (3 Physik Instrumente

[PI, Karlsruhe, Germany] translation stages VT-80) and one degree of rotation (1 PI rotation stage DT-80) controlled by MATLAB. This setup can be used for regular positioning of the probe over the brain of the animals or for performing tomographic scans to produce high-resolution Doppler volumes of the brain. The complete setup is described in detail in Demené et al. (2016).

Frame and magnetic probe holder for transcranial fUS acquisitions in freely moving mice

The challenge for awake and mobile experiments in the mouse is its small size compared with rats. Our setup consists of a new ultralight ultrasonic probe (described above) mounted in a magnetic probe holder, and a metal frame fixed on the mouse head (Fig. 1a,b). All these elements have been designed to minimize size and weight. The metal frame fixed directly on the skull of the mouse (Fig. 1c1) is a rectangle (12 × 23 mm) with an imaging window (6 × 21 mm), cut from a galvanized steel plate, a magnetic material. This property is used for rapid clip-on fixation of the probe holder to the metal frame. For this purpose, four small but strong magnets (Super-magnete, Gottmadingen, Germany, Q-05-1.5-01-N, 1 × 1.5 × 5 mm, NdFeB, adhesive force: 140 g) were built into the base of the probe holder (Fig. 1a), enabling magnetic fixation between the metal frame and the probe holder (Fig. 1b). The probe holder was designed in Auto-desk Inventor software (Autodesk, Inc., San Rafael, CA, USA) and made by a 3-D printer (MakerBot, New York, NY, USA).

Transcranial Doppler acquisitions in anesthetized rat pups and mice

The animal was anesthetized and its head was fixed in a stereotaxic frame. Mice were anesthetized using 1.5% iso-flurane. Rat pups were anesthetized using an intra-peritoneal (ip) injection of a mixture of ketamine (50 mg/kg/h) and medetomidine (0.75 mg/kg/h). Images were acquired through the intact skull and the skin (after hair removal using a commercial depilatory cream, Netline from BIOES Laboratory) without any contrast agent injection or surgery. Thus, under these conditions, the acquisition was fully non-invasive. In rat pups, the fully non-invasive configuration was compared with a minimally invasive configuration, in which the head skin was opened and the probe was placed directly on the skull of the rat.

Probe positioning using vascular landmarks in anesthetized rat pups and mice

To date no complete vascular atlas exists for mouse or rat brain, so a correct positioning of the probe relative to the standard reference bregma landmark is not direct. In young rats, we identified the anterior choroidal arteries, an easily recognizable vascular landmark located approx-imately at bregma −3.8 mm in the adult rat brain (Scremin 2004). For mouse imaging, we acquired a series of coronal Doppler scans of the adult mouse brain with a wire land-mark placed on bregma after skin removal. The image containing the echo of the wire corresponds to the coronal bregma level, allowing localization of the Doppler image scans, acquired through the intact skull. The anterior choroidal arteries in the mouse brain have been identified in the coronal plane corresponding to ~bregma −2.3 mm. This specific vascular landmark can be systematically identified—even through the intact skin in absence of visible skull landmarks—to correctly position the probe at the selected coronal slice. In addition, functional fUS data, for example, barrel-cortex activation during whisker stimulation, may provide additional information to confirm the correct position of the probe. In a preliminary study, we performed systematic scans in developing rat and mouse brains and observed that the relative positions of main veins and arteries do not change between the first post-natal day and adulthood despite significant changes in the size and shape of the developing brain. Conse-quently, here we define locations as 'corresponding to the adult bregma $\pm X$ coordinate'.

Transcranial 3-D Doppler scan in anesthetized mice

The motorized setup was used to perform tomo-graphic scans with a combination of rotation and transla-tion to improve vascular image resolution and to give a 3-D representation of the whole brain (Demené et al. 2016). For a given orientation θ of the probe, a volume of the mouse brain was acquired by translating the probe in steps of 100 μm. Then the probe was rotated along θ in 10° steps. To acquire the whole-brain volume, 18 orienta-tions θ of the probe were used, and for each orientation θ, 70 translation/imaging steps were performed. In each posi-tion of the probe, one ultrafast Doppler image was ac-quired. The acquisition of a 3-D data stack using these parameters takes around 13 min. The entire post-treatment procedure to merge the 18 volumes into one is described in detail in Demené et al. (2016). Briefly the post-treatment consists of the following steps: (i) Each vol-ume is interpolated on an isotropic grid of 50-μm voxels. (ii) Each volume is then transposed to the laboratory land-mark by a θ rotation around the axis Oz. (iii) As the geomet-ric center of the probe does not necessarily coincide with the geometric center of the rotation of the probe around θ, an inter-correlation step is done to compute the offset be-tween this two points and correct it. (iv) After this recalibra-tion step of the 18 volumes, all the volumes are summed.

Frame implantation for experiments on freely moving mice

One week before the fUS imaging acquisition ses-sion on freely moving mice, a flat metal frame was fixed

on the mouse skull (Fig. 1c1) to enable the magnetic fixation of the ultrasonic probe, an approach comparable but not identical to that described in a previous report (Urban et al. 2015). For the surgical implantation of the metal frame, mice were anesthetized using a mixture of ketamine (100 mg/kg, ip) and medetomidine (1 mg/kg, ip) and placed on a stereotaxic frame. After loss of pedal withdrawal reflex, the skin and periosteum were removed to expose the skull. The metal frame was fixed on the skull with two surgical anchoring screws and Superbond C&B (Phymep, Paris, France). The stability of the metal frame was further ensured using dental cement (Henry Schein, Paris, France) (Fig. 1c1). The frame window (ranging roughly from the skull landmarks bregma to lambda) was covered with Kwik-Cast (Phymep) between imaging sessions to ensure protection and maintain integrity of the bone (Fig. 1c2). Anesthesia was then reversed with a subcutaneous injection of atipamezole (1 mg/kg, Antisedan). All mice received a prophylactic administration of penicillin (Extencillin, 100,000 IU/kg, intra-muscularly) and meloxicam (Metacam 5 mg/kg/d, subcutaneously) to prevent post-operative pain. The mice were left for 1 wk to recover from this procedure.

Mice carry chronically only the metal frame and two screws, corresponding to a total weight of 1.4 g (Fig. 1c1,c2). The normal activity of the mice is not notably disturbed (Fig. 1c2).

Transcranial fUS acquisitions in freely moving mice

At the start of the fUS imaging session, mice were slightly anesthetized with isoflurane inhalation. The Kwik-Cast layer protecting the skull was removed, the skull was cleaned with saline and echographic gel was applied to the window. The probe in the magnetic holder was then fixed on the metal frame, aligned with the edge of the frame (Fig. 1d; Supplementary Video S1, online only). During the experiment, the mice carry the total setup (metal frame + probe holder + probe) for a total weight of around 8 g (Fig. 1d). In this current proof of concept study, the experimenter manually lightened the weight of the setup by holding the cable of the probe and following mouse movements. See Supplementary Video 1 to visualize the clip-on positioning of the probe and the complete setup fixed on the mouse head.

The mouse was placed in an empty cage (without sawdust) to facilitate access to its whiskers during whisker stimulation acquisitions. The probe was manually positioned over the coronal plane corresponding to the anteroposterior coordinate bregma −1.5 mm that contains the somatosensory barrel field cortex (S1 BF). After a 30-min habituation period, mouse whiskers were stimulated manually using a cotton swab for fUS imaging under awake conditions. The stimulation pattern consisted of three manual stimulations (30-s ON periods, 5–7 Hz,

1-cm amplitude) of the right or left whiskers, separated by 60-s OFF periods, for a total acquisition duration of 5 min. The temporal correlation between the stimulation pattern and the hemodynamic signal was computed in each pixel of the brain image. Only pixels with correlations higher than three times the noise level were considered as significantly activated by the stimulus. These activated pixels (hot colors) were superimposed on the transcranial vascular image of the brain (gray-scale colors).

RESULTS

Non-invasive 3-D whole-brain imaging in mice

First, using our novel miniaturized ultrasonic probe, we investigated the possibility of performing fully non-invasive ultrafast Doppler imaging of the brain vascular system in 8-wk-old adult mice. Tomographic ultrafast Doppler head scans of anesthetized mice were acquired through the intact skull and the intact skin using the motorized stage. Figure 2a is a schematic representation of the mouse skull landmarks and the extent of the scan in the coronal direction from bregma −5 mm to bregma +1 mm (Fig. 2b). Doppler images obtained completely non-invasively through the intact skull and skin are high quality, similar to those obtained through the skull but with the skin removed (data not shown). The anterior striate arteries (astr), anterior choroidal artery (ach), middle cerebral artery (mcer), posterior medial choroidal artery (pmch) and supracollicular arterial network (scol), as described previously in Scremin (2004) and Demené et al. (2016), are identified in Figure 2b. In the coronal images from bregma −3.5 mm to bregma +0.5 mm, the internal carotids of the circle of Willis are visible, first below the anterior choroidal arteries and then below the middle cerebral arteries. Figure 2c illustrates the full 3-D reconstruction of the mouse brain using the tomographic scan (Demené et al. 2016). (See also Supplementary Video S2 [online only] for a full 3-D visualization.)

In conclusion, the vasculature of the whole brain (entire depth and width) of the young adult mouse can be imaged using fully non-invasive ultrafast Doppler imaging, opening the possibility for longitudinal studies of brain vascularization and function.

Age dependence of non-invasive imaging quality during post-natal development of mice and rats

Transcranial Doppler imaging of the mouse brain during post-natal development. Next, we investigated the possibility of longitudinal studies of brain vasculature, from the post-natal period to late adulthood in mice. Anesthetized mice from post-natal day 1 (PND 1) up to 1 y were imaged through their intact skull and intact
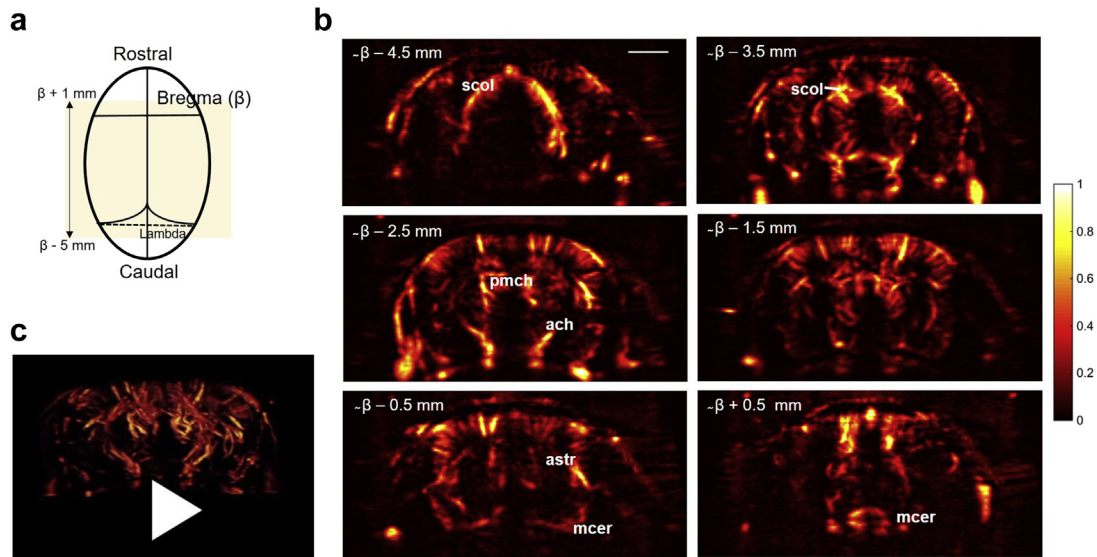
Fig. 2. The entire brain of an 8 wk-old mouse can be imaged fully non-invasively through the intact skull and skin. (a) Schematic of the mouse skull with the bregma and lambda landmark positions. The colored rectangle represents the extent of the scan in the caudorostral direction. (b) Ultrafast Doppler images of six different coronal slices of the mouse brain acquired during the caudorostral scan. Bar = 2 mm. astr = anterior striate arteries; ach = anterior choroidal artery; mcer = middle cerebral artery; pmch = posterior medial choroidal artery; scol = supracollicular arterial network. (c) Screenshot of Supplementary Video S2 revealing 3-D reconstruction of the mouse brain using ultrafast Doppler tomography.

skin (Fig. 3). The same coronal slice of the brain was acquired (around bregma −2.3 mm) to compare image quality between ages. We obtained high-quality images using fully non-invasive transcranial Doppler imaging for all ages tested.

In conclusion, our results indicate that the relative transparency of the mouse skull allows non-invasive ultrafast Doppler imaging in a wide range of ages in this species.

Transcranial Doppler imaging of the young rat brain during post-natal development. The aforementioned results indicate that the relative transparency of the mouse skull allows high-quality ultrafast Doppler imaging up to late adult stages. The skull of adult rats is considerably thicker, leading to the attenuation of ultrasound insonification and requiring either craniotomy (Sieu et al. 2015), a thinned-skull window (Osmanski

et al. 2014) or intra-venous injection of contrast agents (Errico et al. 2016) for Doppler imaging. However, the skull of young post-natal rats is relatively thin, similar to that of adult mice; that is why we investigated the feasibility of fully non-invasive transcranial vascular imaging in the early post-natal period in this species. Ultrafast ultrasound Doppler imaging of five young rats from the same litter was performed at the same coronal position (around bregma −3.8 mm) to compare image quality at different ages. Two conditions were tested for each young rat: a first acquisition through the intact skull and intact skin (Fig. 4, top), and a second one after removal of the skin (Fig. 4, bottom). We found that fully non-invasive Doppler imaging is possible in young rats until PND 35 (Fig. 4, top), with acceptable imaging quality to observe vessels both in the cortex and at the bottom of the brain (at 8-mm depth). However, the quality of the fully non-invasive image (through skull and skin) decreases
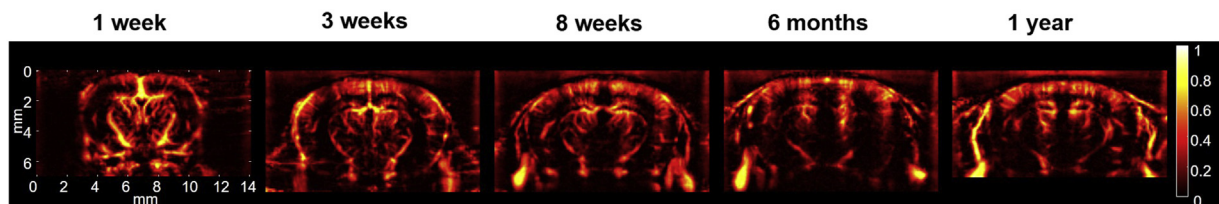

Fig. 3. Cerebral blood volume (CBV) maps of 1-w-old to >1-y-old mice. Approximately the same coronal slice (corresponding to ~bregma −2.3 mm in adult mouse atlas) has been imaged at different ages, non-invasively through intact skull and skin.
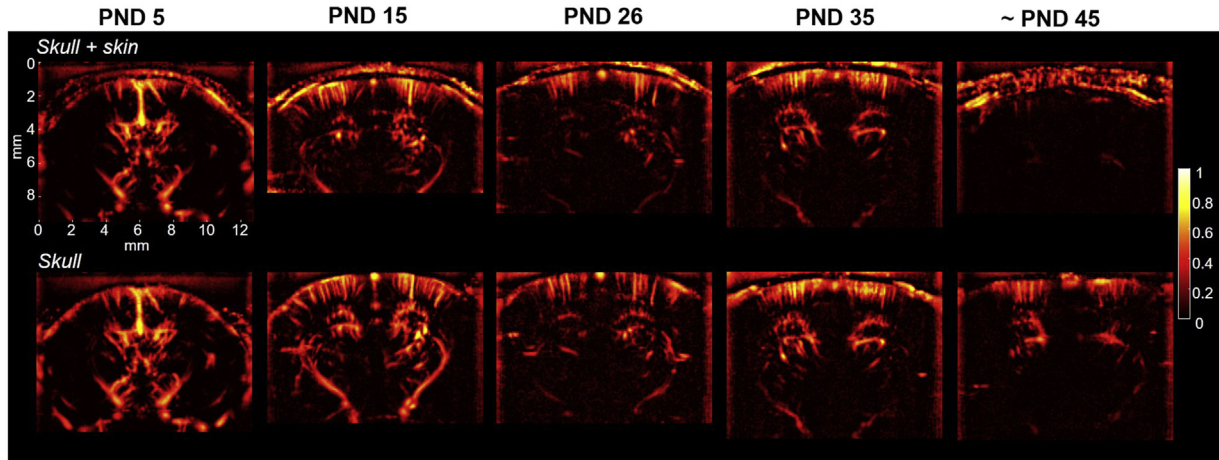
Fig. 4. Cerebral blood volume (CBV) of young rats between 5 and 45 post-natal days (PND) old. Approximately the same coronal slice (corresponding to ~bregma −3.8 mm in adult rat brain atlas) has been imaged at different ages, first through skull and skin (top) and then only through the skull after skin removal to improve image quality (bottom). High-quality images of transcranial vessels can be obtained in rat brain until 1 mo of age without contrast agent or invasive surgery.

gradually with age in rats because of the thickening of the skull during development: the brain Doppler image in the 45-d-old rat through skull and skin does not allow the visualization of blood vessels, even in the cortex.

Removing the skin slightly improves image quality, especially in the 45-d-old rat: several large vessels in cortex and deeper in the brain, invisible through skull and skin, are distinguishable after skin removal (Fig. 4, bottom).

Transcranial fUS imaging in freely moving mice

Transcranial ultrafast Doppler imaging in awake and freely moving mice. Based on the above results for fully non-invasive Doppler imaging in anesthetized mice, we investigated the possibility of recording functional activity in awake and freely moving mice in a minimally invasive way. As described above, Doppler brain imaging in the anaesthetized mouse is possible through the intact skull and intact skin. However, as the skin is not rigidly fixed to the skull, fully non-invasive imaging, by, for example, using glue to fix the probe to the skin, is not possible. Indeed, to avoid movement artifacts in ex-

periments on freely moving mice, robust stabilization of the probe is required. For this purpose, we developed a rapid and reversible fixation system using magnetic clipping of the probe to a small metal frame, chronically fixed on the skull (Fig. 1). After magnetic fixation of the probe holder (enclosing the ultralight probe) to the metal frame, high-quality ultrafast Doppler images revealing the vasculature of a coronal slice of the brain and surrounding tissues could be obtained through the intact skull in the awake and freely moving mouse. Figure 5 illustrates the imaging of several coronal slices of the brain through the imaging window of the metal frame between the bregma and lambda landmarks, by manual adjustment of the probe over the metal frame.

Although we could obtain high-quality individual power Doppler images in awake animals using the same acquisition sequence employed for anesthetized mice, for continuous monitoring of brain activity, optimization of the ultrasonic acquisition sequence was necessary to suppress a previously unreported and relatively frequent artifact we detected specifically in awake mice. The image artifact corresponds to a strong Doppler
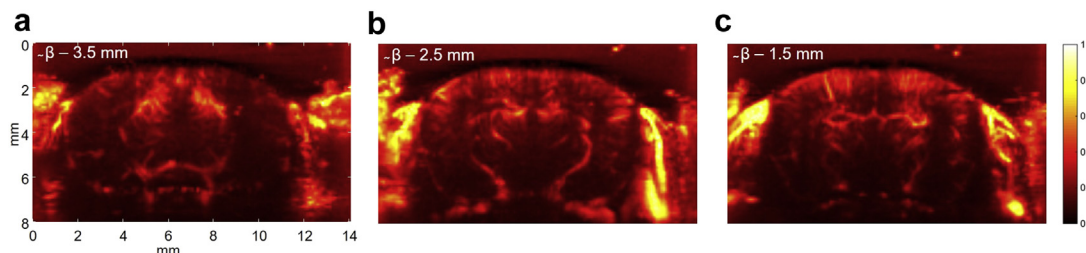

Fig. 5. Brain vascularization maps obtained by power Doppler imaging in a freely moving mouse. Different coronal slices were imaged between the bregma and lambda skull landmarks, by manually moving the probe over the metal frame. These coronal slices were identified as corresponding to bregma −3.5 (a), −2.5 (b) and −1.5 mm (c).

clutter related to temporal muscle movement on both sides of the head during what seems to be mastication, yielding frequent sharp increases up to +120% of the Doppler signal over the brain (Fig. 6c). This artifact is particularly undesirable in the case of fUS imaging, in which the key information corresponds to the variation of the Doppler signal caused by the functional activity of the brain. We thus reduced this artifact by acoustically blinding those muscles both in transmit and in receive modes using apodization based on a hamming weighting window (Fig. 6a,b). This improvement of the sequence led to significant suppression of the artifact, yielding a stable and high-quality Doppler image with amplitude variation compatible with brain activity-induced cerebral blood volume (CBV) changes (<20%) in awake and freely moving mice (Fig. 6d).

Transcranial imaging of barrel-cortex activation during whisker stimulation in awake and freely moving mice. Finally, we investigated the feasibility of task-activated functional ultrasound imaging in awake and freely moving mice. The probe was positioned over the coronal plane corresponding to the anteroposterior coordinate bregma −1.5 mm that contains the somato-sensory barrel field cortex (S1 BF). The activation map in Figure 7a illustrates the spatial distribution of the hemodynamic response to manual stimulation of the left whiskers. The gray-scale background image is the transcranial vascular image of this coronal slice of the brain (∼bregma −1.5 mm). The correlation level represents the temporal correlation between the stimulus pattern and the hemodynamic response (Fig. 7b). Only pixels with correlation higher than three times the noise level were considered significantly activated by the stimulus and are represented (Fig. 7a). As expected, whisker stimulation leads to activation of the contralateral barrel cortex: stimulation of the left whiskers is highly correlated with an increase of the cerebral blood volume in the right S1 BF (Fig. 7b). The average correlation value in the activated area was 0.56. The
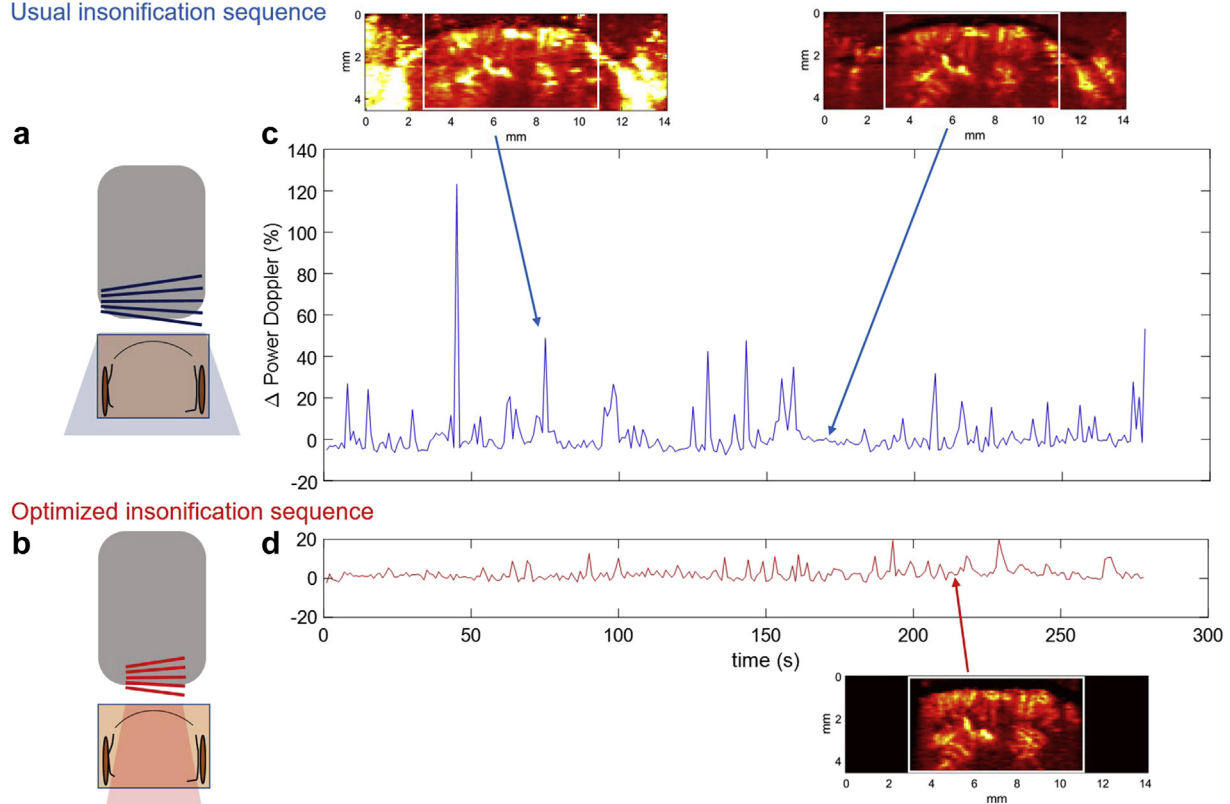

Fig. 6. Ultrasonic sequence optimization by targeting ultrasound energy to and from the brain only to reduce an important artifact originating from the temporal muscles. One power Doppler image was acquired every 1 s over 5 min with either (a) standard insonification sequence using the 128 elements of the probe or (b) the optimized insonification sequence using Hamming apodization in transmission and reception. (c) Clutter Doppler signal originating from the temporal muscles introduces important artifacts inside the brain region and yields a sharp increase, up to +120%, of the Doppler signal, as illustrated by the Doppler images during a peak Doppler amplitude (left image) and during a stable phase (right image). (d) The targeted insonification sequence, which uses apodization of the lateral elements over 3.5 mm in both transmit and receive modes, yields an overall more stable Doppler trace with amplitude variation <20%, compatible with brain-activity induced cerebral blood volume increase.
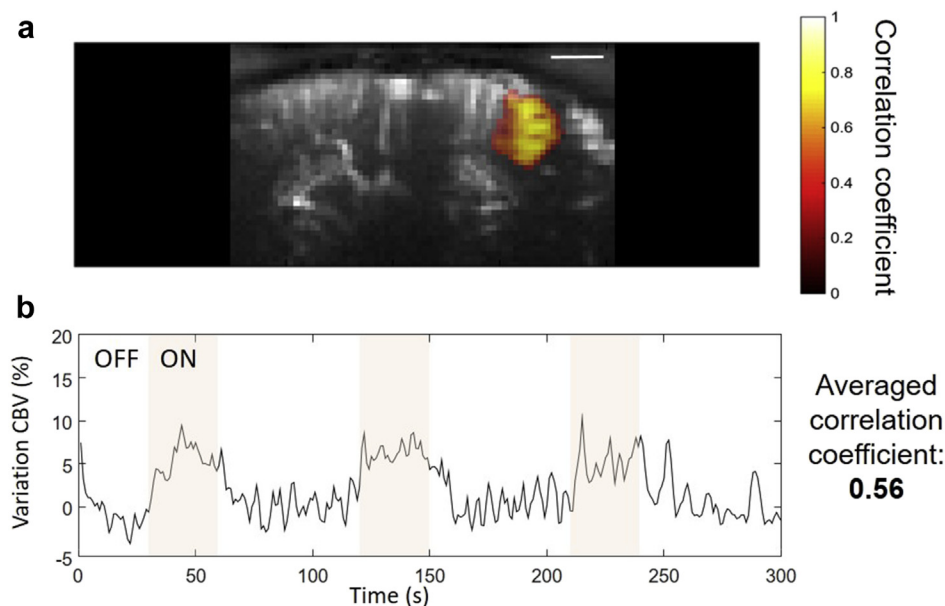
Fig. 7. Transcranial functional ultrasound (fUS) imaging of task-induced changes in cerebral blood flow in the barrel cortex after whisker stimulation in a freely moving awake mouse. (a) The gray-scale background image is the transcranial vascular image of this coronal slice of the brain (∼bregma −1.5 mm). Bar = 1 mm. (b) The correlation level represents the temporal correlation between the stimulus pattern and the hemodynamic response (no averaging, single acquisition).

same experiment was done in N = 3 mice, with an average correlation coefficient of 0.55 ± 0.15. In conclusion, with use of a newly developed ultralight probe with magnetic clip-on fixation, functional ultrasound (fUS) imaging reliably reports task-induced hemodynamic changes in awake and freely moving mice, through the intact skull, without thinning surgery or contrast agent injection (see also Supplementary Video S1 concerning experiments and setup for freely moving mice).

DISCUSSION AND CONCLUSION

Our study represents, to the best of our knowledge, the first description of transcranial and non-invasive functional ultrasound imaging of cerebral vasculature and hemodynamics in both mice and young rats.

We first report that fully non-invasive ultrasensitive Doppler imaging of the vasculature of the entire brain can be performed directly through the intact skin and skull in mice and young rats that are anesthetized and stabilized in a stereotactic frame.

In mice, non-invasive 3-D whole-brain imaging was performed: the vasculature of the entire brain of the mouse can be imaged through the skull and skin in a fully non-invasive manner. A brief description of key vascular landmarks is proposed. In future work, a whole-brain vascular atlas of the mouse brain could be compiled based on this non-invasive 3-D imaging tomographic approach.

In the coronal planes represented in Figure 2b, a well-known effect called stripe artifacts (Vignon et al. 2010), caused by skull bone aberrations, can be observed. This aberration effect could be corrected in further studies by using adaptive focusing approaches, such as time reversal focusing in speckle noise or blood flow proposed in Montaldo et al. (2011) and Osmanski et al. (2012).

We also report that mice can be imaged up to at least 1 y of age, the last time point investigated, whereas young rats can be imaged up to 35 d of age with a gradual reduction in image quality afterward. After that age, either surgical skull thinning (Osmanski et al. 2014) or craniotomy (Sieu et al. 2015) will have to be employed, or the imaging sequence signal-to-noise ratio (SNR) can be improved by using either injected ultrasound contrast agents (Errico et al. 2016) or a novel high-SNR ultrafast sequence (Tiran et al. 2015). Although the skull is the main barrier to the propagation of ultrasonic waves, we also observed that the skin layer influence is not negligible in young rats, principally in those older than 30 d. The signal attenuation observed through the intact skin in the oldest rats may be explained by the gradual increase in the thickness of the skin, which is multiplied by threefold between the post-natal days 5 and 45 (0.5 mm for PND 5 vs. 1.6 mm for PND 45). Another possible explanation could be the presence of a gap, filled by conjunctive tissue, increasing this attenuation even more. Indeed, in rat

experiments, when the conjunctive tissue is not properly cleaned after thinning the skull, the signal is disturbed in a non-homogeneous way.

We also report the feasibility of minimally invasive functional ultrasound imaging in awake and freely moving mice through the intact skull using a novel clip-on fixed ultralight ultrasound probe. Doppler images through the intact mouse skull were obtained with adequate quality for visualization of entire coronal planes of the brain from cortical vessels down to deep vessels at the base of the brain, without surgery or contrast agent injection. Indeed, although the use of contrast agents is an excellent alternative in transcranial functional imaging, injection of contrast agents during experiments on freely moving animals would be rather complex. While contrast agent injection through a previously catheterized vein is possible, this approach requires surgical skills and, because of its invasiveness, might induce infections and/or pain and suffering. Consequently, the possibility of performing transcranial and non-invasive brain imaging in mice without contrast agent injection may bring a real benefit for behavioral and longitudinal studies.

For functional ultrasound imaging, we developed an optimized ultrafast sequence that minimizes unwanted ultrasonic signals both transmitted toward the parietal muscles and extracted in the receive beamforming mode from these parietal muscles. Such ultrasonic signals are indeed an important source of Doppler image clutter and artifacts because of both their strong signal amplitude and rapid movement. The loss of spatial resolution caused by the apodization must be interpreted with respect to the original sequence. In the original sequence, only roughly 80 of 128 elements of the probe contributed at the maximal imaging depth, because of their directivity pattern. In the optimized ultrafast sequence using the Hamming apodization process, 64 of 128 lateral elements are used at less than 50% maximal amplitude. This optimization effectively reduces the artifact at the expense of only a 20% loss in spatial resolution.

In the experiments in freely moving mice, a rigid connection between the skull and the probe was obtained by screwing and cementing a light metal frame to the skull. This lightly invasive approach leaves the cranial cavity undisturbed, reducing the risk of infection. Importantly, the brain is also preserved in a more physiologic state compared with craniotomy, our previous experimental approach used for awake rats (Sieu et al. 2015), which may provoke important biochemical, morphologic, vascular and behavioral changes in the rat brain (Cole et al. 2010; Forcelli et al. 2013; Lagraoui et al. 2012).

Compared with our previous studies performed in much more advantageous configurations for Doppler signal detection (*i.e.,* adult rats with craniotomy or thinned skulled window) (Macé et al. 2011; Osmanski et al. 2014; Sieu et al. 2015), the challenges in the present study were the smaller size of the brains investigated and the transcranial attenuation of ultrasound. As the ultrasonic frequency of the probe used in these experiments is the same as in our previous studies, the Doppler images have slightly lower quality. In future studies, further miniaturized probes dedicated to experiments in freely moving in mice or young rats could be envisaged with a reduced number of elements, as the required field of view in the adult mouse brain is about half of that required in the adult rat brain. However, the optimal frequency will represent a trade-off between resolution and signal attenuation. Indeed, higher frequencies would enable better resolution (which would enable better visualization of some very small vessels in the mouse brain), but also higher attenuation of the signal because of the presence of the skull. Regarding the question of future probe design, capacitive micro-machined ultrasonic transducer (cMUT) technology is a very attractive solution for small rodent imaging as it will enable smaller and lighter probes and 2-D matrices (Oralkan et al. 2003), which could help to overcome several current limitations.

Our findings pave the way for minimally invasive longitudinal studies in anesthetized or awake mice and young rats, two models of great pre-clinical interest because these animals are well-suited for genetic or developmental studies, respectively. We found that functional ultrasound imaging works similarly in awake and anesthetized mice (unpublished data). In young rats, here we only performed ultrasensitive Doppler imaging of the brain vasculature because fUS imaging at early ages requires further work to optimize anesthesia. Future work will also be dedicated to further improve the signal-to-noise ratio to extend the imaging to young adult rats. Further improvement of the freely moving setup toward even lighter and easier systems and development of 3-D functional ultrasound imaging strategies will hopefully enable us to measure functional brain connectivity in mice and young rats. Such functional connectivity imaging approaches in pre-clinical disease models may significantly advance the characterization of drug or disease effects in the brain. By fulfilling these promises, functional ultrasound imaging in both anesthetized and awake rodents could represent a disruptive change to significantly advance pre-clinical studies in neuroscience.

SUPPLEMENTARY DATA

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.ultrasmedbio.2017.03.011.

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