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Sana Hannan¹, Mayo Faulkner¹, Kirill Aristovich¹, James Avery² and David Holder¹¹ Department of Medical Physics and Biomedical Engineering, University College London, London, United Kingdom² Department of Surgery and Cancer, Imperial College London, London, United KingdomE-mail: sana.hannan.14@ucl.ac.uk**Keywords:** electrical impedance tomography, cerebral cortex, cortical stimulation, safety, rat brain**Abstract**

Objective: Electrical impedance tomography (EIT) can be used to image impedance changes which arise due to fast electrical activity during neuronal depolarisation and so holds therapeutic potential for improving the localisation of epileptic seizure foci in patients with treatment-resistant epilepsy to aid surgical resection of epileptogenic tissue. Prolonged cortical stimulation may, however, induce neural injury through excitotoxicity and electrochemical reactions at the tissue–electrode interface. The purpose of this work was to assess whether current levels used in fast neural EIT studies induce histologically detectable tissue damage when applied continuously to the rat cerebral cortex. **Approach:** A 57-electrode epicortical array was placed on one or both hemispheres of adult Sprague Dawley rats anaesthetised with isoflurane. In an initial series of experiments, current was injected simultaneously at 10, 25, 50, 75 and 100 μA for 1 h at 1.725 kHz through five electrodes across two epicortical arrays to provide a preliminary indication of the safety of these current levels. Since no obvious cortical damage was observed in these rats, the current level chosen for further investigation was 100 μA , the upper-bound of the range of interest. In a separate series of experiments, 100 μA was applied through a single electrode for 1 h at 1.725 kHz to verify its safety. Following termination of stimulation, brain samples were fixed in formalin and histologically processed with Haematoxylin and Eosin (H&E) and Nissl stains. **Main results:** Histological analysis revealed that continuous injection of 100 μA current, equating to a current density of 354 Am^{-2} , into the rat cortex at 1.725 kHz does not cause cortical tissue damage or any alterations to neuronal morphology. **Significance:** The safety of current injections during typical EIT protocols for imaging fast neural activity have been validated. The current density established to be safe for continuous application to the cortex, 354 Am^{-2} , exceeds the present safety limit of 250 Am^{-2} which has been complied with to date, and thus encourages the application of more intensified fast neural EIT protocols. These findings will aid protocol design for future clinical and *in vivo* EIT investigations aimed at imaging fast neural activity, particularly in situations where the signal-to-noise ratio is considerably reduced.

1. Introduction**1.1. Background**

Electrical impedance tomography (EIT) is a medical imaging technique that can be used to generate images of internal impedance changes within an object by injecting current and recording boundary voltage measurements from peripherally placed electrodes (Holder 2005). Recent developments in EIT have enabled imaging of the impedance changes which arise during fast electrical activity during neuronal depolarisation in somatosensory evoked potentials (SEPs) and epileptiform events at a high spatiotemporal resolution (Aristovich *et al* 2014, 2016, Hannan *et al* 2018). EIT, therefore, holds therapeutic potential for improving the localisation of epileptic seizure foci in patients with treatment-resistant epilepsy to aid surgical resection of epileptogenic tissue (Fabrizi *et al* 2006). In these individuals, intracranial electrode mats are often placed on the cortical surface as part of an extensive presurgical evaluation comprising video-EEG telemetry to localise the epileptogenic zone and

determine the likelihood of performing a successful surgery with minimal functional deficits (Duncan 2011). In conjunction with these conventional methods, fast neural EIT may be performed by injecting current through electrode pairs on these intracranial mats, sequentially or in parallel, and recording the resulting boundary voltages from all remaining electrodes (Dowrick *et al* 2015). To image fast impedance changes due to neuronal activity in the brain with EIT, it is desirable to inject as high a current as possible to maximise the signal-to-noise ratio (SNR). Of equal importance, however, is the need to avoid significant current-induced influences on neuronal excitability, particularly those that result in irreversible structural damage.

1.1.1. Mechanisms of stimulus-induced neuronal damage

The principal mechanisms by which electrical stimulation may cause tissue damage fall into one of three main categories. The first relates to the effects of injecting current across the tissue–electrode interface, including cytotoxicity through accumulation of products arising from electrical dissolution and electrochemical reactions there (Brummer and Turner 1977). The second category describes the physiological processes associated with passage of the stimulus current through the tissue, which may cause damage through excitotoxicity, induced by neuronal hyperactivity, and electroporation (Butterwick *et al* 2007, Grill 2008). McCreery *et al* demonstrated that it is this latter mechanism that is dominant in causing neuronal damage during electrical stimulation of the brain surface, by giving rise to phenomena such as neuronal hyperexcitability (McCreery *et al* 1988). As such, direct physical contact between the electrode and tissue is not necessarily required for the generation of neuronal damage. The final mechanism of stimulus-induced damage relates to the transfer of charge through tissue, which can induce heating due to thermal energy dissipation (Elwassif *et al* 2006, Liebetanz *et al* 2009).

1.1.2. Safety limits of low-frequency cortical stimulation

The safety limits for application of current to the cortex have been proposed mainly in relation to transcranial direct current stimulation (tDCS), a non-invasive brain stimulation therapy whereby low currents are delivered to brain regions of interest through scalp electrodes for the purpose of modifying neuronal activity in an array of neurological disorders (Fregni *et al* 2006a, 2006b, Brunelin *et al* 2012). The current levels used in typical tDCS protocols are based on the following animal studies which have characterised stimulation parameters at which overt brain damage starts to occur. McCreery *et al* investigated the effects of prolonged electrical stimulation of the surface of the cat parietal cortex for 7 h with charge-balanced pulse pairs delivered at 50 Hz (McCreery *et al* 1990). They concluded that the threshold of stimulus-induced neural injury, characterised by morphological alterations to neurons subjacent to the stimulating electrode, equated to a current density of 250 Am^{-2} . In another study, rats were subjected to sessions of continuous tDCS through a single epicranial electrode fixed above the frontal cortex for up to 9 h; here, the current density threshold for histologically detectable brain lesions was 142.9 Am^{-2} for stimulation durations greater than 10 min (Liebetanz *et al* 2009). The conflicting results from these animal studies can be attributed to the wide variability in the experimental parameters employed—namely, species, electrode geometry and material, anatomical region of electrode implantation, and stimulation protocol used—and highlight the difficulties associated with extrapolating safety data for application beyond constrained experimental setups. Thus, the safety limits of specific stimulation protocols must generally be determined on a case-by-case basis.

1.1.3. Current amplitudes used in EIT of fast neural activity

The amplitude of applied current used in a typical EIT protocol for imaging fast impedance changes during neuronal depolarisation is an important consideration. Electric fields induced by external stimulation or endogenous extracellular currents, such as those associated with hippocampal theta rhythms and sharp waves, are known to modulate neural network activity (Anastassiou *et al* 2010, Fröhlich and McCormick 2010). With increasing frequency, higher current levels are tolerated due to the more rapid fluctuation of electric charge across cell membranes, leading to less accumulation of charge and thus less physiological effects of the injected current (Holder 2005). Although the effects of these non-synaptic ephaptic interactions on the membrane potential of neurons is considerably greater at low frequencies, the possibility of such alterations arising as a result of the cortical current injections employed in fast neural EIT experiments using higher frequencies, usually in the 1–2 kHz range, cannot be dismissed (Fröhlich and McCormick 2010).

The neuromodulatory effects of injected current have previously been addressed in the context of imaging SEPs in the rat cerebral cortex with EIT using current amplitudes ranging from 2–100 μA at a constant carrier frequency of 225 Hz (Oh *et al* 2011). Here, it was demonstrated that (a) the SNR increased with increasing amplitude of applied current, and (b) the size of the impedance changes associated with SEPs remained unaffected by current injected at amplitudes up to 100 μA (Oh *et al* 2011). A similar analysis showed that current amplitudes up to 60 μA at a higher frequency of 1025 Hz did not significantly alter the amplitude or latency of SEPs or the magnitude of the impedance change recorded with a planar epicortical electrode array, placed on the rat brain (Vongerichten 2014). Previous in-house studies involving the use of higher-frequency EIT to image fast neu-

ral activity from the rat cortex, in the form of SEPs and epileptiform activity, have generally utilised constant sinusoidal current injections ranging from 50–100 μA in amplitude (Vongerichten 2014, Aristovich *et al* 2016, Vongerichten *et al* 2016). The aim of the present study is, therefore, to provide an indication of the safety of these current levels on neuronal tissue, when injected at a constant carrier frequency of 1.725 kHz, which has previously been shown to yield a high SNR and allows for an improved temporal resolution of 2 ms for imaging fast neural activity (Aristovich *et al* 2016, Vongerichten *et al* 2016, Hannan *et al* 2018).

1.1.4. Assessment of current-induced neuronal damage

The extent of neural injury induced by electrical stimulation is often assessed through histological evaluation of tissue after the stimulation period. Yuen *et al* developed a method for quantifying stimulus-induced neural damage under light microscopy using two standard histological stains, Haematoxylin and Eosin (H&E) and Nissl, which respectively enable visualisation of cortical tissue structure and neuronal morphology (Yuen *et al* 1981). Whereas the somas of healthy cortical neurons are marked by a round appearance, electrical stimulation above safe thresholds can cause neurons to shrink and become hyperchromic to the histological stain (Yuen *et al* 1981, McCreery *et al* 1990). This damage can thus be characterised by assessing neuronal morphology with respect to the degree of neuronal shrinkage and hyperchromism. Using H&E, Liebetanz *et al* also demonstrated the occurrence of such stimulus-induced histopathological alterations, in addition to a loosened and pale cortical tissue texture transcending multiple cortical layers (Liebetanz *et al* 2009).

1.2. Purpose

The purpose of this study was to investigate whether continuous electrical stimulation of the cerebral cortex at current amplitudes used for fast neural EIT in the rat brain induces histologically detectable structural damage. To address this, the safety of current amplitudes in the 10–100 μA range, which encompasses the current levels utilised in typical EIT protocols for imaging neural activity *in vivo*, was evaluated.

1.3. Experimental design

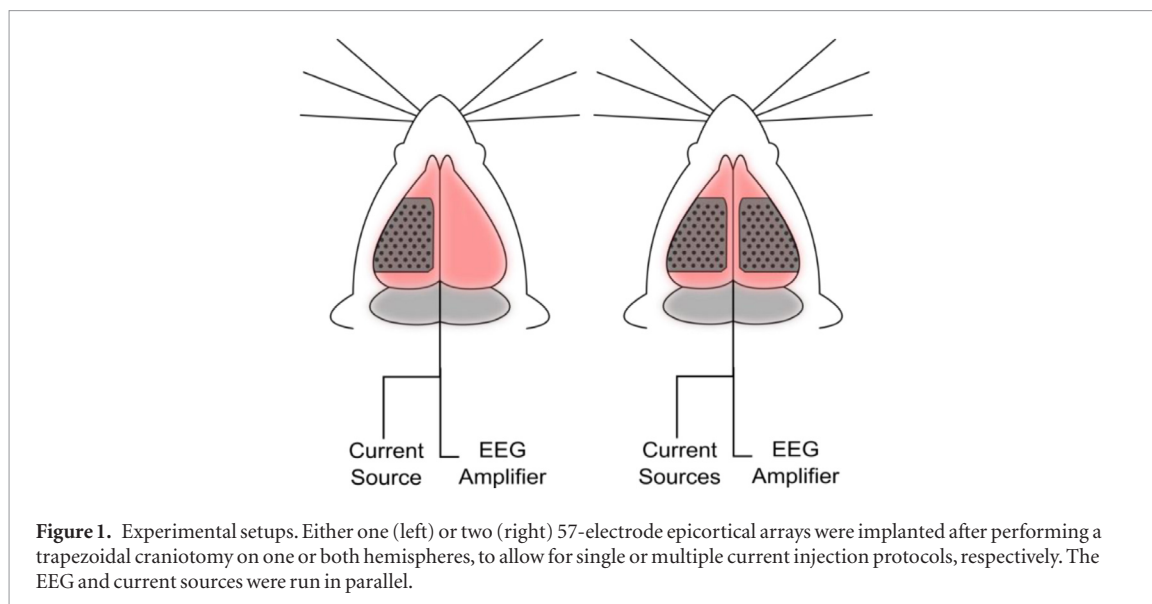
The extent of current-induced neuronal damage will depend on the amplitude, carrier frequency and duration of the current injection. In the present study, the latter two conditions were kept constant: the carrier frequency at 1.725 kHz, typical of *in vivo* fast neural EIT protocols (Aristovich *et al* 2016, Vongerichten *et al* 2016, Hannan *et al* 2018), and the stimulation duration at 1 h. This stimulation time was chosen as it is sufficient to allow for any histopathological alterations associated with current-induced neuronal injury to manifest, provided that the intensity of stimulation surpasses the safety threshold in question (Liebetanz *et al* 2009). Additionally, a stimulation duration of one hour was a reasonable compromise between the total time a single electrode on the array would be stimulated in serial and parallel EIT protocols.

A typical *in vivo* experimental setup for fast neural EIT comprises placement of one or two 57-electrode epicortical arrays on the exposed cortical surface of an anaesthetised rat (Faulkner *et al* 2018, Hannan *et al* 2018). Thus, a total of 114 electrode contacts are available for current application. In order to maximise the information obtained from every animal and thus minimise overall animal usage in accordance with the 3Rs philosophy (reduction, refinement and replacement) as outlined in the Animals (Scientific Procedures) Act 1986, the possibility of testing more than one current level in the same animal was initially investigated by simulating the propagation of current at five amplitudes of interest in the 10–100 μA range through the rat brain. Since this modelling revealed that it is possible to evaluate the effects of current application at 10, 25, 50, 75 and 100 μA independently within a single rat, initial experiments comprised current injection at these five current amplitudes in parallel. The purpose of these experiments was to efficiently provide a preliminary indication of the safety at each current level using fewer animals, thus directing the focus of more thorough investigation towards a single current level at which cortical damage may begin to occur.

2. Materials and methods

2.1. Animal preparation

All animal handling and experimental investigations undertaken in this study were ethically approved by the UK Home Office and performed in accordance with its regulations, as outlined in the Animals (Scientific Procedures) Act 1986. Twelve adult female Sprague Dawley rats (320–410 g) were used. Anaesthesia was induced with 4% isoflurane in 2 l min^{-1} O_2 and an endotracheal intubation was performed to enable mechanical control of ventilation with 1.5%–3% isoflurane in a 30/70 mixture of oxygen/air using an SAV03 small animal ventilator (Vetronic Services Ltd, Abbotskerswell, UK). Exhaled gases, respiratory rate, tidal volume, heart rate and SpO_2 were monitored regularly using an anaesthetic monitor (Lightning; Vetronic Services Ltd, Abbotskerswell, UK). Core body temperature was maintained at $36.5\text{ }^\circ\text{C} \pm 0.5\text{ }^\circ\text{C}$ using a homeothermic heating unit comprising a blanket wrapped around the rat and a rectal thermistor probe (Harvard Apparatus, Edenbridge, UK). Rats were



then fixed in a stereotaxic frame (Narishige International Ltd., London, UK), the skin of the head shaved and the scalp incised. The insertion of the temporal muscle on each side was cauterised using a bipolar coagulation unit (Codman Malis CMC-II; Codman, Raynham, MA) and incised with a scalpel. The cerebral cortex was exposed through a craniotomy in one or both hemispheres, depending on cortical stimulation protocol, using a veterinary bone drill (Ideal Micro-Drill; Harvard Apparatus, Edenbridge, UK). The paramedial edge of the craniotomy extended from 1 mm anterior to lambda to 5 mm posterior to bregma, with the lateral boundary at the junction of the zygomatic arch to the temporal bone, forming a trapezoidal opening. When two craniotomies were performed, a thin strip of bone remained above the midline to protect the superior sagittal sinus. The dura was incised with micro scissors and the brain was kept moist by frequent irrigation of the area with 0.9% sterile saline at 37 °C. A planar custom-designed 57-contact epicortical array, fabricated from stainless steel foil and silicone rubber, was implanted on the exposed cortical surface of each hemisphere. The two experimental setups used for the different cortical stimulation protocols are illustrated (figure 1). The 57-electrode array was trapezoidal in shape and measured 15 × 9 mm at its furthest edges, thus providing coverage of ~90% of the cortical surface of one cerebral hemisphere and has previously been used for fast neural EIT of epileptiform discharges in the rat brain (Hannan *et al* 2018). The 57 electrodes were each 0.6 mm in diameter and were platinised to produce a contact impedance of $\leq 5 \text{ k}\Omega$ across the electrode–electrolyte interface. To minimise the occurrence of inadvertent mechanical damage to the surface of the cerebral cortex, electrodes were never repositioned after their initial placement. In all cortical stimulation protocols, current was injected continuously between the chosen source electrode(s) within the array and a sink electrode in contact with the hard palate in the roof of the mouth. All voltage recordings were made with respect to a reference electrode placed beneath the nuchal skin. Both the sink and reference electrodes comprised a silver–silver chloride plate, 9 mm in diameter.

2.2. Validating a healthy state of the cerebral cortex

To confirm that the rat cortex was in a healthy condition prior to commencing current injection protocols, the following structural and functional tests were conducted. First, the physical appearance of the cortex was examined under light microscopy before electrode implantation to verify that the dura was intact and had not been inadvertently penetrated during the craniotomy. In the rare case that the dura was perforated, the underlying cortical tissue and the blood vessels supplying it were observed to ensure the absence of visible damage. To validate normal cortical functioning, SEPs were induced by electrically stimulating peripheral nerves (median, ulnar and radial) in the contralateral forepaw with 1 mA pulses at 2 Hz (pulse width: 500 μs), using two subdermal silver needle electrodes inserted into the third digit and connected to a NeuroLog current stimulus isolator (NL800A; Digitimer Ltd, Welwyn Garden City, UK). The resulting SEPs were recorded from the somatosensory cortex with the subdural electrode array, averaged for 60 s and subsequently assessed with respect to their amplitude and latency after stimulus application. Voltage changes due to SEPs and normal cortical electrographic rhythms were digitised at a sampling frequency of 25 kHz using a BrainVision actiCHamp 128-channel EEG amplifier and data acquisition was controlled using the BrainVision Recorder program (Brain Vision LLC, Cary, NC). Only rats which did not display any visible cortical damage and in which expected EEG rhythms and SEPs could be successfully recorded were used.

In the 12 rats that were used, the maximum averaged SEP recorded from each hemisphere of interest had an amplitude of $564 \pm 118 \mu\text{V}$ and latency of $5.63 \pm 0.28 \text{ ms}$ after delivery of the forepaw stimulus (mean \pm SD; $n = 15$ hemispheres, 12 rats). All averaged SEPs were typical of that expected during isoflurane anaesthesia (Hayton *et al* 1999, Masamoto *et al* 2007), with respect to their amplitude and latency, and thus confirmed normal functioning of the somatosensory cortex prior to initiating cortical stimulation.

2.3. Evaluating the propagation of current through the rat brain

A modelling study was undertaken to determine the current propagation paths of the five current levels of interest in the 10–100 μA range. As such, the possibility of testing the effects of multiple current levels in the same rat brain could be evaluated to provide an indication of which current levels should be further investigated.

Assuming that a current injecting electrode on the cortical surface behaves as a monopole, the current density is expected to emanate from the electrode centre in a hemispherical configuration, its magnitude decreasing inversely with distance. At a certain distance from the centre, the current density will decrease to a level which is considered to have a negligible effect on neurons. To determine this distance, and ultimately indicate the number of independent current injections possible through two 57-electrode epicortical arrays, an anatomically realistic finite element method (FEM) model of the rat brain generated from MRI images, comprising 2.9 million tetrahedral elements, was used to simulate parallel current injections through source electrodes in the epicortical arrays and a sink electrode, 9 mm in diameter, below the ventral surface of the brain. This sink electrode modelled that which is placed against the hard palate of the mouth in the experimental setup. Simulations assumed background conductivity to be isotropic throughout the cerebral grey matter (0.3 Sm^{-1}) and white matter (0.15 Sm^{-1}) (Ranck 1963, Baumann *et al* 1997, Latikka *et al* 2001), and that current density values below a chosen threshold of 2.5 Am^{-2} had negligible effects on neuronal excitation (Rattay 1998, 1999, Reilly 1998). The radii of hemispherical propagation paths of current density above this negligible threshold were calculated for each current level of interest: 10, 25, 50, 75 and 100 μA . In increasing order, the radii were 0.85, 1.33, 2.03, 2.57 and 3.34 mm (figure 2). These values were used to ensure sufficient separation between injecting electrodes on the 57-electrode arrays to avoid overlap of current propagation paths (figure 3). Provided that such a spatial arrangement of current injecting electrodes within the epicortical arrays was chosen, these simulations demonstrated that it was possible to inject current at these amplitudes in parallel through up to five electrode contacts across the rat brain. Therefore, any current-induced damage subjacent to and around a particular stimulating electrode could be presumed to have resulted exclusively from the current injected through it and independent conclusions could be made regarding the effects of each of these current intensities on cortical tissue in the same animal.

2.4. Cortical stimulation protocols

After validation of normal cortical functioning, rats were divided into three experimental groups based on the cortical stimulation protocol received (table 1 and figure 3). For all stimulation protocols, the Keithley 6221 current source (Keithley Instruments Ltd, Bracknell, UK) was utilised for current injection, which can produce sinusoidal current at 2 pA–100 mA (with an accuracy of $\pm 0.02 \mu\text{A}$ in the current range being tested), within a frequency range of 1 mHz–100 kHz, and has a large output impedance ($10^{14} \Omega$) to ensure stable current delivery with varying loads.

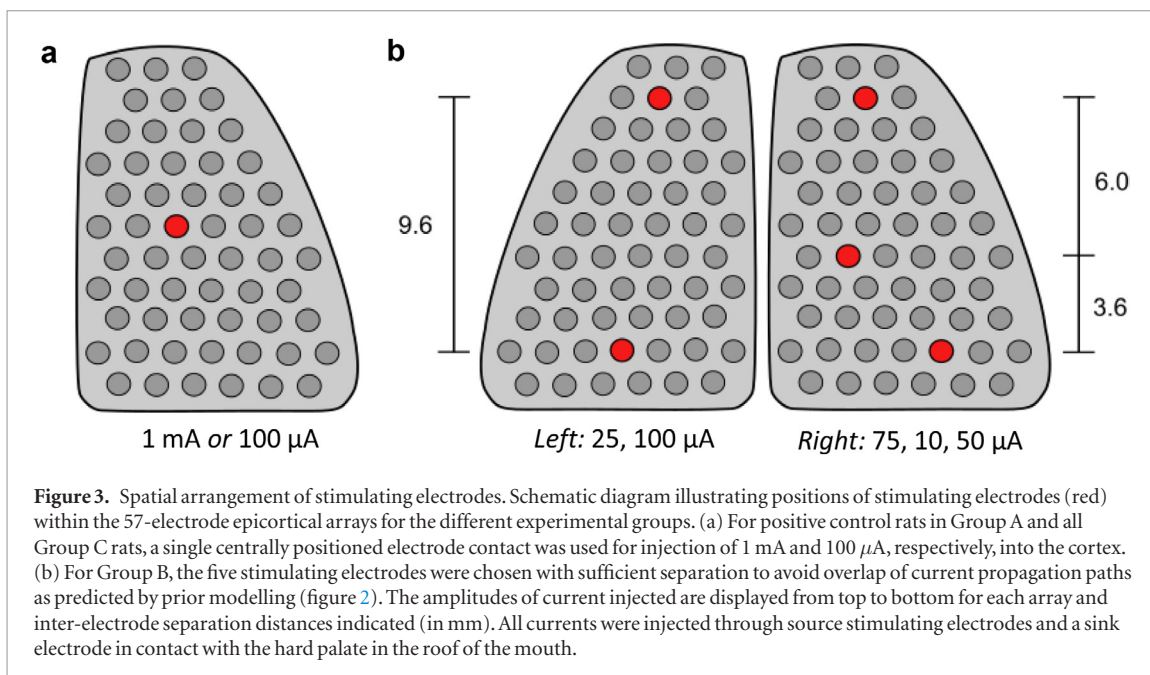
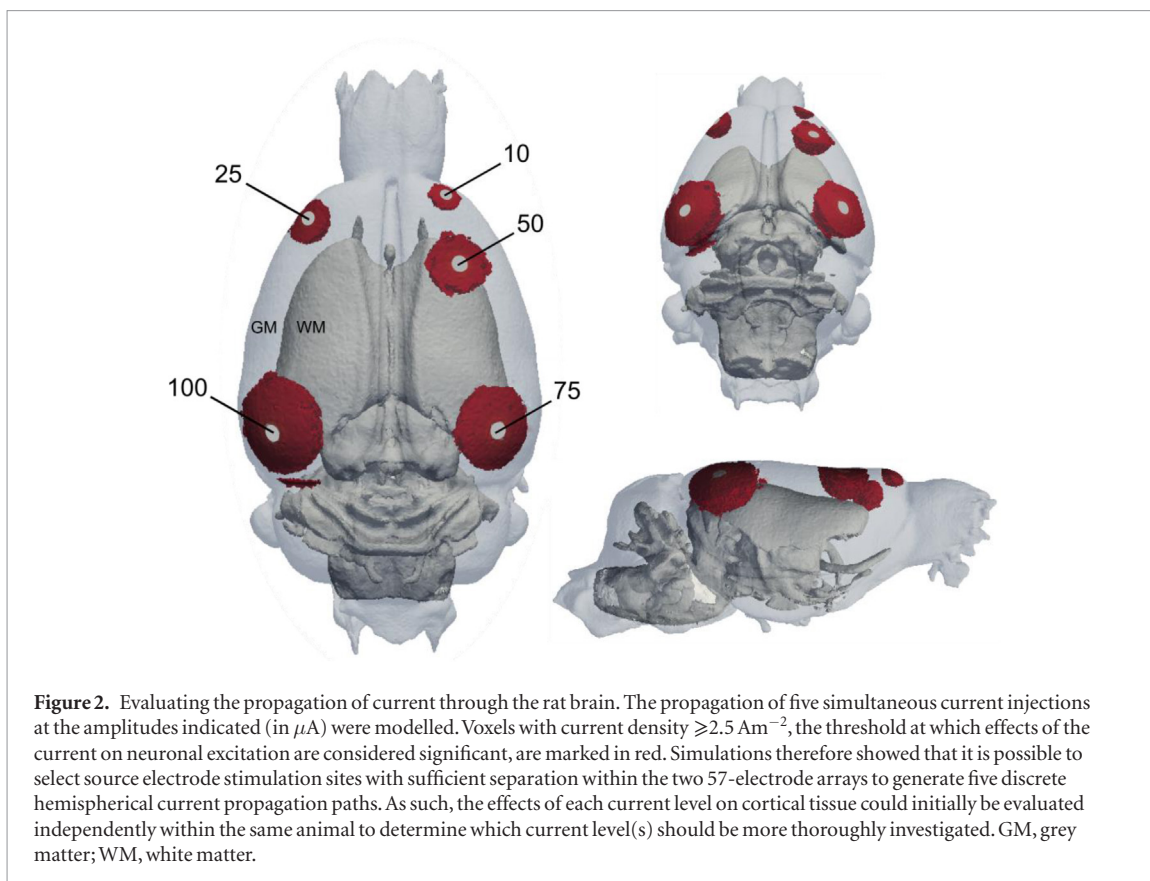
2.4.1. Experimental controls

Positive control experiments were undertaken in three Group A rats to confirm that the histological protocol employed was adequate to enable detection of current-induced damage. For this purpose, current injection at 1 mA through a single electrode within the epicortical array was undertaken for 1 h at 1.725 kHz, corresponding to a current density of 3540 Am^{-2} ; these parameters far exceed the current safety limits of cortical stimulation and so are expected to cause considerable neuronal damage (McCreery *et al* 1990, Liebetanz *et al* 2009).

A single Group A rat was subjected to a sham stimulation procedure by performing a craniotomy on one hemisphere and placing an epicortical array on the cortical surface for 1 h without stimulating the electrode contacts, to assess the degree of inadvertent mechanical damage that occurred during the craniotomy, electrode implantation and removal of the brain from the skull.

2.4.2. Current injection at 10–100 μA

Group B rats received five current injections in parallel—10, 25, 50, 75 and 100 μA —across two epicortical electrode arrays, to provide a rough approximation of the safety of these current levels and demonstrate which amplitude in this range, if any, might start to cause histologically detectable neuronal damage (table 1). The spatial arrangement of the five current injecting electrodes was decided, based on the radii of hemispherical current propagation paths predicted by simulations (figure 2), to ensure that current propagation paths did not overlap (figure 3). Two Keithley 6221 current sources were used to inject current with the lowest amplitudes, 10 and 25 μA . Currents for the remaining levels were injected using three individual voltage generators (TG230; Thurlby



Thandar Instruments, Huntingdon, UK) connected to fixed 100 k Ω resistances in series to the electrodes; the applied voltage was manually adjusted to deliver the desired current value. Results from these multiple current injections determined the stimulation protocol for the final series of experiments in Group C.

Since there were no signs of stimulus-induced cortical damage at any of the current levels tested in Group B and C rats were stimulated at 100 μA , the upper bound of the range of interest, through a single electrode to verify its safety definitively. All current injections for Groups B and C were also delivered continuously for 1 h at 1.725 kHz.

2.5. Histological processing

Immediately after the end of the stimulation period, animals were culled by administering a lethal dose of pentobarbital (150 mg kg $^{-1}$, intraperitoneal). The epicortical electrode arrays were gently removed and the

Table 1. Summary of cortical stimulation protocols. Each current was delivered at a carrier frequency of 1.725 kHz for 1 h through a single electrode site within one (Group A, C) or two (Group B) implanted epicortical arrays in each animal.

Group	Animals (<i>n</i>)	Purpose	Current (μA)	Current density (Am^{-2})
A	4	Positive controls (3)	1000	3540
		Negative control (1)	0	0
B	3	Multiple current injections in parallel	10	35.4
			25	88.4
			50	177
			75	265
			100	354
C	5	Single current injection	100	354

sites of stimulating electrodes marked with a fine paintbrush and a black water-insoluble tissue-marking dye (Cellpath Ltd, Newtown, UK). The brain was then quickly and gently removed from the skull using bone rongeurs and immersed in 10% neutral buffered formalin for tissue fixation at room temperature for 2–4 d. Following tissue processing through a series of solvents (70% IMS (industrial methylated spirit), 90% IMS, absolute IMS and xylene) and subsequent embedding in paraffin wax, brain samples were cut at the electrode positions, into sections 5 μm in thickness using a vibrating microtome. All sections were cut in the coronal plane, with the exception of one brain sample from Group A, which was cut sagittally in order to confirm that there were no planar differences in the appearance of stimulus-induced cortical damage. For Group B rats, the two hemispheres were separated by cutting the brain in the midsagittal plane prior to coronal sectioning. Sections were obtained at 100 μm intervals at stimulating and non-stimulating electrode sites; since the diameter of a single electrode contact is 600 μm , this ensured that the cortical tissue subjected to stimulation was captured several times to allow for more reliable conclusions to be drawn from the histological analysis. In the negative control rat belonging to Group A, coronal sections were obtained at the central row of electrodes within the array. At each required interval, two serial sections were cut and stained respectively with H&E or Nissl stains. Histological evaluation was then performed with brightfield light microscopy to detect pathological changes such as tissue ischaemia, oedema and necrosis, and alterations in cellular morphology, including neuronal shrinkage and hyperchromism, which have been previously described in the context of electrical neurotrauma (Yuen *et al* 1981, McCreery *et al* 1990). Any sections which displayed evidence of considerable mechanical damage, particularly at or around the cortical region of interest, were dismissed from further histological evaluation.

2.6. Statistical analysis

Histological sections obtained at stimulating and non-stimulating electrode sites in Group C rats were blindly evaluated to determine whether current-induced tissue damage, in the form of a cortical lesion and alterations to neuronal morphology, occurred in response to continuous injection of 100 μA current for 1 h at 1.725 kHz. To test the null hypothesis that there are no statistically significant differences between cortical tissue subjacent to stimulating and non-stimulating electrode sites, Fisher's exact test was conducted using a significance level of $\alpha = 0.05$. This statistical test is commonly used for the assessment of statistically significant differences between binary variables (in this case, the presence or absence of structure damage to cortical tissue) determined by semiquantitative histopathological scoring (Gibson-Corley *et al* 2013).

3. Results

3.1. Verifying the effectiveness of methods for histological evaluation

In Group A rats which received 1 mA cortical stimulation for 1 h, there were distinct histopathological changes which took the form of a hemispherical focus of ischaemic damage, 0.8–1.0 mm in diameter, emanating from the known stimulating electrode position, and a morphological transformation of cortical neurons to a shrunken, irregularly-shaped and hyperchromic state (figure 4, $n = 3$ rats). These positive control experiments, therefore, confirmed that the histological protocol used was adequate to enable evaluation of current-induced damage.

3.2. Assessing the extent of mechanical damage caused by surgical procedures

A single rat underwent a sham current stimulation by placing an epicortical array on one hemisphere for 1 h without stimulating any of the electrode contacts. In the 18 histological windows of cortical tissue to be evaluated (obtained from three coronal sections across the central row of six non-stimulating electrodes within the array), mechanical damage was seen in only two windows from the same electrode site (figure 5(a)). This was evidenced by a widespread blood clot and lesions extending across the molecular layer of the cortex (Layer I).

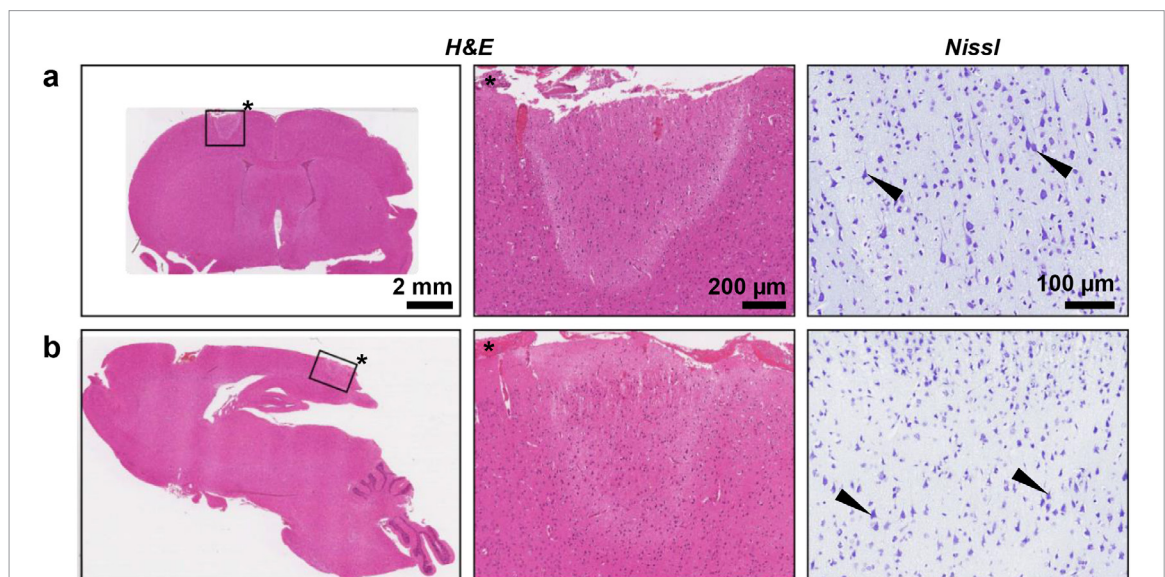


Figure 4. Histological evaluation of brain sections obtained from positive controls. Microscopic nature of cortical lesions induced by 1 mA stimulation consist of a focal ischaemic lesion transcending all layers of the cortex, marked by a loosened and pale tissue texture (H&E stain), and neuronal damage and necrosis (Nissl stain); the latter manifests as histopathological alterations of neurons to a shrunken and hyperchromic state (arrowheads). These changes were the same in sections cut in the coronal (a) and sagittal (b) planes, which agrees with the hemispherical propagation of current predicted by simulations (figure 2).

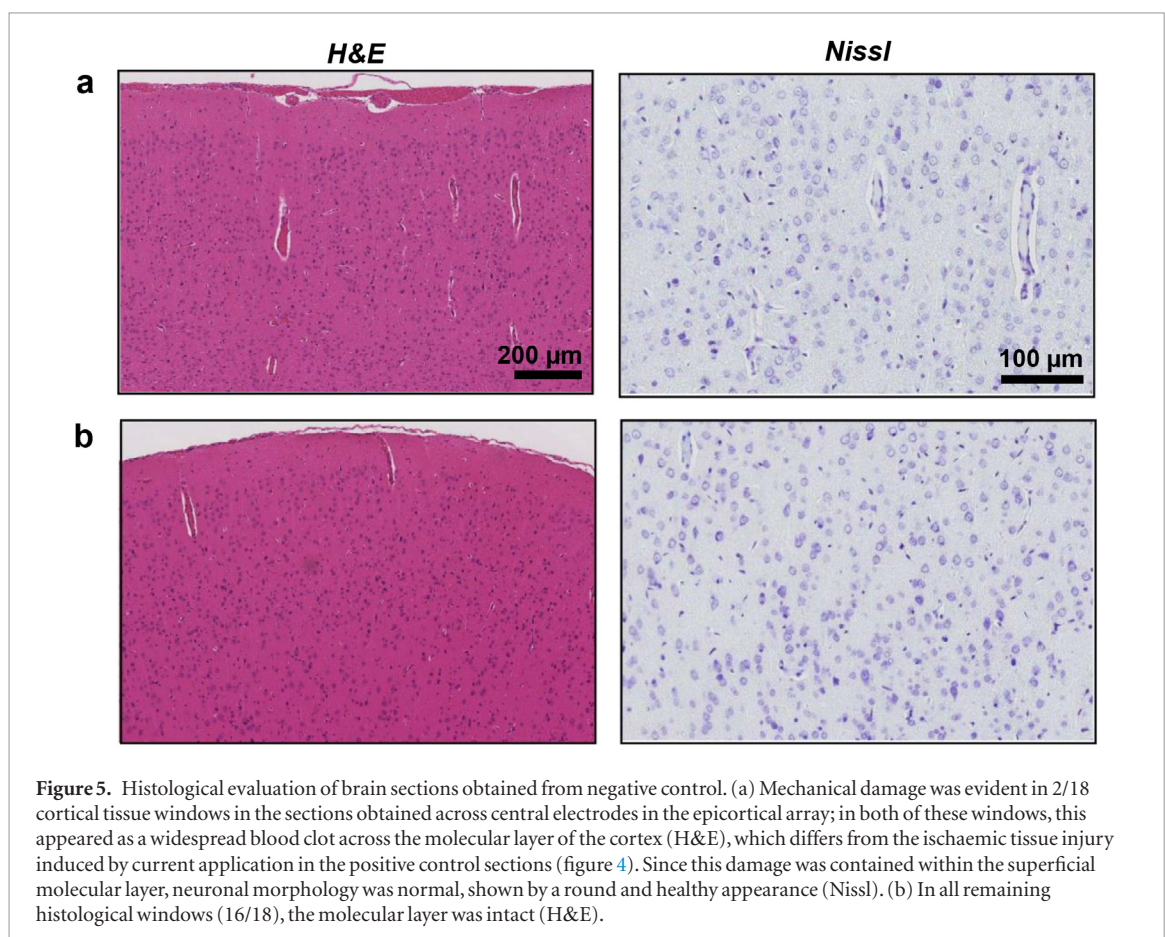
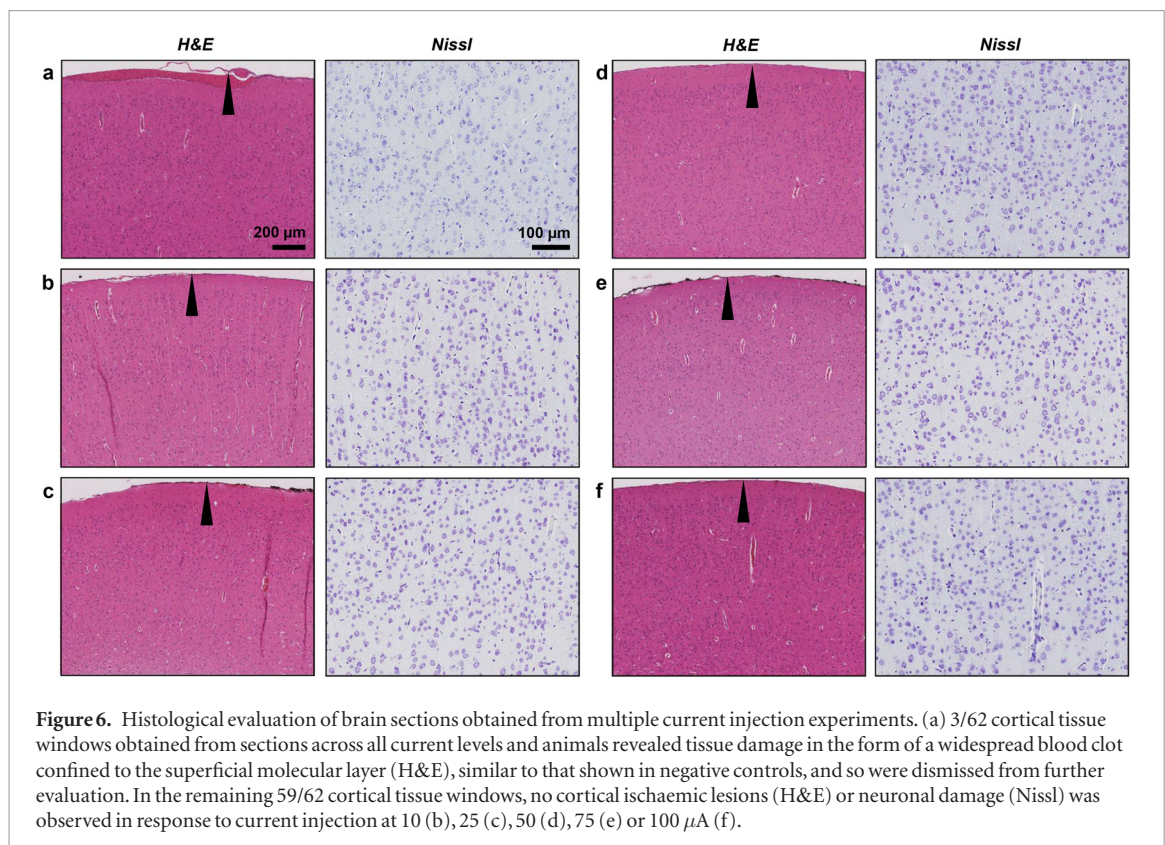


Figure 5. Histological evaluation of brain sections obtained from negative control. (a) Mechanical damage was evident in 2/18 cortical tissue windows in the sections obtained across central electrodes in the epicortical array; in both of these windows, this appeared as a widespread blood clot across the molecular layer of the cortex (H&E), which differs from the ischaemic tissue injury induced by current application in the positive control sections (figure 4). Since this damage was contained within the superficial molecular layer, neuronal morphology was normal, shown by a round and healthy appearance (Nissl). (b) In all remaining histological windows (16/18), the molecular layer was intact (H&E).

The remaining 16/18 windows showed a healthy appearance of cortical tissue (figure 5(b)). As such, it was clear that the possibility of mechanical damage occurring in cortical tissue subjacent to electrode sites, albeit small, could not be dismissed but that this mechanical damage differed in appearance to that induced by electrical stimulation (figure 4).



3.3. Evaluating the effects of continuous cortical stimulation at 1.725 kHz

3.3.1. Multiple current injections in parallel indicate the safety of current levels in the 10–100 μA range

In each Group B rat, 3–5 coronal sections were obtained at each of five electrode sites stimulated with 10, 25, 50, 75 and 100 μA in parallel. Tissue damage was seen in only 3 of the total 62 histological windows of cortical tissue across the stimulating electrode sites in all rats. This took the form of a widespread blood clot in the superficial molecular layer (figure 6(a)), comparable to that which was seen in the negative control and attributed to mechanical damage (figure 5(a)). In the remaining 59/62 tissue windows, there was no evidence of cortical tissue damage or alterations to neuronal morphology at any of the five current levels tested in the 10–100 μA range (figures 6(b)–(f), $n = 3$ rats).

3.3.2. Validating the safety of cortical stimulation at 100 μA

Since histological analysis of Group B sections suggested that all currents tested in the 10–100 μA range did not induce any obvious neuronal injury, the safety of cortical stimulation at 100 μA , the upper bound of currents used in a typical fast neural EIT protocol, was conclusively validated in Group C rats. Histological analysis was conducted blindly between stimulating and non-stimulating electrodes within the epicortical array and revealed that continuous injection of 100 μA current, equating to a current density of 354 Am^{-2} , into the cortex for 1 h at 1.725 kHz did not cause cortical damage (figure 7; $p > 0.05$, $n = 5$ rats, Fisher's exact test). This was evidenced by the absence of an ischaemic lesion and no observable alterations to neuronal morphology in the cortical tissue subjacent to the stimulating electrode site in all animals (figure 7(a)).

4. Discussion

4.1. Summary of results

The safety of continuous electrical stimulation of the rat cerebral cortex with current amplitudes in the 10–100 μA range at 1.725 kHz, parameters which are representative of typical *in vivo* EIT protocols for imaging fast neural activity in the brain, were investigated. The results of this safety study demonstrate that cortical stimulation with current levels up to and including 100 μA , corresponding to a current density of 354 Am^{-2} , do not induce neuronal injury and are therefore safe for continuous application during fast neural EIT.

4.2. Technical considerations

The passive tissue response to the chronic residence of subdural electrode arrays within the brain, due to ongoing mechanical injury to cortical tissue as a result of electrode displacement during free movement, was not investigated as rats remained anaesthetised throughout experiments. Moreover, culling the animal immediately

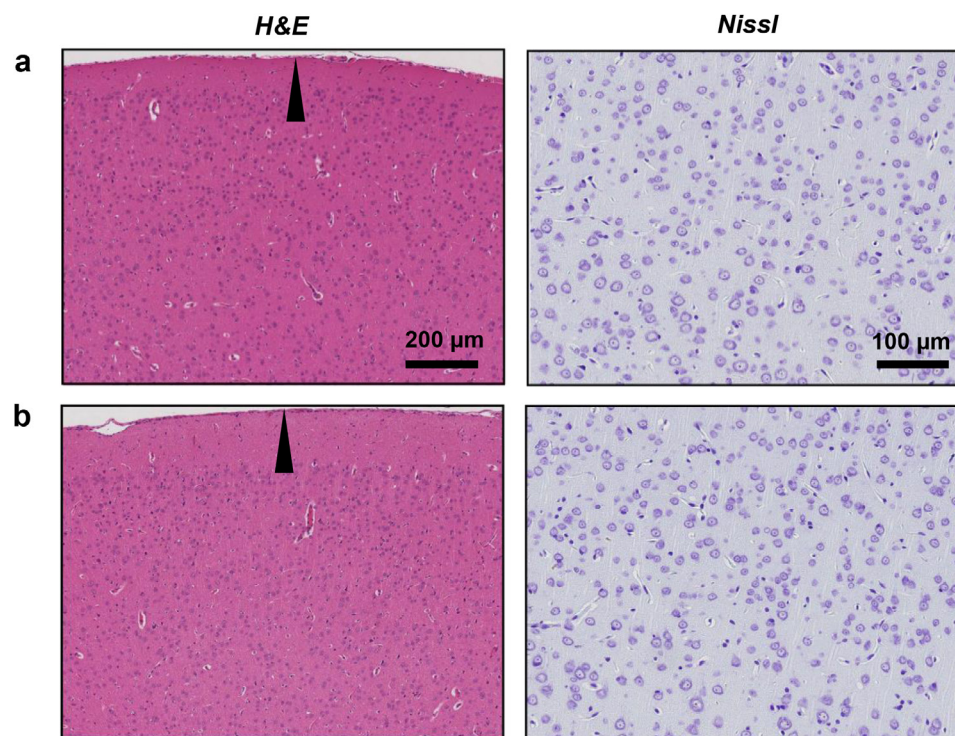


Figure 7. Stimulation of the cortex with $100\ \mu\text{A}$ at $1.725\ \text{kHz}$ for 1 h does not induce cortical tissue damage. Histological evaluation revealed no current-induced ischaemic lesions (H&E stain) or neuronal injury (Nissl stain) subjacent to electrode sites stimulated with $100\ \mu\text{A}$. (a) The appearance of cortical tissue in sections obtained at stimulating electrode sites (a) did not differ significantly from those obtained at control non-stimulating electrode sites (b) ($p > 0.05$, $n = 5$ rats, Fisher's exact test). The centres of electrode contacts, $600\ \mu\text{m}$ in diameter, are indicated (arrowheads).

after the end of stimulation meant that any long-term immunological responses of cortical tissue to the electrode material could not be examined. However, since our aim was to determine the safety of injecting alternating current (AC) itself into the cerebral cortex, addressing these issues would have complicated the evaluation of cortical damage induced exclusively by electrical stimulation and was therefore beyond the scope of this work. Additionally, since the present study specifically investigated whether continuous cortical stimulation in an acute experimental setup causes histologically detectable structural damage, the neurophysiological and behavioural effects of such stimulating paradigms were not assessed. It should also be noted that the histopathological outcomes determined by morphological investigations in this study, namely observation of H&E- and Nissl-stained brain sections under light microscopy, may not have had sufficient resolution to identify more subtle current-induced alterations at the neuronal level. An example of such phenomena is localised stimulation-induced depression of neuronal excitability (SIDNE) which may occur even in the absence of histologically detectable tissue damage and can be evaluated using electrophysiological recordings (McCreery *et al* 1997).

4.3. Comparison of findings to safety data in literature

The current density value established to be safe for continuous injection into the cortex, $354\ \text{Am}^{-2}$, exceeds safety thresholds in the existing literature, namely: (a) $250\ \text{Am}^{-2}$, which was proposed in a study investigating the safety of stimulating the cat parietal cortex with charge-balanced 50 Hz DC pulse pairs for seven continuous hours (McCreery *et al* 1990); and (b) $143\ \text{Am}^{-2}$, concluded from a safety study testing the limits of different cathodal tDCS regimes through an epicranial electrode above the rat frontal cortex (Liebetanz *et al* 2009). The differences in the stimulation parameters employed, as well as the species utilised, between these studies and the present work may explain this apparent discrepancy. For AC at $1.725\ \text{kHz}$, the flux of electric charge rapidly fluctuates with each cycle, leading to charging and discharging of the capacitive cortical tissue membrane; in contrast, the unidirectional charge flow which defines DC cannot cross cell membranes and will therefore cause more rapid accumulation of charge at the cortical surface (Holder 2005). As a result, there is an increased generation of cytotoxic electrochemical reaction products at the tissue–electrode interface, as well as increased excitotoxicity due to neuronal hyperactivation, at lower current density levels for DC currents compared to their higher-frequency AC counterparts (Holder 2005, Grill 2008). This highlights the fact that safety data are often limited to applications that conform to the specific experimental paradigms from which they were derived.

4.4. Implications for EIT of fast neural activity

The results of this study relate to the effects of continuous electrical stimulation of the surface of the rat cerebral cortex for one hour. Standard time-difference serial EIT protocols entail frequent switching of the current-injecting electrode pairs to enable current to be sequentially directed from multiple angles, thus providing uniform sensitivity to impedance changes in the brain region of interest. In such protocols, the cortical tissue subjacent to any given electrode site would typically be stimulated for only up to 30 s at a time and would thus receive current for a fraction of the total duration of the EIT protocol. The intermittent patterns of stimulation used in these serial protocols can be expected to have an even lower likelihood of inducing neuronal injury compared to continuous stimulation for prolonged time periods; the present work, therefore, encourages the application of more intensified current injection protocols for time-difference EIT imaging of fast neural activity both *in vivo* and clinically. Since previous EIT studies have demonstrated that it is possible to image fast neural impedance changes throughout the rat cerebral cortex at a high spatiotemporal resolution using current injection at 50 μA (Aristovich *et al* 2016, Hannan *et al* 2018), the increase in SNR obtained by using 100 μA is expected to improve the depth sensitivity of EIT and thus may also enable imaging of fast neural activity in deeper subcortical brain regions from the surface of the cerebral cortex. Potential applications of this improved depth sensitivity would be to image epileptic activity originating in the hippocampus, for example, both in animal models, to aid understanding of the mechanisms of seizures, and in patients with treatment refractory epilepsy, to aid presurgical localisation of epileptogenic foci. Furthermore, a custom-designed parallel multi-frequency EIT system, in which current is simultaneously injected at different frequencies, is currently under development to enable continuous recording of boundary voltages in individuals with treatment-refractory epilepsy without introducing low-frequency artefacts in the EEG due to switching of the current-injecting electrode pair (Fabrizi *et al* 2006, Dowrick *et al* 2015). Since use of this system will entail prolonged AC injection at multiple electrode sites in parallel, the present study has provided a valuable initial indication of the safety of such protocols.

5. Conclusion

This study has demonstrated that the current levels used in typical EIT experiments, equating to a current density of up to 354 Am^{-2} , are safe for continuous injection into the cerebral cortex through epicortical electrodes at a carrier frequency of 1.725 kHz, with regard to histologically detectable structural indicators of current-induced tissue damage. Since fast neural EIT protocols are already restricted to using a current amplitude below the level at which stimulus-induced artefacts are evident in the resulting impedance changes due to the effects of external electric fields in modulating neural network activity (Fröhlich and McCormick 2010), defining an absolute safety threshold for the occurrence of neurotrauma evoked by electrical stimulation during EIT would be of limited value and so is not the present priority. Therefore, future studies should be aimed instead at establishing the effects of current injections at $>100 \mu\text{A}$ on the boundary voltages measured by recording electrodes; this would indicate the current density threshold at which non-linear alterations of the impedance response due to current-induced neuronal activity begin to occur. Additionally, it would be of interest to investigate the current density thresholds for the occurrence of significant functional deficits and whether these correlate to specific mechanisms of stimulus-induced neurotrauma in awake, freely-moving rats over several days. This would provide a more accurate representation of the proposed use of EIT for imaging fast neural activity in a clinical setting and ultimately enable the assessment of any behavioural and long-term morphological effects of cortical stimulation during EIT protocols.

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