

Received:
8 August 2016
Revised:
22 September 2016
Accepted:
12 October 2016

Heliyon 2 (2016) e00181



Functional neuroimaging of post-mortem tissue: lithium-pilocarpine seized rats express reduced brain mass and proportional reductions of left ventral cerebral theta spectral power

Nicolas Rouleau^{a,b}, Brady S. Reive^b, Michael A. Persinger^{a,b,*}

^a Biomolecular Sciences Program, Laurentian University, Sudbury, Ontario P3E 2C6, Canada

^b Behavioural Neuroscience Program, Laurentian University, Sudbury, Ontario P3E 2C6, Canada

* Corresponding author.

E-mail address: mpersinger@laurentian.ca (M.A. Persinger).

Abstract

Structural imaging tools can be used to identify neuropathology in post-mortem tissue whereas functional imaging tools including quantitative electroencephalography (QEEG) are thought to be restricted for use in living subjects. We are not aware of any study which has used electrophysiological methods decades after death to infer pathology. We therefore attempted to discriminate between chemically preserved brains which had incurred electrical seizures and those that did not using functional imaging. Our data indicate that modified QEEG technology involving needle electrodes embedded within chemically fixed neural tissue can be used to discriminate pathology. Forty ($n = 40$) rat brains preserved in ethanol-formalin-acetic acid (EFA) were probed by needle electrodes inserted into the dorsal and ventral components of the left and right cerebral hemispheres. Raw microvolt potentials were converted to spectral power densities within classical electroencephalographic frequency bands (1.5 Hz to 40 Hz). Brain mass

differences were shown to scale with left hemispheric ventral theta-band spectral power densities in lithium-pilocarpine seized rats. This relationship was not observed in non-seized rats. A conspicuous absence of pathological indicators within dorsal regions as inferred by microvolt fluctuations was expected given the known localization of post-ictal damage in lithium-pilocarpine seized rats. Together, the data demonstrate that post-mortem neuroimaging is both possible and potentially useful as a means to identify neuropathology without structural imaging techniques or dissection.

Keywords: Biological sciences, Neuroscience

1. Introduction

Post-mortem neuroimaging is currently performed by the use of structural imaging tools such as MRI [1]. These techniques have even been used to image ancient tissues preserved by mummification [2]. Functional neuroimaging tools such as quantitative electroencephalography (QEEG), however, are used exclusively in living subjects. Rouleau and Persinger [3] have recently demonstrated that a modified QEEG technique using needle electrodes and a vascular electrical reference can be used to identify intrinsic laterality of frequency-dependent microvolt fluctuation power in coronal sections of human brain tissue. The experiments also demonstrated that select areas including the parahippocampal gyrus could be “reactivated” by frequency-modulated 2 μ V pulsed current applied directly to the brain. These results indicate that post-mortem tissue can be measured by use of functional imaging technologies and that structural-functional relationships can be investigated post-mortem in chemically fixed tissue specimens. Whether or not this method is of any practical consequence with respect to pathological analysis or autopsy can now be considered.

For more than three decades we [4, 5] explored the structural bases of epilepsy as well as the multiple means of chemical-procedures by which seizures could be induced in rats. During that period both quantitative and semi-quantitative measurements of the proportion of neuronal dropout within the approximately 150 Paxinos and Watson structures within the telencephalon and diencephalon of hundreds of rats were documented [6]. Most of the severe neuronal damage occurred within ventral structures that included the pyriform lobe, CA1 of the hippocampus, portions of the amygdala and directly connected thalamic structures. The mosaics of those proportions of damage were found to be correlated with the animals’ subsequent histories of developing spontaneous seizures and unusual behaviours [7] as well as blood chemistry and alterations in specific organ weights [8, 9]. We hypothesized that structural changes at the level of the cerebrum would be paired with quantifiable fluctuations of electric potential differences within the fixed tissue samples. If, as Rouleau and Persinger [3] demonstrated, the normal

human brain's regional structure is intimately tied to unique, spatially-specific microvolt potential fluctuations, the pathological rat brains should display an equivalent structural-electrical relationship. Here we present evidence that long after brains have been removed and fixed there are residual, site-specific electrophysiological anomalies that reflect the history of the animals. This novel approach might be considered a technique for first screening of the potential anomaly within a brain before the costly and labour-intense histopathological analyses are pursued.

2. Methods and materials

2.1. Specimens

Forty ($n = 40$) rat brains (Wistar, albinos), which were extracted within 5 min after death (usually decapitation) and preserved in ethanol-formalin-acetic acid (EFA) at various points over the course of the past 20 years, were utilized for the present study. The brains were sequestered in compliance with ethical standards outlined by Laurentian University's Research Ethics Board. Five ($n = 5$) were laboratory teaching specimens, and thirty-five ($n = 35$) were involved in one of 12 experiments supervised by Dr. Michael A. Persinger (primary investigator) and conducted by the Neuroscience Research Group at Laurentian University between 1996 and 2008. Data presented by Michon and Persinger [10] as well as Peredery and colleagues [11] are examples of the primary studies from which the tissue collection protocols were originally approved by the University Research Ethics Board. Of the preserved specimens, eight ($n = 8$) were known females and nine ($n = 9$) were known males. Within the full sample, thirteen ($n = 13$) brains had been extracted from rats in which seizures had been induced by a single system injection of lithium-pilocarpine. Twenty three ($n = 23$) were not seized and four ($n = 4$) were excluded from the analysis due to group uncertainty. The brains shared common gross morphology and were structurally unremarkable by visual detection without the assistance of histological tools. Behavioural metrics and identifying characteristics associated with each animal including weight and sex were included as part of the data analysis. Brain wet weights were collected and included as part of the analysis.

2.2. Quantitative electroencephalography

A quantitative electroencephalography (QEEG) electrical amplifier (Model 201) by Mitsar was coupled to a series of 5 needle electrodes (probes). Four probes were inserted into the brainstem where the average microvolt value was computed and served as an electrical reference. The fifth probe was inserted directly into 1 of 4 regions of interest which will be discussed in a later section. Measurements were recorded from an HP ENVY laptop computer running Windows 8. WinEEG

software version 2.93.59 (07.2013) was employed for data collection. Gain was set to 150 μ V while low- (1.6 Hz) and high-cut (50 Hz) filters were applied in addition to a notch filter (50 Hz – 70 Hz and 110 Hz – 130 Hz) to exclude electrical artefacts. Each measurements was preceded by a surface clearing of the tissue with 10% EFA to avoid tissue damage and changes of conductivity associated with the electrophysiological measurement device.

2.3. Procedure

Brains were probed directly by the modified electrophysiological measurement device (QEEG). A similar procedure has been previously applied to human tissue [3]. Microvolt fluctuations within brains were measured by insertion of 1/3 of the needle probe (6.6 mm) into the dorsal or ventral surface of the left and right hemispheres of the rat cerebrum (Fig. 1). The dorsal probes were inserted dorso-ventrally at equidistance between the rostral- and caudal-most poles of the cerebrum and at its apex on the dorsal surface. Ventral probes are inserted caudo-rostrally at the caudal- and ventral-most extension of the cerebrum anterior to the cerebellum and inferior to the rhinal fissure which included the pyriform region involving the entorhinal cortices and underlying hippocampal formation. The reference consisted of an average of probes inserted into the spinal cord. If the spinal cord was not spared during extraction from the animal, the medulla or pons served as the reference point and these exceptions were noted within the dataset. Each brain was washed with 10% EFA and measured by QEEG for 40 s. The order of measurements was counterbalanced so that confounding factors of time could be eliminated.

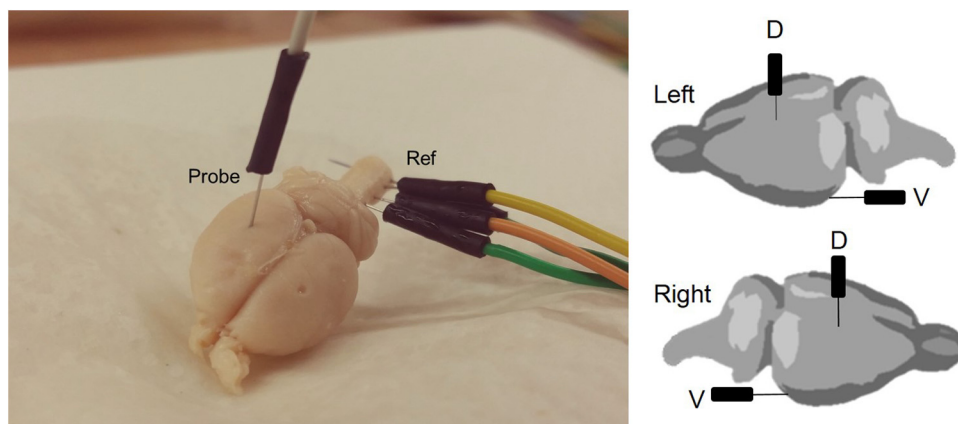


Fig. 1. A probe, referenced to the spinal cord, is inserted 6.6 mm into the dorsal (D) or ventral (V) surface of the left or right cerebral hemisphere.

2.4. Analysis

Spectral power densities (SPDs) derived from QEEG microvolt fluctuations were extracted directly from WinEEG, computed from 30 second segments of raw microvolt fluctuations with 4 second epochs. Classic electroencephalographic bands were selected: Delta (1.5 Hz – 4 Hz), theta (4 Hz – 7.5 Hz), alpha (7.5 Hz – 14 Hz), beta1 (14 Hz – 20 Hz), beta2 (20 Hz – 30 Hz), gamma (30 Hz – 40 Hz). Data were entered into an SPSS v19 dataset with all relevant experimental and quasi-experimental variables obtained from laboratory logs. Sex, age of the rat, time elapsed since death, whether the rat was seized or not, and brain wet weight were included into the analysis. Basic differences were computed by the use of t-tests as well as parametric and non-parametric correlational analyses. Equality of variances were satisfied. Measures of effect sized were included to demonstrate the relative magnitude of each finding beyond the simple presence of differences between groups.

3. Results

The results were surprisingly robust and site-specific suggesting minimal contribution from generalized measurement epiphenomena. As expected the weight of the male brains ($M = 2.01$ g, $SEM = 0.02$ g) were significantly heavier than female brains ($M = 1.83$ g, $SEM = 0.01$ g) [$t(14) = 3.94$, $p < 0.001$, $r^2 = 0.53$]. This difference was equivalent to a proportion of 9% or approximately 200 mg. This is within the range expected for general sexual dimorphism. However the brains of rats in which seizures had been induced by a single, systemic injection of lithium-pilocarpine demonstrated lighter brains ($M = 1.81$ g, $SEM = 0.04$ g) compared to non-seized rats ($M = 1.91$, $SEM = 0.02$) [$t(32) = -2.20$, $p < 0.05$, $r^2 = 0.13$ (Fig. 2A)]. The difference was equivalent to about 5% or 100 mg.

Theta-band (4 Hz – 7.5 Hz) spectral power densities were decreased in seized ($M = 66.37$, $SEM = 1.56$) rat brain measurements relative to non-seized ($M = 73.22$, $SEM = 2.10$) rat brain measurements [$t(142) = 2.26$, $p < 0.05$, $r^2 = 0.03$]. Those which were obtained within the left ventral quadrant of seized rat cerebra (Fig. 2B) expressed theta-band SPDs which positively correlated with brain mass, $r = 0.61$, $p < 0.05$, $\rho = 0.81$, $p < 0.001$ (Fig. 2C). As the brain weight decreased in seized brains, so did the left hemispheric ventral theta rhythms. Non-seized rat cerebra did not display an analogous relationship within the left ventral quadrant ($r = 0.02$, $p = 0.94$, $\rho = 0.04$, $p = 0.85$). These results are shown in Fig. 2D. There were no differences observed as a function of the electrical reference point ($p > 0.05$).

4. Discussion

The results demonstrate that voltage potential differences obtained from deceased, chemically fixed brains can be used to discriminate between animals which

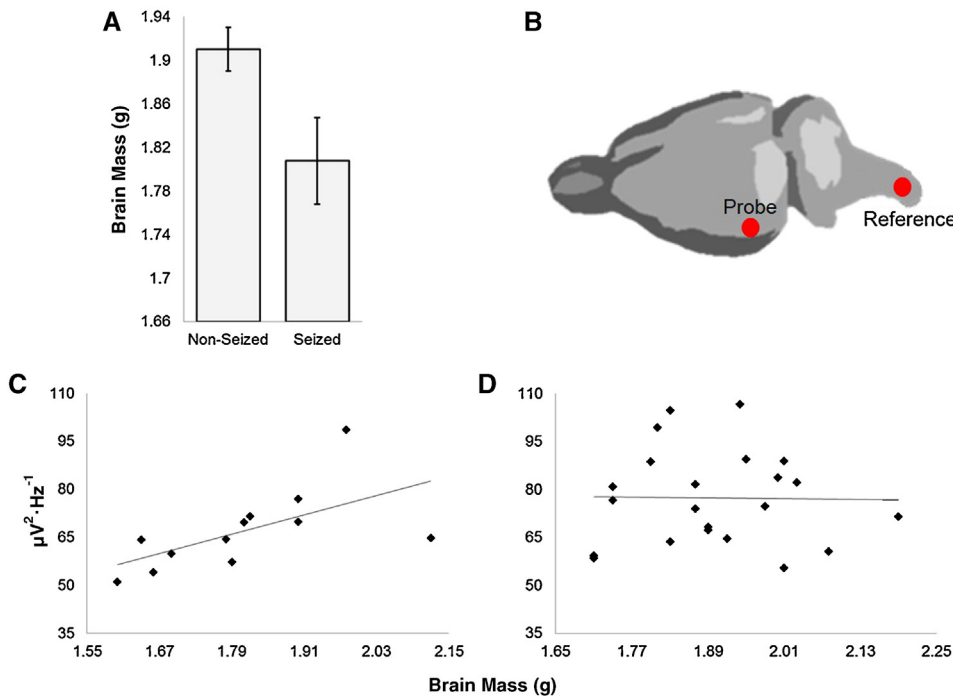


Fig. 2. Seized rats demonstrated a statistically significant ($p < 0.05$) 5% decrease in brain mass relative to non-seized rats (A). Theta-band spectral power densities obtained by probing the left hemispheric ventral component of the rat cerebrum (B) demonstrated a linear relationship with brain mass in seized rats (C) which was not observed for non-seized rats (D).

displayed a history of seizure activity and those that did not whilst alive. The average decrease of 5% cerebral mass compared to normal rats would be equivalent to $\sim 10^8$ neurons and other cells assuming about 50% of the space is occupied by cells immersed in interstitial fluid. This is estimated by assuming each cell with a width of $10 \mu m$ and a density of water would weigh about $5 \cdot 10^{-10} g$ and 5% decreased weight would be about $10^{-1} g$ (100 mg).

There is quantitative evidence that numbers of cells associated with that mass (volume) of brain tissue could have contributed to the shift in electroencephalographic power. The slope from Fig. 2C was $25.2 \mu V^2 \cdot Hz^{-1}$. The theta band included 3.5 Hz; as a result the total potential change was $9.4 \cdot 10^{-6} V$ (the square root of $88.2 \mu V^2$). The numbers of water molecules with a charge (such as H^+) at a pH of ~ 5 within $5.6 \cdot 10^{-3} M$ (0.1 cc) would be $3.4 \cdot 10^{14}$ charges. About 10^6 charges (static) are functionally associated with the resting plasma membrane potential of a neuron [12]. Consequently the shift would be equivalent the integrated loss of $\sim 10^8$ cells. This is the same order of magnitude as the estimated loss of neurons inferred from the brain mass differences between the seized and non-seized rats. Alternatively, 10^{14} charges multiplied by the unit charge ($1.6 \cdot 10^{-19} A \cdot s$) per s

(1 Hz) would be $5.4 \cdot 10^{-5}$ A. Considering the enhanced conductivity of the fixative ($\sim 10^{-1} \Omega$) the voltage equivalence would be within the microvolt range.

The measurements showed that only the power within theta band was significantly correlated with the weight of the brains if the animals had histories of seizures. Other QEEG bands did not display this relationship. In addition, the normal, non-seized brains did not display any differential shift in theta power density as a function of brain weight. That brain tissue, specifically grey matter, in mammals may display an intrinsic resonance within the theta range has been suggested by the calculations of Tsang et al. [13]. Employing a LC (impedance-capacitance) formula for these values for grey matter they found that the intrinsic resonance frequency was about 7 Hz. If this frequency band is a material property of brain matter, within nature or fixed environments, then its ubiquitous presence within structures such as the hippocampal formation-entorhinal cortices might be accommodated. It may also explain one possible advantage for gamma ripples to be superimposed upon this fundamental theta rhythm and for the pervasive neocortical connections and influences that originate from the entorhinal cortices.

The occurrence of the brain weight-theta power within the left lower caudal quadrant (piriform region) for the seized brains would be expected because of the relatively greater proportion of grey matter and hence the greater range of decimation from the progressive consequences of the seizures. Hemispheric asymmetries have been well established for both function and structure. For example Adamec and Morgan [14] found that right hemispheric kindling within the basolateral amygdala increased behaviours consistent with anxiety in rats while left hemispheric kindling of this structure diminished these behaviors. Schubert et al. [15] demonstrated differential effects in plasticity within the rat amygdala and hippocampus following theta pulse stimulation that reflected hemispheric differences. Asymmetrical compromise of the blood brain barrier can be induced by seizures according to Kutlu et al. [16]. The observation by Kawakami et al. [17] that strong asymmetrical distributions of epsilon 2 subunits of NMDA receptors that strongly influenced if not determined the differential plasticity between the left and right hippocampus. This might have the capacity to produce subtle changes in intrinsic theta elicitation when activated by specific proton donors such as EFA.

Left ventral cerebral theta power scaled with the mass of seized rat brains where decreases in mass would have been associated with linear decreases in power. This relationship could be relevant to entorhinal-hippocampal connections. Peredery and colleagues [6] reported that neuronal dropout following lithium-pilocarpine seizures in the rat were most evident within CA1 fields of the hippocampus and the piriform cortex in addition to select thalamic regions. The distribution of said structures is skewed toward the ventral half of the cerebrum. The entorhinal cortex, which is localized to the ventral components of the rat cerebrum, is known to both

project to CA1 fields and generate theta rhythms [18]. Neuronal loss within the CA1 field could have promoted afferent degeneration, resulting in mass-scaled theta power dropout. However, it remains unclear as to why this relationship was only present within the left hemisphere. It may be relevant that we [19] found that the amount of damage within the left dentate gyrus was the driving factor for the numbers of spontaneous monthly seizures subsequent to induction of lithium-pilocarpine epilepsy.

Declarations

Author contribution statement

Nicolas Rouleau, Brady S. Reive, Michael A. Persinger: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Funding statement

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

References

- [1] P.D. Griffiths, M.N.J. Paley, E.H. Whitby, Post-mortem MRI as an adjunct to fetal or neonatal autopsy, *The Lancet* 365 (2005) 1271–1273.
- [2] S.J. Karlik, R. Bartha, K. Kennedy, R. Chhem, MRI and multinuclear MR spectroscopy of 3,200-year-old Egyptian mummy brain, *Am. J. Roentgenol.* 189 (2007) W105–W110.
- [3] N. Rouleau, M.A. Persinger, Differential Responsiveness of the Right Parahippocampal Region to Electrical Stimulation in Fixed Human Brains: Implications for Historical Surgical Stimulation Studies? *Epilepsy Behav.* 60 (2016) 181–186.
- [4] M.A. Persinger, K. Makarec, J.C. Bradley, Characteristics of limbic seizures evoked by peripheral injections of lithium and pilocarpine, *Physiol. Behav.* 44 (1988) 27–37.

- [5] M.A. Persinger, Y.R. Bureau, M. Kostakos, O. Peredery, H. Falter, Behaviors of rats with insidious multifocal brain damage induced by seizures following single peripheral injections of lithium and pilocarpine, *Physiol. Behav.* 53 (1993) 849–866.
- [6] O. Peredery, M.A. Persinger, G. Parker, L. Mastrosov, Temporal changes in neuronal dropout following inductions of lithium/pilocarpine seizures in the rat, *Brain Res.* 881 (2000) 9–17.
- [7] L.S. St-Pierre, M.A. Persinger, Extreme obesity in female rats following prepuberal induction of lithium/pilocarpine seizures and a single injection of acepromazine, *Epilepsy Behav.* 7 (2005) 411–418.
- [8] K.R. George, T. Rico, L.S. St-Pierre, M.J. Dupont, C.G. Blomme, A. Mazzuchin, M.A. Persinger, Large differences in blood measures, tissue weights, and focal areas of damage 1 year after postseizure treatment with acepromazine or ketamine, *Epilepsy Behav.* 15 (2009) 98–105.
- [9] M.A. Persinger, L.S. Stewart, P.M. Richards, T. Harrigan, R.P. O'Connor, Y. R.J. Bureau, Seizure onset times for rats receiving systemic lithium and pilocarpine: sources of variability, *Pharmacol. Biochem. Behav.* 71 (2002) 7–17.
- [10] A.L. Michon, M.A. Persinger, Experimental simulation of the effects of increased geomagnetic activity upon nocturnal seizures in epileptic rats, *Neurosci. Lett.* 224 (1997) 53–56.
- [11] O. Peredery, M.A. Persinger, G. Parker, L. Mastrosov, Temporal changes in neuronal dropout following inductions of lithium/pilocarpine seizures in the rat, *Brain Res.* 881 (2000) 9–17.
- [12] M.A. Persinger, 10^{-20} Joules as a neuromolecular quantum in medicinal chemistry: an alternative approach to myriad molecular pathways, *Curr. Med. Chem.* 17 (2010) 3094–3098.
- [13] E.W. Tsang, S.A. Koren, M.A. Persinger, Power increases within the gamma range over the frontal and occipital regions during acute exposure to cerebrally counter clockwise rotating magnetic fields with specific derivatives of change, *Int. J. Neurosci.* 114 (2004) 1183–1193.
- [14] R.E. Adamec, H.D. Morgan, The effect of kindling of different nuclei in the left and right amygdala on anxiety in the rat, *Physiology Behav.* 55 (1993) 1–12.
- [15] M. Schubert, H. Siegmund, H.C. Pape, D. Albrecht, Kindling-induced changes in plasticity of the rat amygdala and hippocampus, *Learn. Mem.* 12 (2005) 520–526.

- [16] N. Kutlu, H.S. Vatansever, T.O. Bayazit, N. Ekerbicer, U. Tan, Blood brain barrier in right- and left-pawed female rats assessed by a new staining method, *Int. J. Neurosci.* 112 (2002) 1037–1046.
- [17] R. Kawakami, Y. Shinohara, Y. Kato, H. Sugiyama, R. Shigemoto, I. Ito, Asymmetrical allocation of NMDA receptor (2 subunits in hippocampal circuitry), *Science* 300 (2003) 990–994.
- [18] A. Alonso, E. Garcia-Austt, Neuronal sources of theta rhythm in the entorhinal cortex of the rat, *Exp. Brain Res.* 67 (1987) 493–501.
- [19] M.A. Persinger, M.J. Dupont, Emergence of spontaneous seizures during the year following lithium/pilocarpine-induced epilepsy and neuronal loss within the right temporal cortices, *Epilepsy Behav.* 5 (2004) 440–445.