



Research article

Increased oxidation of RNA despite reduced mitochondrial respiration after chronic electroconvulsive stimulation of rat brain tissue

Birgitte Nielsen^{a,*}, Vanja Cejvanovic^b, Gitta Wörtwein^a, Allan R. Hansen^a, Kasper K. Marstal^c, Allan Weimann^b, Peter N. Bjerring^d, Flemming Dela^e, Henrik E. Poulsen^b, Martin B. Jørgensen^f

^a Laboratory of Neuropsychiatry, Copenhagen University Hospital, Copenhagen, Denmark

^b Laboratory of Clinical Pharmacology, Bispebjerg and Frederiksberg Hospital, Copenhagen, Denmark

^c Biomedical Imaging Group Rotterdam (BIGR), Department of Radiology & Medical Informatics, Erasmus Medical Center, Rotterdam, the Netherlands

^d Department of Hepatology, Copenhagen University Hospital, Copenhagen, Denmark

^e Xlab, Centre of Healthy Aging, Department of Biomedical Sciences, University of Copenhagen, Copenhagen, Denmark

^f Psychiatric Center Copenhagen and Institute of Clinical Medicine, University of Copenhagen

ARTICLE INFO

Keywords:

Mitochondria
Reactive oxygen species (ROS)
Hydrogen peroxide
Major depression
8-oxoGuo

ABSTRACT

Major depressive disorder (MDD) affects 350 million people worldwide and is a serious socio-economic burden. The most efficient treatment of MDD is electroconvulsive therapy (ECT), which has been shown to influence the oxidative status believed to be part of the pathophysiology of MDD. We investigated the effects of chronic electroconvulsive stimulation (ECS) on mitochondrial respiration and mitochondrial hydrogen peroxide production, RNA oxidation, and the content of mitochondria in the piriform cortex of the rat. We found reductions of mitochondrial respiration in respiratory states 2 and 3 by 33% and 32%, respectively, and a 23% reduction in electron transfer capacity. RNA oxidation, as measured by 8-oxo-7,8-dihydroguanosine, was increased by 58%, while mitochondrial production of H₂O₂ was unaffected. The increased oxidative stress may thus be ascribed to extra-mitochondrial sources.

1. Introduction

Depression affects 350 million people worldwide and is expected to be among the heaviest economic and social burdens in 2020 [1,2]. Its pathogenesis has been associated with neurotransmitter alterations, neuroplasticity, inflammation, and increases in reactive oxygen species (ROS) [3]. The most harmful ROS, hydrogen peroxide (H₂O₂), leads to oxidative stress when in excess [4]. As the most important source of H₂O₂, the mitochondria are especially susceptible to disturbances in the oxidant system, leading to the oxidation of protein, RNA, and DNA [5]. The high metabolism and generally low level of antioxidants in the brain render it particularly vulnerable to oxidative stress [6], making this a key factor in diseases of the brain. In comparison to a control group, the mitochondrial electron transfer system (ETS) of depressed patients has been found to have inhibited complexes [7,8], possibly linked to the observed high oxidative status. Our group has previously found severely depressed patients to have increased urinary excretion of 8-oxo-7,8-dihydroguanosine (8-oxoGuo), a product of RNA oxidation [9]. RNA is furthermore believed to be more susceptible to oxidative stress when compared to DNA, proteins, and lipids [10]. Despite strong

evidence linking depression and mitochondrial dysfunction, there is no clear understanding of the pathological development, and hence no treatment for the underlying mitochondrial dysfunction.

The introduction of electroconvulsive therapy (ECT) in the 1930s led to the first successful treatment of depression. Today, ECT remains the most effective treatment for the condition [11]. Despite intense efforts, its mechanism of action is still not fully understood [12–16]. Our aforementioned study [9] surprisingly found a further increase (rather than a decrease) in 8-oxoGuo in depressed patients after a series of ECTs, emphasizing that ECT affects oxidative stress levels. The present study aimed to investigate the effects of ECS on mitochondrial respiration and mitochondrial H₂O₂ production, the oxidation of RNA, and the content of mitochondria in piriform cortex from rats [17]. We hypothesized that ECS would impair mitochondrial respiration and increase 8-oxoGuo formation and H₂O₂ production, possibly through increased mitochondrial content [18].

* Corresponding author.

E-mail address: pvf484@alumni.ku.dk (B. Nielsen).

<https://doi.org/10.1016/j.neulet.2018.09.061>

Received 21 April 2018; Received in revised form 9 September 2018; Accepted 28 September 2018

Available online 01 October 2018

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2. Material and methods

2.1. Animals

Male Sprang-Dawley rats (Taconic Europe A/S, Lille Skensved, Denmark; weight 290–300 g) were group-housed in plexiglass cages (two per cage) with ad libitum access to food pellets (Altromin 1319, Brogaarden, Denmark) and acidified tap water. The rats were kept on a 12-hour light-darkness cycle with 30 min of twilight (lights on at 7 a.m.), at a constant temperature of $21 \pm 1^\circ\text{C}$ and humidity of $52 \pm 2\%$. The animals were weighed four days after arrival and every week thereafter. At arrival, they were randomly distributed into two groups: 10 rats were assigned to sham ECS (*control*), and 10 rats were assigned to ECS (*ECS*). The animals were sacrificed without anesthesia by decapitation with a rodent guillotine three days after the last treatment. This was done immediately after removal from the home cage to avoid stress responses. After decapitation, the brain was dissected and placed in an ice-cold buffer (MiR05; 60 mM K-lactobionate, 0.1% BSA essentially fatty acid free, 0.5 mM EGTA, 3 mM MgCl_2 , 20 mM taurine, 10 mM KH_2PO_4 , 20 mM HEPES, 110 mM D-sucrose) for 30 s. The brain was then placed on an ice-cold cutting board and piriform cortex was dissected from one of the hemispheres. Approximately 20–30 mg of piriform cortex was homogenized in ice-cold MiR05 using a glass Potter-Elvehjem homogenizer to a concentration of 1 mg/ml for immediate measurements of oxygen consumption and H_2O_2 production in an Oxygraph-2k (OROBOROS Instruments, Innsbruck, Austria). Five minutes after decapitation the homogenate was injected into its chamber in the Oxygraph. Another 50 mg of piriform cortex and the other hemisphere were immediately placed on dry ice and stored at -80°C for later measurement of 8-oxoGuo and citrate synthase (CS) activity, respectively.

2.2. Electroconvulsive stimulation

ECS was administered to unanesthetized rats using a pair of auricular electrodes soaked in saltwater. The current was administered through a UGO Basile (57,800) ECT unit with the following stimulus parameters: duration 1 s; width 0.9 ms; frequency 200 Hz; current 50 mA, generating a charge of 9 mC. The sham groups were restrained with the auricular electrodes with no current passing through. Commencing the ECS session one week after arrival, the rats received 9 treatments in total over 3 weeks, mimicking a clinical ECT treatment course. The treatment was considered successful when inducing a generalized tonic-clonic seizure. To avoid sonic and ultrasonic vocalizations from influencing the rat's stress level, the ECS treatments were performed in a separate room.

2.3. Mitochondrial respiration and H_2O_2 production measurements

Mitochondrial respiration (measured by oxygen consumption) and H_2O_2 production were determined using the Oxygraph-2k (OROBOROS Instruments, Innsbruck, Austria), combined with the O2k-Fluo LED2 module using the Fluorescence-Sensor Green [19]. Two oxygraphs, each with two separate chambers, were used simultaneously. Each instrument contained one control and one ECS sample; the other instrument serving as a duplicate. All experiments were performed in 37°C MiR05. Reoxygenation was performed at an oxygen concentration of 80–100 μM . Mitochondrial respiration and H_2O_2 production in relation to specific complexes of the electron transfer system were determined using a substrate-uncoupler inhibitor titration (SUIT) protocol. Before administering the homogenate to the chambers, Amplex Red (Amp, 10 μM), horse radish peroxidase (HRP, 1 U/ml), and superoxide dismutase (SOD, 5 U/ml) were added. SOD converts superoxide into H_2O_2 , whose reaction with Amplex Red is catalyzed by HRP to produce resorufin. The fluorescence intensity of resorufin is known to be nearly proportional to the concentration of H_2O_2 [19,20]. The fluorescence signal

was sensitivity-tested through a one-time addition (just after adding the homogenate) and with repeated titrations of H_2O_2 . As no difference in sensitivity was detected, we accepted a one-time H_2O_2 calibration to avoid the accumulation of resorufin, which inhibits HRP at concentrations above 3 μM [19]. In each experiment the fluorescence signal was calibrated with H_2O_2 titration (0.1 μM) after adding the homogenate. In the present SUIT protocol, routine respiration was obtained by adding the homogenate to the buffer solution and controlled by endogenous substrates. Routine respiration was used as baseline; its values were thus subtracted from the ensuing respiratory states. Leak state (L_N) was induced by adding pyruvate (5 mM), malate (2 mM), and glutamate (10 mM) (PMG). In this non-phosphorylating respiratory state (state 2), oxygen flux compensates for proton leak. The OXPHOS capacity of CI-linked activity (CI_p) was measured after adding a saturating concentration of ADP + Mg (1.9 mM). Further addition of succinate (S, 10 mM), which is a direct substrate for complex II, provided measurement of OXPHOS capacity with both CI and II-linked substrates (CI + CII_p), also termed respiratory state 3. By stepwise titration of the ionophore carbonyl cyanide m-chlorophenylhydrazone (CCCP, 0.5 μM steps), experimental uncoupled respiration was obtained. This expresses electron transfer system capacity (ETS), the result of proton leakage into the mitochondrial matrix. CI, CII and CIII were inhibited by rotenone (0.5 μM), malonate (5.0 mM), and antimycin A (Ama, 2.5 μM), respectively, reflecting residual oxygen consumption (ROX). The ROX value was subtracted from mitochondrial respiratory state values [21]. All chemicals were purchased from Sigma-Aldrich. Pyruvate, rotenone, malonate, and H_2O_2 were freshly prepared within one hour of experimentation. Amplex Red was not exposed to light during storage or experiments [22].

2.4. 8-oxoGuo and guanosine measurement

The content of 8-oxoGuo and guanosine (Guo) was quantified in the piriform cortex (50 mg) using UPLC-MS/MS as described in [23]. Chromatographic separation was performed using an Acquity UPLC system (Waters Corp., Milford, USA), while mass spectrometric detection was performed using a Xevo TQ-S triple quadrupole mass spectrometer (from the same supplier). Electrospray ionization in the negative mode was employed for both analytes. The results are reported as the ratio of 8-oxoGuo per 10^6 Guo.

2.5. Citrate synthase activity

Approximately 2 mg of piriform cortex was homogenized and processed for the determination of citrate synthase (CS) activity as previously described [24]. CS activity is expressed as micromoles substrate per minute per gram of brain tissue and is used for the normalization of mitochondrial respiration and H_2O_2 production and as a biomarker of mitochondrial content.

2.6. Ethics

The animal experiments were approved by the Animal Experiments Inspectorate under the Danish Ministry of Justice (license number 2012-25-2934-00038). All procedures were performed in a fully AAALAC-accredited facility in accordance with Guide for the Care and Use of Laboratory Animals (2011) and the European Communities Council Directive of 24 November 1986 (86/609/EEC).

2.7. Statistical analyses

All statistical analyses were programmed in open source code R (version 3.3.2), available at <https://github.com/birgittenielsen/antidepressants>.

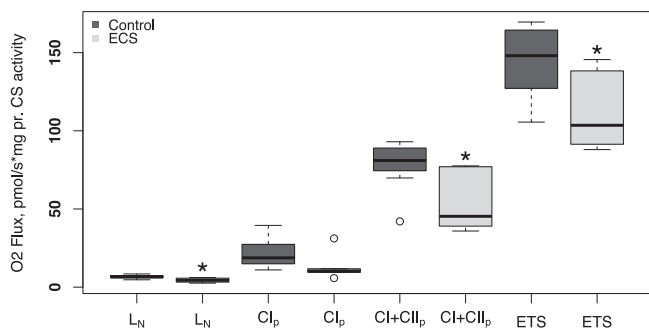


Fig. 1. Mitochondrial respiration.

Oxygen Flux (pmol/sec*mg) in different respiratory states normalized by citrate synthase (CS) activity in control group ($n = 8$) and electron convulsive stimulation (ECS) group ($n = 6$). Ln (Leak State), CI_p (Oxphos capacity, Complex I), CI & CIIP (Oxphos capacity, Complex I + II), ETS (Electron Transfer System Capacity). The box plots the interquartile range (IQR), the line inside the box shows the median, “whiskers” show the locations of the minimum and maximum, and the circles above represent suspected outliers (1.5xIQR from the third quartile). * = $p < 0.05$, Student’s T-test.

2.8. Normal distribution was tested by Shapiro-Wilk test

Differences in mitochondrial respiration, H₂O₂ production, and mitochondrial content were normally distributed between the ECS-treated rats and the controls. The results were tested using Student’s *t*-test and reported as means \pm standard error with corresponding CI and *p*-values.

Due to deviation from normal distribution, differences in 8-oxoGuo per 10⁶ Guo ratios were tested using the Mann–Whitney Test. Results are reported as *W*- and *p*-values and medians. by using a normal approximation, The Mann–Whitney test determines the *p*-value based on the *W*-value, which is the sum of the ranks of the first sample.

3. Results

This study showed decreases in mitochondrial respiration in the brains of ECS-treated rats when compared to the control rats in three different respiratory states (Fig. 1). Leak respiration (Ln, respiratory state 2) was reduced by 33% (control: 6.6 ± 0.4 pmol O₂/mg*s per CS, ECS: 4.4 ± 0.6 pmol O₂/mg*s per CS, CI: 0.6; 4, $n = 8$; 10, $p < 0.05$). The OXPHOS capacity of CI-linked activity was unchanged (control: 22 ± 3 pmol O₂/mg*s per CS, ECS: 13 ± 4 pmol O₂/mg*s per CS, CI: -3; 19, $n = 8$; 10, $p = 0.12$), whereas the OXPHOS capacity of CI + CIIP-linked activity (respiratory state 3) was decreased by 32% (control: 78 ± 6 pmol O₂/mg*s per CS, ECS: 53 ± 8 pmol O₂/mg*s per CS, CI: 3; 46, $n = 8$; 10, $p < 0.05$). A 23% decrease in ETS was likewise found (control: 144 ± 9 pmol O₂/mg*s per CS, ECS: 111 ± 10 pmol O₂/mg*s per CS, CI: 4; 62, $n = 8$; 10, $p < 0.05$). We demonstrated a 58% increase in the ratio of 8-oxoGuo per 10⁶ Guo for the ECS-treated rats when compared to controls (Fig. 2). In the control group, the median 8-oxoGuo per 10⁶ Guo ratios were 4.8, with 7.6 in the ECS group. Mann–Whitney testing showed a significant difference ($W = 12$, $n = 10$, $p < 0.05$) between the two groups, both when outliers were included and when excluded (Fig. 2b).

In no respiratory state did the ECS treatment significantly influence H₂O₂ production (Fig. 3). Neither was there any difference in CS activity between ECS-treated rats and controls (control: $69 \mu\text{mol}/(\text{min}\cdot\text{g})$, ECS: $72 \mu\text{mol}/(\text{min}\cdot\text{g})$, $n = 12$; 16, CI: -8.5 ; 2.8 , $p = 0.29$), indicating no effect of ECS on mitochondrial content. Mitochondrial respiration and H₂O₂ production were furthermore normalized by CS activity.

At the time of decapitation, the ECS rats weighed 12% less than controls. The data were not adjusted for weight as no correlation with other parameters was found.

4. Discussion

This study showed a decrease in mitochondrial respiratory states 2 and 3 by 33% and 32%, respectively. The total electron capacity of the mitochondrial membrane was reduced by 23% after chronic ECS (Fig. 1). To our knowledge, no previous study has measured mitochondrial respiration following ECS treatment. When they measured enzyme activity in complexes II and IV in rat brain tissue after a course of ECS, Burigo et al. found no difference between control rats and ECS-treated rats [13]. Our measurements reflect real-time oxygen consumption in mitochondria, thus providing a more accurate reflection of the complex cell physiology. It follows that comparison between the results of Burigo et al. and our results are inexpedient. The decreased respiration demonstrated by this study could be consistent with Nobler et al., who used fluorodeoxyglucose positron emission tomography (FDG-PET) to show that ECT had reduced the brain glucose metabolism in depressed patients [25]. While several areas of the brain have been investigated for changes in glucose metabolism after ECT, there is growing consensus that the frontal lobe is the main locus of ECT-associated decreases in glucose metabolism [25–29]. Since various antidepressant treatments have been shown to correlate with reduced glucose metabolism, this is a possible mechanism of action for ECT as well as for other antidepressant treatments [30,31].

Our finding of lower oxygen consumption may also have been caused by the increase in oxidized RNA after ECS. The oxidation may disturb the translational process through ribosomal stalling, leading to fewer and/or dysfunctional proteins [32], even though Burigo et al. demonstrated no change in the enzyme activity of complexes II and IV [13].

This is the first study to demonstrate increased RNA oxidation in brain tissue after chronic electroconvulsive stimulation (ECS) in rats. We showed a 58% increase in the 8-oxoGuo/Guo ratio as a marker of oxidative damage to RNA (Fig. 2). Contrary to our expectations we found no significant effect on H₂O₂ production (Fig. 3). In one of the few studies that have investigated oxidative stress and antioxidant status following ECS treatment in rats, a group reported decreased frontal cortex SOD and glutathione peroxidase (GPX) activity following both acute single ECS and chronic ECS. The group also observed reduced SOD and GPX activity in the hippocampus following acute single ECS, while GPX was reduced in only one of two chronic ECS settings. Only the chronic ECS rats showed reduced antioxidant activity in the cerebellum and the pons [14]. Measuring at different times after the last ECS, another study reported decreased lipid peroxidation and protein carbonylation in the hippocampus, cerebellum and striatum after both single acute ECS and chronic ECS. Increases in lipid peroxidation after single and chronic ECS were observed in the cortex [12]. These findings were supported in a study demonstrating increased lipid peroxidation in the frontal cortex and increased SOD and GPX activity in the hippocampus and the cerebellum after acute single ECS [33]. These results appear together to support a theory of an adaptive system with region specificity in which the cortex is more susceptible to oxidative stress than is the hippocampus. Time is another important parameter. The measurements after acute ECS indicate the results of the seizures, while chronic ECS measurements demonstrate the effect of an adapted system.

Since this study has demonstrated decreased OXPHOS capacity, increased RNA oxidation but no increase in mitochondrial production of H₂O₂, extra-mitochondrial components could be the origin of oxidative stress after ECS [18]. The pentose phosphate pathway (PPP) located in the cytosol is an important source of glutathione-reducing NADPH. Reduced glucose metabolism is known to weaken PPP, resulting in less NADPH and thereby a reduced antioxidant status. Several studies agree on the importance of this pathway even though the quantitative contribution is small in non-diving cells, such as neurons [34]. This may partly explain the extra-mitochondrial oxidative stress induced by ECS. We also speculate that RNA oxidation may affect the

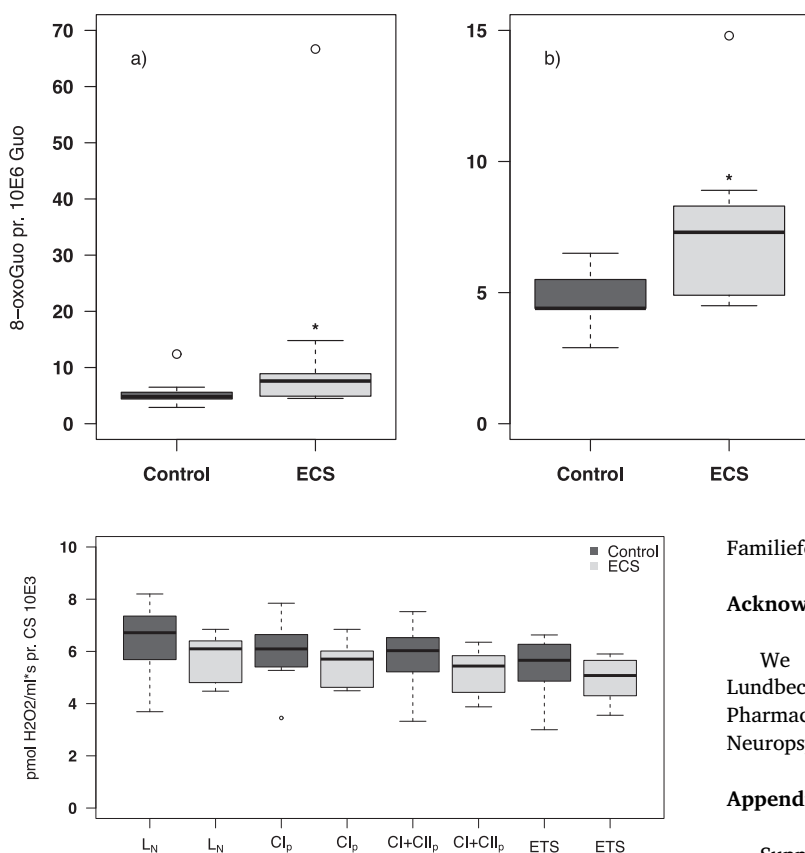


Fig. 3. Hydrogen peroxide production in mitochondrial respiratory system. Mitochondrial production of hydrogen peroxide (H₂O₂) (pmol H₂O₂/ml*sec) in piriform cortex of rats in control group ($n = 10$) and chronic electroconvulsive stimulation (ECS) group ($n = 6$). L_N (Leak State), Cl_p (Oxphos capacity, Complex I), Cl + Cl_p (Oxphos capacity, Complex I + II), ETS (Electron Transfer System Capacity). The box plots the interquartile range, IQR, the line inside the box shows the median, “whiskers” show the locations of the minimum and maximum, and the circles above represent suspected outliers (1.5xIQR from the third quartile).

regulation of the adenosinergic and/or glutamatergic transmitter systems, as was recently demonstrated for guanosine [35].

Our hypothesis that mitochondrial content would increase after ECS found no support. Similarly, a recent study employing electron microscopy and stereology identified no mitochondrial changes in Flinders-resistant rats after chronic ECS [36], mitochondria are critical. Several studies of neuroplasticity in both animal and human subjects have demonstrated neurogenesis, synaptogenesis, angiogenesis, and gliogenesis following ECS/ECT [37]. Chen et al. thus found spine, rather than shaft synapse, activity to increase after ECS [38]. Shaft synapses are located directly at mitochondria-filled dendrites, whereas spine synapses are located at the small mitochondria-free protrusions of dendrites. The increase in the number of spine synapses, rather than shaft synapses, is therefore compatible with the unchanged density of mitochondria demonstrated here. In conclusion, we found decreased mitochondrial respiration and increased RNA oxidation, as measured by 8-oxoGuo, while the production of H₂O₂ and mitochondrial content was unchanged. Despite the clarification provided on the mechanism of action for ECS, the puzzle of ECT and depression remains unfinished. Research into the causes of oxidative stress and the adaptive antioxidant system should provide further pieces to this puzzle.

Funding

This study was funded by grants from Simon Fougner Hartmanns

Fig. 2. 8-oxoGuo ratio.

8-oxoGuo/10⁶ guanosine (Guo) ratio in piriform cortex of rats in control group and in chronic electroconvulsive stimulation (ECS) group ($n = 10$). The box plots the interquartile range, IQR, the line inside the box shows the median, “whiskers” show the locations of the minimum and maximum, and the circles above represent suspected outliers (1.5xIQR from the third quartile). *: P-value < 0.05, $W = 12$, Mann-Whitney Test. a) All data are included. b) Data without the two suspected outliers.

Familiefond and Lundbeckfonden.

Acknowledgements

We wish to thank Simon Fougner Hartmanns Familiefond, Lundbeckfonden, and the lab technicians at the Laboratory of Clinical Pharmacology, Copenhagen, and co-workers at the Laboratory of Neuropsychiatry at Psychiatric Center, Copenhagen.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.neulet.2018.09.061>.

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