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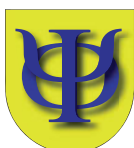

Markers of HPA-axis activity and nucleic acid damage from oxidation after electroconvulsive stimulations in rats

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Abstract

Objective: Oxidative stress has been suggested to increase after electroconvulsive therapy (ECT), a treatment which continues to be the most effective for severe depression. Oxidative stress could potentially be mechanistically involved in both the therapeutic effects and side effects of ECT. *Methods:* We measured sensitive markers of systemic and central nervous system (CNS) oxidative stress on DNA and RNA (urinary 8-oxodG/8-oxoGuo, cerebrospinal fluid 8-oxoGuo, and brain oxoguanine glycosylase mRNA expression) in male rats subjected to electroconvulsive stimulations (ECS), an animal model of ECT. Due to the previous observations that link hypothalamic–pituitary–adrenal (HPA)-axis activity and age to DNA/RNA damage from oxidation, groups of young and middle-aged male animals were included, and markers of HPA-axis activity were measured. *Results:* ECS induced weight loss, increased corticosterone (only in middle-aged animals), and decreased cerebral glucocorticoid receptor mRNA expression, while largely leaving the markers of systemic and CNS DNA/RNA damage from oxidation unaltered. *Conclusion:* These results suggest that ECS is not associated with any lasting effects on oxidative stress on nucleic acids neither in young nor middle-aged rats.

Significant outcomes

- We tested if young and middle-aged male rats subjected to an animal model of ECT (ECS) had increased whole-body and brain levels of free radical damage to DNA and RNA.
- While ECS caused weight-loss and increased stress hormone (corticosterone) levels, we found no evidence that ECS causes increased free radical damage to DNA and RNA.
- The study does not support that ECS is associated with any major changes in systemic or CNS oxidative stress on nucleic acids.

Limitations

- We did not include measurements immediately after ECS, and thus we cannot rule out that the seizures could cause a transient effect on the parameters investigated.
- Direct measures of neuronal 8-oxodG/8-oxoGuo in DNA/RNA were not obtained, and thus we potentially could have overlooked a small effect of locally increased oxidative stress in the brain that did not manifest itself in the systemic or CSF markers or affect OGG1 mRNA expression.
- The study did not include any behavioural measurement to validate the ECS effects. However, ECS treatments did induce weight loss, age-dependent CORT changes, and decreased cerebral GluR expression, strongly suggesting that the treatments were effective.

Introduction

Electroconvulsive therapy (ECT) continues to be the most effective treatment for severe depression and can be life-saving in a range of other acute psychiatric conditions

(UK_ECT_Review_Group, 2003; Nielsen *et al.*, 2014; Dessens *et al.*, 2016). The mechanism underlying the powerful clinical effects of ECT continues to be unknown (Bolwig, 2011). ECT is associated with cognitive side effects, which is a concern for clinicians and patients and a major cause of treatment discontinuation (Semkovska & McLoughlin, 2013). However, these side effects are usually transient (Semkovska & McLoughlin, 2010), and there are no indications that ECT causes long-term damage to the brain. On the contrary, on a neuronal level ECT causes increased neurogenesis, dendritic growth, and synaptic sprouting (Madsen *et al.*, 2000; Hageman *et al.*, 2008; Chen *et al.*, 2009). A consistent finding is that the hippocampus, as well as other areas of the brain, is increased in volume after ECT (Jorgensen *et al.*, 2016; Joshi *et al.*, 2016). Many cognitive domains improve beyond baseline levels after ECT, probably due to the negative effect on cognition by depression in itself (Semkovska & McLoughlin, 2010), and finally, a new study found no evidence that ECT increases the risk for dementia (Osler *et al.*, 2018).

Preclinical studies suggest that various markers of oxidative stress, including protein and lipid oxidation markers, are increased in the brain after ECT (Feier *et al.*, 2006; Zupan *et al.*, 2008), although other studies did not find such an association (Barichello *et al.*, 2004; Jornada *et al.*, 2007). Such a phenomenon could potentially be causally involved in both the antidepressant effects and the cognitive side effects of ECT. We previously observed an increase in systemic RNA damage from oxidation in severely depressed patients after ECT (Jorgensen *et al.*, 2013b). However, many other factors than ECT *per se* could cause a change in a systemic marker of oxidative stress, for example, increased calorie intake and weight gain following the relief of depressive symptoms.

Hence, in the present study, we wished to test whether systemic and central nervous system (CNS) markers of oxidative damage to DNA/RNA would increase in a preclinical model of ECT (i.e. electroconvulsive stimulations, ECS). We measured urinary and cerebrospinal fluid (CSF) contents of 8-oxo-7,8-dihydro-2-deoxyguanosine (8-oxodG) and 8-oxo-7,8-dihydroguanosine (8-oxoGuo) by ultraperformance liquid chromatography with tandem mass spectrometry (UPLC-MS/MS). 8-OxodG/8-oxoGuo are sensitive markers of cellular DNA/RNA damage from oxidation, respectively, and their detection by UPLC-MS/MS has been validated by us and others in a range of studies (Henriksen *et al.*, 2009; Barregard *et al.*, 2013; Poulsen *et al.*, 2014). We also measured the cerebral mRNA expression of a key enzyme in the DNA base excision repair pathway, oxoguanine glycosylase 1 (OGG1). Because we and others have previously found associations between hypothalamic–pituitary–adrenal (HPA)-axis activity and oxidative stress (Epel, 2009; Joergensen *et al.*, 2011; Jorgensen *et al.*, 2017), selected markers of HPA-axis activity were determined. Finally, the role of age, which we previously found to modify the association between glucocorticoid and DNA/RNA oxidative stress marker excretion (Jorgensen *et al.*, 2017), was addressed by including a group of young and middle-aged animals, respectively.

Materials and methods

Animals and housing

Forty-eight male Sprague–Dawley rats (Taconic, Denmark) were used for the study: 24 young rats (≈8 weeks of age) and 24 retired breeders (7–8 months old). Animals were group-housed (two rats per cage) in Tecniplast cages (Scanbur, Denmark), 1291H for young animals and 1500U for middle-aged animals. Cages were lined with aspen bedding and enriched with aspen nesting material, aspen bricks, and red polycarbonate rodent huts (Scanbur, Denmark). Food pellets (Altromin 1319, Brogaarden, Denmark) and acidified tap water were provided *ad libitum*. They were kept at a 12-h light–dark cycle with 30 min of twilight (lights on at 7 a.m.), temperature at 21 ± 1°C, and humidity at 52 ± 2%.

General experimental procedure

Upon arrival, the animals were left to acclimatise for 1 week. On day 8, animals were single-housed in metabolic cages for 24 h to obtain baseline food-intake and urine samples. They were then, within each age group, matched by weight into two treatment groups [ECS and sham treatments (SHAM)], yielding four experimental groups: Young SHAM animals (Y-SHAM), young ECS animals (Y-ECS), middle-aged SHAM animals (M-SHAM), and middle-aged ECS animals (M-ECS). The rats then went through a 3-week treatment period (days 12–30) receiving either ECS or SHAM. On day 34, they were placed in metabolic cages for another 24 h. After each 24-h metabolic cage housing, the 24-h food intake was recorded and the 24-h calorie intake calculated. The 24-h urine output was weighed and stored at −80°C until further analysis. Animals were weighed on treatment days and on days of metabolic cage housing.

Electroconvulsive stimulations

Animals received ECS or SHAM (no current in the apparatus) three times per week for three consecutive weeks, mimicking a clinical ECT protocol. A unidirectional square wave current (50 mA, 50 Hz, 10 ms) was administered using forceps electrodes for the ears with a stimulus duration of 0.5 s, as previously described (Hageman *et al.*, 2008; Hageman *et al.*, 2009). ECS was given between 9 a.m. and 12 a.m. and was considered successful when generalised tonic-clonic seizure was induced.

CSF sampling, euthanasia, and retrieval of brains

On day 35, animals were anesthetised with an i.p. injection of pentobarbital 200 mg/ml with lidocainhydrochloride 20 mg/ml (Glostrup Apotek, Denmark). Under anaesthesia, CSF from the rats was collected *ad modum* (Mahat *et al.*, 2012), as previously described (Jorgensen *et al.*, 2017). Briefly, after the induction of anaesthesia, animals were placed in a stereotaxic instrument with the nose tipped downwards at 75°. A 21G needle mounted on a needle holder was slowly lowered to puncture the skin at −1.5 mm in the anteroposterior plane. CSF was extracted from the cisterna magna into a silicone tube by creation of a slight vacuum with a syringe. The CSF was collected in 1.5-ml Eppendorf tubes. The silicone tube was cleaned with isotonic sodium chloride and air between each sampling. Samples were kept on dry ice and stored at −80°C until analysis. Immediately following the retrieval of CSF, the animals were decapitated; the brains were taken out and snap frozen at −40°C in isopentane. The frozen brains were wrapped in parafilm and stored in individual plastic containers at −80°C until sectioning.

In situ *hybridisation*

In situ hybridisation of brain mRNAs was performed as previously described (Hjaeresen *et al.*, 2012). Briefly, brains were cut in 15-μm coronal sections throughout the prefrontal cortex (CTX)

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(+5.46 mm to +4.20 mm relative to bregma, according to Paxinos & Watson 2nd ed.) and dorsal hippocampus (−2.40 mm to −4.20 mm relative to bregma). Sections were mounted on Superfrost-Plus® slides and hybridised with a radiolabelled oligonucleotide probe. A previously used oligonucleotide probe for the glucocorticoid receptor (GluR) (DNA Technology, Denmark) was used (Hageman *et al.*, 2009). The probe had the following sequence.

5′-CATATCCTGCATACAACACCTCGGGTTCAATCACCTCC-3′

For the present study, a new synthetic oligonucleotide OGG1 probe (DNA Technology, Denmark) was designed and tested in control tissue before the experiment. The probe had the following sequence.

5′-TGGACCAGCCAGGGCATGAAGGTTTGGGAAGCCATGATAA-3′

Competitive controls with an excess of unlabelled probe were used to test signal specificity. The optical density of the radioactive probes was analysed using the software Scion Image® (RRID: SCR_008673). Based on background signal and ^{14}C-standards, a calibration curve was determined. The densities, expressed as greyscale values in Bq/g, were measured by manually marking the areas of interest bilaterally in four sections of the hippocampus and five sections of the CTX. Areas of interest were the dentate gyrus (DG), cornu ammonis (CA) 1, CA3, and CTX. The density data were corrected for background, and the average value of the section densities for each of the areas of interest was calculated.

Quantification of urinary and CSF markers

Corticosterone (CORT), 8-oxodG, and 8-oxoGuo concentrations were determined as previously described (Jorgensen *et al.*, 2017). Urinary CORT was measured by a commercial ELISA kit (DRG, Germany, RRID: AB_2636819). The kit was developed for detection of CORT in serum and plasma, but it has been tested by us in several previous studies for the reliable use in urine (Jorgensen *et al.*, 2013c, 2017). The kit is based on a polyclonal antibody directed towards an antigenic site on the corticosterone molecule. According to the manufacturer, the kit has a mean intra- and interassay variability of 3.1% and 6.1%, respectively.

Urinary and CSF 8-oxodG and 8-oxoGuo were measured by UPLC-MS/MS. The equipment consisted of an Acquity UPLC system connected to a Xevo TQ-S triple quadrupole mass spectrometer equipped with a standard electrospray (ESI) inlet probe, all obtained from Waters (Waters, Milford, MA, USA). In both methods, stable isotope-labelled 8-oxoGuo (M+5) and 8-oxodG (M+5) were used as internal standards. The urinary content of 8-oxodG/8-oxoGuo was quantified using a modification of the previously described mass spectrometry method (Rasmussen *et al.*, 2016). The chromatographic separation was performed on an Acquity UPLC BEH Shield RP18 column (1.7 μm, 2.1 × 100 mm) from Waters. The column temperature was 4°C. The mass spectrometry detection was performed in the negative ionisation mode. The MS/MS transition for detection of 8-oxoGuo was m/z 298→208, and m/z 282→192 for 8-oxodG. The CSF samples were added internal standards filtered through pre-washed filters (VectaSpin 3 centrifuge filters, 10 K molecular weight cut-off, Whatman, Kent, UK) before injection. The chromatographic separation was performed on an Acquity UPLC HSS T3 column (1.8 μm, 2.1 mm x 100 mm) protected with an HSS T3 pre-column (1.8 μm, 2.1 mm x 5 mm) both obtained from Waters. The analytes were separated by gradient elution using 0.5% acetic acid and acetonitrile. The column temperature was 1°C. MS/MS detection was performed in the positive ionisation mode. The MS/MS transition for detection of 8-oxoGuo was m/z 300→168, and m/z 284→168 for 8-oxodG. For all the urinary markers, 24-h excretion was calculated as nmol/24 h. Although 8-oxodG was detected in some CSF samples, the concentration (<1/1000 than the concentration in urine) was below the limit of quantification (≈5 pmol/l) in most of the samples, and therefore the CSF 8-oxodG concentration is not reported.

Statistics

All data were analysed by two-way analysis of variance (ANOVA) with treatment and age as explanatory variables, followed by relevant *post hoc* comparisons of groups by independent samples t test in case of significance in the ANOVA. Normality was tested by visual inspection of the data distributions. Pearson's product-moment correlation coefficients were utilised for the correlation analyses. Two extreme CSF 8-oxoGuo values (>2.5 times above the 95 percentile of the data set) were omitted from the analyses. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS, RRID: SCR_002865) software version 24.0 (IBM Corporation, Armonk, NY, USA). Statistical significance was defined as $p < 0.05$. All statistical tests were two-sided.

Ethics

The study design was approved by the Animal Experiments Inspectorate under the Danish Ministry of Food, Agriculture and Fisheries (license number 2012-15-2934-00038). Procedures were performed in agreement with the EU directive 2010/63/EU.

Results

Two animals (one from each ECS group) were euthanised before the endpoint of the experiment due to compromised well-being, yielding a total of 11–12 animals in each experimental group. ECS caused a significant reduction of body weight gain/weight loss ($F_{(1,42)} = 27.41$, $p < 0.001$), which was most prominent in the middle-aged animals ($p < 0.001$). There was no significant effect of ECS on 24-h calorie intake (Fig. 1).

At both pre- and post-treatment, middle-aged animals had a higher 24-h urinary excretion of CORT ($F_{(1,42)} = 11.48$, $p = 0.002$, and $F_{(1,42)} = 5.20$, $p = 0.03$, respectively). There was a significant effect of ECS on the pre–post treatment difference in 24-h excretion of CORT ($F_{(1,42)} = 4.74$, $p = 0.035$), and a borderline significant interaction between age and treatment ($F_{(1,42)} = 3.86$, $p = 0.056$), reflecting a significant effect of ECS in middle-aged animals only in the *post hoc* test ($p = 0.03$). There were no significant differences between the groups with respect to 24-h 8-oxodG or 8-oxoGuo excretion at baseline. Furthermore, there were no significant group differences in the pre–post treatment difference of the 24-h excretion of 8-oxodG or 8-oxoGuo (Table 1).

With respect to the CNS markers, we found no effect of age or treatment on CSF 8-oxoGuo concentration, but a significant age × treatment interaction ($F_{(1,40)} = 4.87$, $p = 0.033$), reflecting a trend towards reduced levels after ECS in young animals and increased levels in middle-aged animals. However, this was not significant in direct *post hoc* comparisons of the groups (Fig. 2(A)). We found no significant main effects or interactions in the cerebral expression of OGG1 mRNA (Fig. 2(C)). Across all brain areas of interest, and most prominent in the DG and CA1, we found a reduction of GluR mRNA expression after ECS in young animals ($p < 0.05$) (Fig. 2(D)).

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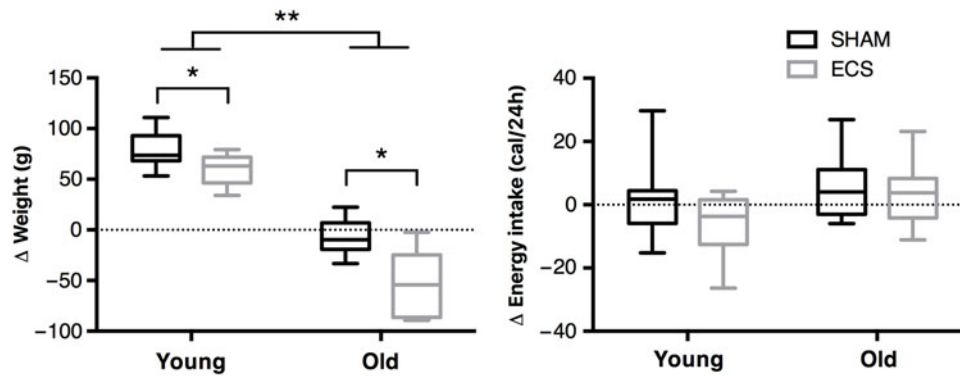

Fig. 1. Pre–post changes in body weight and calorie intake in young and middle-aged animals subjected to ECS: Data are presented as means with 5–95 percentiles and were analysed by two-way ANOVA with age and treatment as fixed factors. In case of significance in the ANOVA, relevant *post hoc* comparisons were performed by independent samples *t* test. $N = 11–12$ animals in each group. *Significant effect of treatment ($p_{young} = 0.008$, $p_{middle\text{-}aged} < 0.001$). **Significant effect of age ($p < 0.001$).

Table 1. Urinary markers of HPA-axis activity and nucleic acid damage from oxidation after ECS: 24-h urinary excretion of CORT and 8-oxodG/8-oxoGuo in young versus middle-aged rats subjected to ECS or sham treatments ($N = 11–12$ animals in each group). Values from pre- and post-treatment as well the pre–post difference are presented as means (standard deviation) and were analysed by two-way ANOVA with age and treatment as fixed factors. In case of significance in the ANOVA, relevant *post hoc* comparisons were performed by independent samples *t* test

	Young						Middle-aged					
	SHAM			ECS			SHAM			ECS		
Variable	Pre-treatment	Post-treatment	Change	Pre-treatment	Post-treatment	Change	Pre-treatment	Post-treatment	Change	Pre-treatment	Post-treatment	Change
U-CORT (nmol/24 h)	2.05 (0.64)	2.61 (0.69)	0.563 (0.682)	1.87 (0.42)	2.49 (0.54)	0.621 (0.547)	2.61 (0.78)*	2.61 (0.73)*	0.0005 (0.481)	2.68 (0.82)*	3.81 (1.63)*	1.135 (1.598)†
U-8-oxodG (nmol/24 h)	0.10 (0.02)	0.13 (0.04)	0.026 (0.021)	0.11 (0.03)	0.12 (0.02)	0.017 (0.037)	0.11 (0.02)	0.13 (0.03)	0.03 (0.03)	0.13 (0.04)	0.15 (0.06)	0.019 (0.028)
U-8-oxoGuo (nmol/24 h)	0.41 (0.08)	0.50 (0.09)	0.084 (0.051)	0.44 (0.10)	0.51 (0.08)	0.073 (0.119)	0.44 (0.06)	0.45 (0.10)	0.02 (0.09)	0.47 (0.06)	0.54 (0.10)	0.072 (0.118)

*Significant effect of age ($p < 0.05$).
†Significant effect of treatment in middle-aged animals ($p = 0.03$).

In exploratory correlation analyses, we found significant correlations between the 24-h urinary excretion of CORT and 8-oxoGuo at baseline ($r = 0.331$, $p = 0.02$), as well as between the pre–post treatment difference in the 24-h excretion of CORT and 8-oxoGuo ($r = 0.406$, $p = 0.005$). Finally, post-treatment 24-h urinary CORT excretion correlated with the CSF 8-oxoGuo concentration ($r = 0.398$, $p = 0.008$) (Fig. 3). There were no significant correlations between CORT and 8-oxodG (data not shown).

Discussion

In the present study, we investigated the effects of ECS on body weight and calorie intake, on markers related to the activity of the HPA-axis, as well as on markers of systemic and CNS nucleic acid damage from oxidation. ECS caused a significant weight loss or stalling of weight gain, in particular in middle-aged animals. This occurred in spite of a sustained 24-h calorie intake. One possible interpretation of this finding is that the effect of ECS on body weight is a consequence of a peripheral catabolic state induced by increased circulating glucocorticoids (Bodine & Furlow, 2015), rather than a centrally mediated reduction in food intake.

At baseline, middle-aged animals had a higher excretion of 24-h CORT, and this is in line with existing evidence that the overall glucocorticoid output increases with age (Nater *et al.*, 2013; Gaffey *et al.*, 2016). A proposed therapeutic mechanism of ECT is normalisation of HPA activity (Bolwig, 2011), but a recent clinical study did not find any correlations between baseline salivary cortisol, illness severity, and ECT treatment response in older, depressed patients (Suijk *et al.*, 2018). In our study, the excretion of CORT was unaltered by ECS in young animals and increased by ECS in middle-aged animals, suggesting that ECS in itself causes a biological stress response in older individuals. In young animals, ECS caused a reduction in the brain expression of GluR mRNA, and this is a replication of a previous finding (Hageman *et al.*, 2009). Because there was no increase in urinary CORT after ECS in the young animals, the reduced expression of GluR is unlikely to be a downregulation caused by negative feedback, but rather a direct effect on the brain by ECS *per se*. It should be noted that we did not include a group of animals with experimentally induced depressive symptoms, and thus we cannot conclude on what the net effect of ECS on HPA-axis markers in ‘depressed’ animals would be. We have previously found that in animals subjected to chronic restraint stress, concomitant ECS causes a normalisation of the hippocampal GluR expression and depressive behaviour (Hageman *et al.*, 2009).

We found no effects of ECS on any of the oxidative stress markers, that is, urinary 8-oxodG/8-oxoGuo excretion, CSF 8-oxoGuo concentration, or cerebral OGG1 RNA expression. Previous studies on alternative markers of oxidative stress, for example, protein

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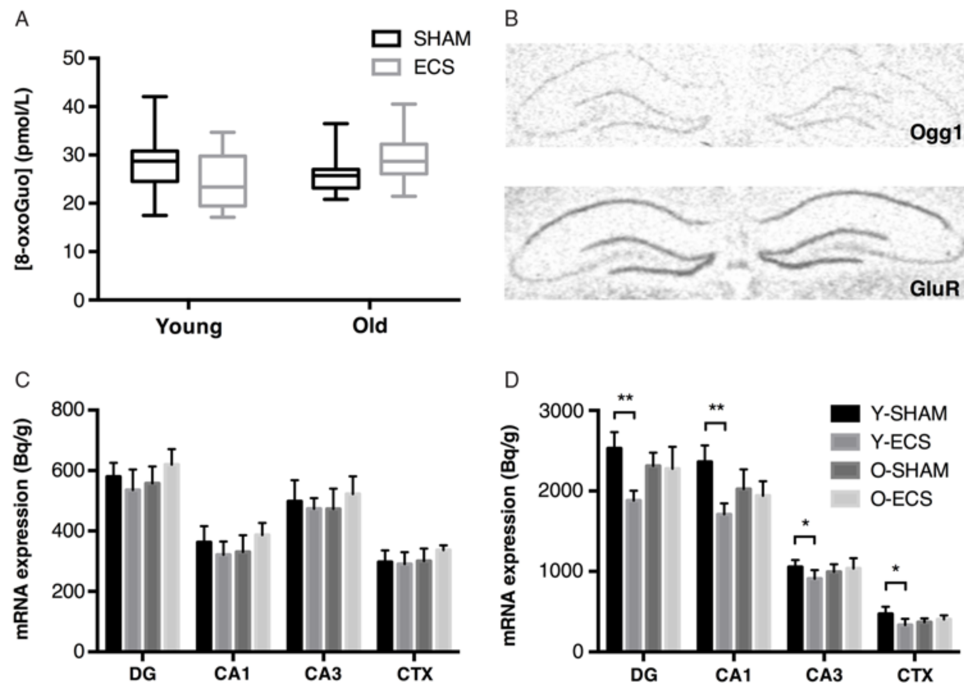

Fig. 2. CNS markers of HPA-axis activity and nucleic acid damage from oxidation in young and middle-aged animals subjected to ECS: (A) CSF concentrations of 8-oxoGuo. (B) Representative micrographs of *in situ* hybridisation of the GluR and OGG1 mRNA in the rat hippocampus (both from a young animal given sham treatments). (C and D) mRNA expression levels of OGG1 (C) and GluR (D) in the DG, CA1, and CA3 area of the hippocampus, as well as the frontal CTX. Data are presented as means with 5–95 percentiles (A) or 95% confidence intervals (C and D) and were analysed by two-way ANOVA with age and treatment as fixed factors. In case of significance in the ANOVA, relevant *post hoc* comparisons were performed by independent samples *t* test. $N = 11–12$ animals in each group. *Significant effect of treatment ($p < 0.05$). **Significant effect of treatment ($p < 0.001$).

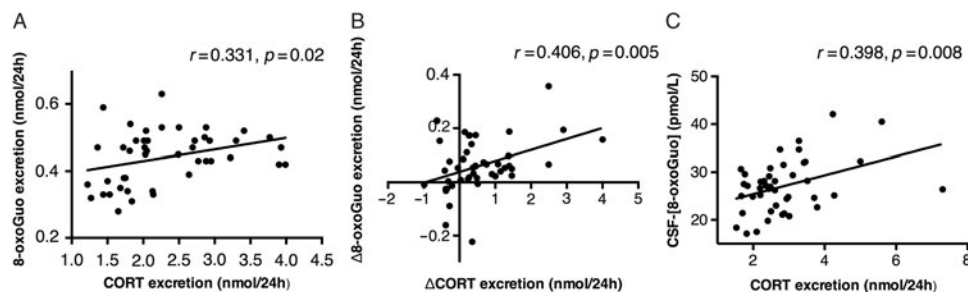

Fig. 3. Correlations between corticosterone and 8-oxoGuo in all animals: (A) Baseline urinary corticosterone (CORT) versus 8-oxoGuo excretion. (B) Pre–post treatment differences in urinary CORT versus 8-oxoGuo excretion. (C) Post-treatment urinary CORT excretion versus CSF 8-oxoGuo concentration.

oxidation and lipid peroxidation markers, as well as antioxidant enzymes, have shown conflicting results. Barichello *et al.* (2004) found a reduction in hippocampal markers of protein and lipid oxidation and an increase in antioxidant enzymes such as superoxide dismutase and catalase, both after acute and chronic ECS (Barichello *et al.*, 2004). In contrast, Feier *et al.* (2006) found increases in the same markers of oxidative stress and decreases in the antioxidant enzymes in the hippocampus and striatum after ECS (Feier *et al.*, 2006). Finally, using a protocol mimicking maintenance ECT, Jornada *et al.* measured oxidative damage parameters (thiobarbituric acid reactive species (TBARS) and protein carbonyls) in the hippocampus, CTX, cerebellum, and striatum immediately, 48 h and 7 days after the last ECS, and found no alteration in lipid peroxidation and protein damage in these four structures (Jornada *et al.*, 2007). Our study indicates that ECS does not cause any significant or lasting changes in systemic or brain DNA/RNA damage from oxidation. The major advantage of the markers used in our study compared to, for example, the TBARS and protein carbonyls assay, is that they have a much higher specificity for the measured target of oxidation (i.e. guanine residues in DNA/RNA) and a high analytical sensitivity and precision (Barregard *et al.*, 2013). The negative findings with respect to effects of ECS are unlikely to be due to insensitivity of the markers, which we have previously demonstrated to be sensitive to a range of interventions, including induced DNA damage from oxidation (Deng *et al.*, 1998; Jorgensen *et al.*, 2017).

Furthermore, we found no effect of age on any of the oxidative stress markers. This is in contrast with studies showing increased cellular levels of DNA damage with age (in tissues with limited cell proliferation) (Moller *et al.*, 2010), as well as our own previous

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finding (Jorgensen *et al.*, 2017). However, in the latter study, the old animals were 48 weeks of age, whereas the older animals in the present study were only up to 32 weeks of age. We speculate that this is the main reason why we did not find an age effect in the present study.

In exploratory analyses, we found positive correlations between CORT and 8-oxoGuo. This is a partial replication of previous findings (Joergensen *et al.*, 2011, Jorgensen *et al.*, 2013a). The reasons for this correlation between CORT and a marker of RNA damage from oxidation are not known. *In vitro* studies have indicated that CORT may induce oxidative stress (Flint *et al.*, 2007). However, in a previous study in which we induced a physiological increase in circulating CORT, we found a *reduction* in urinary nucleic acid oxidation markers (Jorgensen *et al.*, 2017), and hence the association does not appear to be a direct or causal one.

Some limitations of the study should be mentioned. We did not include measurements immediately after ECS, and thus we cannot rule out that the seizures could cause a transient effect on the parameters investigated. Furthermore, we did not include any direct measure of neuronal 8-oxodG or 8-oxoGuo, and thus we potentially could have overlooked a small effect of locally increased oxidative stress in the brain that did not manifest itself in the systemic or CSF markers or affect OGG1 expression. Because we aimed to study the biochemical effects of ECS rather than those of depression, groups of animals with experimentally induced depressive behaviour were not included. Female animals were not included and the results can not necessarily be extrapolated to females.

Finally, the study did not include any behavioural measurement to validate the ECS effects. However, ECS treatments did induce weight loss, age-dependent CORT changes, and decreased cerebral GluR expression, strongly suggesting that the treatments were effective.

In conclusion, we found that ECS caused a stalling of weight gain and age-dependent changes in HPA-axis activity, while largely leaving sensitive and validated markers of systemic and CNS DNA/RNA damage from oxidation unaltered. These results suggest that ECS is not associated with any lasting effects on oxidative stress on nucleic acids.

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Author contributions. All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: AJ, MBJ, and GW. Acquisition of data: AJ, KB, OK, AW, TH, HEP, and GW. Analysis and interpretation of data: AJ, KB, MBJ, and GW. Drafting of the manuscript: AJ, MBJ, and GW. Critical revision of the manuscript for important intellectual content: All authors. Statistical analysis: AJ and GW. Obtained funding: MBJ and GW. Administrative, technical, and material support: OK, AW, TH, HEP, MBJ, and GW. Study supervision: MBJ and GW.

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Conflict of interest. None of the authors have any financial or other conflicts of interest.

Animal welfare. The study design was approved by the Animal Experiments Inspectorate under the Danish Ministry of Food, Agriculture and Fisheries (license number 2012-15-2934-00038). Procedures were performed in agreement with the EU directive 2010/63/EU.

Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals.

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