



A chronic increase of corticosterone age-dependently reduces systemic DNA damage from oxidation in rats



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ABSTRACT

Stress and depression are associated with an acceleration of brain and bodily aging; effects which have been attributed to chronic elevations of glucocorticoids. We tested the hypothesis that a three week administration of stress-associated levels of corticosterone (CORT, the principal rodent glucocorticoid) would increase systemic and CNS DNA and RNA damage from oxidation; a phenomenon known to be centrally involved in the aging process. We also hypothesized that older individuals would be more sensitive to this effect and that the chronic CORT administration would exacerbate age-related memory decline. Young and old male Sprague-Dawley rats were non-invasively administered CORT by voluntary ingestion of nut paste containing either CORT (25 mg/kg) or vehicle for a total of 22 days. CORT increased the 24 h urinary excretion of the hormone to the levels previously observed after experimental psychological stress and caused a downregulation of the glucocorticoid receptor in the CA1 area of the hippocampus. Contrary to our hypothesis, 24 h excretion of 8-oxodG/8-oxoGuo (markers of DNA/RNA damage from oxidation) was reduced in CORT-treated young animals, whereas old animals showed no significant differences. In old animals, CORT caused a borderline significant reduction of RNA oxidation in CNS, which was paralleled by a normalization of performance in an object location memory test. To our knowledge, this is the first demonstration that chronic stress-associated levels of CORT can reduce nucleic acid damage from oxidation. These findings contradict the notion of elevated CORT as a mediator of the accelerated aging observed in stress and depression.

1. Introduction

An accumulating body of evidence suggests that prolonged psychological stress and stress-associated mental illnesses such as depression accelerate various aspects of aging. Both stress and depression are associated with an increased occurrence of age-related medical conditions, such as the metabolic syndrome, type 2 diabetes and cardiovascular disease [1–3]. Furthermore, stress and depression have been linked to accelerated cognitive decline [4], an increased risk of

dementia [5,6], and age-related changes in the brain, including hippocampal atrophy and neuronal dendritic retractions [7,8]. Consistent with these observations, both perceived psychological stress, as well as suffering from depression or other mental disorders, have been shown to be associated with increased non-suicide mortality [9,10]. Biochemically, prolonged stress and depression have been associated with increased oxidative stress and telomere attrition; key mechanisms in the cellular aging process [11–13]. However, the biological mechanisms that mediate the connections between psycho-

List of abbreviations: CORT, Corticosterone; CNS, Central Nervous System; DNA, Deoxyribonucleic Acid; RNA, Ribonucleic Acid; HPA-axis, Hypothalamic-Pituitary-Adrenal axis; ROS, Reactive Oxygen Species; 8-oxodG, 8-oxo-7,8-dihydro-2-deoxyguanosine; 8-oxoGuo, 8-oxo-7,8-dihydroguanosine; Y-VHC, Young vehicle-treated rats; Y-CORT, Young CORT-treated rats; O-VHC, Old vehicle-treated rats; O-CORT, Old CORT-treated rats; CSF, Cerebrospinal Fluid; FCM, Fecal Corticosterone Metabolites; GluR, Glucocorticoid Receptor; CA1, Cornu Ammonis of the hippocampus, area 1; CA3, Cornu Ammonis of the hippocampus, area 3; ELISA, Enzyme-linked Immunosorbent Assay; UPLC-MS/MS, Ultra-Performance Liquid Chromatography with Tandem Mass Spectrometry; ANOVA, Analysis of Variance

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logical states and accelerated aging remain largely unknown.

The hypothalamic-pituitary-adrenal (HPA) axis is activated upon psychological stress exposure, resulting in the release of glucocorticoids from the adrenal glands. Prolonged increase and/or dysregulation of glucocorticoid secretion are key components in the concept of allostatic load, in which prolonged stress leads to brain and bodily damage through metabolic dysregulation [14]. This has been suggested to be a central mediator of the age-advancing effects of stress and mental disorders [15,16]. Therefore, a critical question is to what extent circulating glucocorticoids at stress-associated physiological levels influence molecular aging mechanisms.

Oxidatively generated damage to DNA is an important mechanism in the aging process *per se*, as well as a putative early pathogenic event in age-related medical conditions such as the metabolic syndrome, type-2 diabetes, and dementia [17–19]. Reactive oxygen species (ROS) induced damage to DNA increases with age [20], and the inability to repair DNA damage from oxidation leads to a progeric phenotype in both animals and humans [19]. Furthermore, oxidative stress on telomeric DNA is an important negative regulator of telomere length [21]. Recently, an increased attention to the effects of RNA oxidation has emerged, with studies indicating that the oxidation of non-coding RNAs and mRNA negatively influences gene regulation and protein synthesis/folding [22,23], respectively; observations which could bear relevance to the pathogenesis of a range of medical disorders [24].

The main cellular source of ROS is the mitochondria, and mitochondrial dysfunctions may lead to increased ROS leakage [25]. High physiological levels of glucocorticoids have been shown to negatively influence a range of mitochondrial functions, e.g. levels of functional mitochondria and cytochrome *c* oxidase in muscle cells [26,27] and both *in vivo* and *in vitro* experiments have indicated that exposure to glucocorticoids increases oxidation of mitochondrial DNA in the liver [28] and causes DNA strand breaks [29].

In an aged human population, we previously observed a positive association between markers of oxidatively generated DNA and RNA damage (8-oxo-7,8-dihydro-2-deoxyguanosine (8-oxodG) and 8-oxo-7,8-dihydroguanosine (8-oxoGuo), respectively), and the urinary excretion of cortisol, the principal human glucocorticoid [15]. The purpose of the present study was to determine the effect of an experimental physiological increase in glucocorticoid exposure on markers of nucleic acid damage and on hippocampus-dependent memory in rats, and to study the influence of age on these effects. We hypothesized that elevated circulating levels of CORT would lead to greater oxidative damage of DNA/RNA, evident as increases in both systemic and CNS concentrations of 8-oxodG/8-oxoGuo, and attenuated performance in a spatial memory test. Furthermore, we hypothesized that these effects would be more pronounced in aged rats than in their younger conspecifics.

2. Material and methods

2.1. Animals and housing conditions

24 young (6 weeks old) and 24 old (46 weeks old; retired breeders) male Sprague-Dawley rats (Taconic, Ry, Denmark) were housed singly in individually ventilated standard cages (Tecniplast, Varese, Italy), and left to acclimatize for two weeks. They were provided pelleted feed (Altromin 1319; Brogaarden, Gentofte, Denmark) and acidified tap water *ad libitum*. Cages were lined with aspen chips (Tapvei Oy, Kortteinen, Finland) and enriched with nest building material (Lilico, Horley, UK), bite blocks (Tapvei Oy), plastic shelters (Lilico) and cardboard tubes (Lilico). Cage temperature was maintained at 22 ± 2 °C and 55% humidity with 63 h^{-1} airchanges. The light regimen was a 12/12 h dark/artificial light cycle with 30 min of “twilight” and the lighting period starting at 7:00 AM.

2.2. General experimental procedure

At the start of the study, the animals were randomized into four experimental groups: Young vehicle-treated rats (Y-VHC), young CORT-treated rats (Y-CORT), old vehicle-treated rats (O-VHC), and old CORT-treated rats (O-CORT) ($n=12$ in each group). The animals were CORT treated for 22 days (days 1–22). On day 21, the animals were habituated to the behavioral testing apparatus and then returned to their home cages. On day 22, the animals were tested for their spatial memory, and, immediately following the behavioral test, transferred to metabolism cages for collection of urine and feces. On day 23, after 24 h of metabolism cage housing, cerebrospinal fluid (CSF) was collected, and the animals were euthanized while still in anesthesia.

2.3. Corticosterone dose titration

To match the 24 h CORT excretion to the levels previously observed in rats subjected to severe psychological stress [30], a dose titration study was conducted. Six animals (body weight 300–350 g) were administered corticosterone by voluntary ingestion of nut paste (Nutella[®]; Ferrero, Pino Torinese, Italy) dosed with corticosterone (Sigma-Aldrich, St. Louis, USA) dissolved in dimethyl sulfoxide (DMSO). The nut paste administration method has previously been validated for buprenorphine administration [31]. The animals were administered 0, 2, 4, 6, 8 or 10 mg/day for five consecutive days (one animal per dose), divided into two doses per day at 8 AM and 4 PM, respectively. Subsequently, 24 h urine and fecal samples were collected by placing the animals in metabolism cages, and the samples were assayed for CORT/corticosterone metabolite excretion (see below). We found that a dose of 10 mg/day, corresponding to a weight-adjusted dose of 25 mg/kg/day, resulted in 24 h urinary CORT and fecal corticosterone metabolites (FCM) levels observed in a previous study of rats subjected to severe psychological stress [30]. Accordingly, this dose was used in the subsequent experiments.

2.4. Corticosterone treatment

The animals were dosed for the entirety of the study, including during the metabolism cage housing on day 22. All animals were weighed daily. CORT (25 mg/kg/day) was administered as two voluntary ingestions – at 8 AM and 4 PM – of nut paste containing corticosterone (12.5 mg/kg) dissolved in sesame oil. The control groups received only nut paste with sesame oil. The amount of vehicle used per dose was 1 g of nut paste and 0.625 g/kg body weight of sesame oil. Throughout the experiment, the dose of CORT was continuously adjusted individually to the animal's weight. To avoid recording acute effects of the last dose, behavioral tests began at a minimum of 3 h after the 8 AM CORT administration.

2.5. Behavioral test

The animals were tested for spatial memory performance by an object location test [32], as previously described [33]. The test was chosen because of the dependence of spatial memory on hippocampal function [34], and because it does not involve training or reinforcement, which could act as a stressor in itself [32]. An open arena consisting of a black box measuring 77 (l) × 56 (w) × 41 cm (h) was used, allowing the animal spatial orientation using cues in the environment outside of the arena. The arena was constantly and evenly illuminated. Behavior was recorded by a video camera mounted vertically above the test arena and analyzed using the video tracking program EthoVision[®] (Noldus Information Technology, Wageningen, The Netherlands). In all experiments the computer automatically initiated registration of the rat's position (in four animals, the tracking was not successful). During testing, the room was sealed to minimize disturbing noise. Two arenas were used simultaneously. The arena was

wiped down with water between tests to remove smell and excrements from the previous animal. On day 21, animals were habituated to the arena by placing them in the empty arena for 5 min. On day 22, two identical objects (tin cans sufficiently heavy to not be displaced by the animals) were placed in separate corners of the arena, and again, the animals were allowed to freely explore the arena for 5 min (T1). The animals were taken out of the arena for one hour, during which time the T1 objects were replaced by two new, but identical, objects, one of which was placed in a new corner (new objects were used to avoid olfactory traces attached to the T1 objects). After one hour, animals were again allowed to freely explore the arena (T2). Total exploration time (s) of the two objects at T1 and T2, respectively, was measured. The discrimination between the object in the familiar position (A1) vs. the object in the new position (A2) was calculated as: Discrimination index = (Time spent at A2 (s) – time spent at A1 (s)) / total exploration time (A1 + A2) (s) [32].

2.6. Urine, feces, CSF and tissue sampling

Immediately following the behavioral test, animals were housed for 24 h in metabolism cages as previously described [30]. The method allows for a reliable and complete collection of 24 h urine and fecal production. Upon collection, on day 23, all samples were weighed and stored at -80°C until analyses. The 24 h food intake was recorded, and total energy intake, taking into account nut paste and sesame oil, was calculated.

Immediately after the 24 h metabolism cage housing, animals were anaesthetized with a 7 mL/kg s.c. injection of a mixture of Fentanyl (0.02 mg/mL), Droperidol (1.3 mg/mL), and Midazolam (0.13 mg/mL). CSF was isolated as described by Mahat et al. [35]. Briefly, the fur above the cisterna magna was removed and the animal was placed in a stereotaxic frame. The head was fixed with ear bars. A 21 G needle, attached to a silicone tube and a 1 mL syringe, was slowly inserted into the cisterna magna at -1.5 mm from stereotaxic zero in the anteroposterior plane. Mild negative pressure was maintained with the syringe until CSF was seen in the tube. CSF was drawn until the fluid became unclear, at which time the sampling was immediately terminated to minimize the contamination of the CSF with blood. Approximately 100–150 μL of CSF was obtained from each animal. The CSF samples were snap frozen on dry ice and subsequently stored at -80°C until analyses. In four animals, randomly distributed across groups, CSF was not successfully obtained. Immediately after the CSF extraction, animals were decapitated and the brains were removed, snap frozen on dry ice and stored at -80°C until analyses.

2.7. In situ hybridization of glucocorticoid receptor messenger RNA

In situ hybridization of Glucocorticoid Receptor (GluR) mRNA was performed as previously described [36]. Briefly, 15 μm coronal sections were cut throughout the hippocampus at -2.40 mm to -4.20 mm relative to bregma. The sections were thaw-mounted onto Superfrost Plus slides and stored at -80°C . Sections were fixed in 4% paraformaldehyde and stored in 96% ethanol until further use. A synthetic oligonucleotide probe for GluR mRNA (Gen-Bank Accession no. Y12264, probe sequence: CATATCCTGCATACAACACCTCGGGTTCAATCACCTCC) (DNA Technology, Risskov, Denmark) labelled with [$\alpha^{35}\text{S}$]dATP (Perkin Elmer) at the 3' end using terminal deoxynucleotidyl transferase (Roche Diagnostics) was used for hybridization. Slides were incubated at 42°C overnight in hybridization mixture containing labelled oligonucleotide, DTT and hybridization buffer (50% formamide, 4x saline sodium citrate (SSC) (0.15 M NaCl and 0.015 M $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$, pH 7) and 10% dextran sulphate) in ratio 1:0.75:75. Controls with unlabelled "cold" probe were used to test the specificity of the signal. Slides were washed in 1x SSC for 30 min at 60°C , rinsed at room temperature in 1x SSC, 0.1x SSC, 70% ethanol, and finally 95% ethanol. The slides were air-dried for x h before exposure to Kodak Biomax MR autoradiography films (Sigma-Aldrich).

The slides were kept in hypercassettes at 5°C for the entire exposure (14 days). The density of the bound radioactive probes (expressed as Bq/g) was estimated using image analysis software (Scion Image, <http://scion-image.software.informer.com/4.0/>). Based on the background exposure of the films and ^{14}C -standards (Amersham) a calibration curve was constructed. The densities, expressed as greyscale values, were measured for the areas Cornu Ammonis 1 (CA1), CA3 and dentate gyrus, bilaterally in the hippocampus on each slide. Mean values for the two hemispheres were calculated for each area of interest using four consecutive sections. One brain had very low densities ($< 50\%$ of the sample average) in the investigated brain areas, and was deemed failed and excluded from analyses.

2.8. Urine corticosterone and fecal corticosterone metabolite quantification

The total 24 h urinary CORT and FCM excretions were quantified as described previously [30,37]. Briefly, fecal samples were extracted in 96% (v/v) ethanol (5 mL/g solid matter) overnight. The extracted medium was then evaporated to dryness under vacuum at room temperature (Genevac EZ-2 personal evaporator; Genevac Ltd., Stone Ridge, USA) and resuspended in PBS. Precipitating material was removed by centrifugation and the clear liquid was analyzed together with urine samples for CORT/FCM using a commercial ELISA kit (EIA-4164; DRG Diagnostics GmbH, Marburg, Germany). The kit has been verified to have a crossreactivity equivalent to 7.4% with progesterone, 3.4% with deoxycorticosterone, 1.6% with 11-dehydrocorticosterone, 0.3% with cortisol and pregnenolone, and less than 0.1% with other steroids.

2.9. Urine and CSF 8-oxodG/8-oxoGuo determination by UPLC-mass spectrometry

The oxidatively modified guanine nucleosides were quantified using ultra-performance liquid chromatography/tandem mass spectrometry (UPLC-MS/MS). The chromatographic methods for the detection of oxidized nucleosides are superior to ELISA-based methods in both sensitivity, specificity and precision and have been optimized and applied by our group in several previous studies [13,15,18,38,39]. The equipment consists 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 8oxoGuo (M+5) and 8oxodG (M+5) were used as internal standards. The urinary content of the oxidatively modified guanine nucleosides was quantified using a modification of the previously described ultra-performance liquid chromatography/tandem mass spectrometry method [40]. Briefly, 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 ionization mode. The MS/MS transition for detection of 8oxoGuo was m/z 298 \rightarrow 208, and m/z 282 \rightarrow 192 for 8oxodG.

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 , 2.1×100 mm) protected with a HSS T3 pre-column (1.8 μm , 2.1×5 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 ionization mode. The MS/MS transition for detection of 8oxoGuo was m/z 300 \rightarrow 168, and m/z 284 \rightarrow 168 for 8oxodG. 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.

2.10. Ethics

The animal experiments were approved by the Animal Experiments Inspectorate under the Danish Ministry of Food, Agriculture and Fisheries (license number 2012-15-2934-00038). All procedures were performed in accordance with the EU directive 2010/63/EU in a fully AAALAC accredited facility under the supervision of a local animal welfare committee. All efforts were made to minimize pain or discomfort as well as the number of animals used during the experiment.

2.11. Statistics

Hypothesis testing was carried out using analysis of variance (ANOVA) with treatment and age offered as explanatory variables. For longitudinal data on body weights a repeated measures design was used. Urinary CORT, FCM, urinary 8-oxodG/8-oxoGuo and CSF 8-oxoGuo data were log-transformed before variance analyses in order to make them conform to a normal distribution (verified using QQ plots and the Shapiro-Wilk test). Where relevant, and if significant effects were found in the ANOVA, post-hoc comparisons of individual groups were performed by independent samples *t*-tests. For exploratory correlation analyses Spearman's rank correlations were constructed between urinary CORT data and oxidized DNA/RNA markers. Pooled non-transformed data from young and old animals in the VHC and CORT groups, respectively, were used. Robustness of the findings was subsequently verified using linear regression models with corrections for age (regression residuals were confirmed to approximate a normal distribution). All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) software version 20.0 (IBM Corporation, Armonk, NY, USA). Statistical significance was defined as $p < 0.05$. All statistical tests were two sided.

3. Results

3.1. Urinary and fecal excretion of corticosterone

Results from the dose titration study are summarized in Fig. 1A. For the main study, with a few randomly distributed exceptions, we observed that the animals reliably ingested all doses of nut paste throughout the treatment period. There was a significant effect of CORT treatment on the urinary CORT ($F_{(1,44)}=9.74$, $p=0.003$) and FCM ($F_{(1,44)}=99.33$, $p < 0.001$) on day 22 (Figs. 1B and 1C). We also found a significant effect of age on FCM ($F_{(1,44)}=7.93$, $p=0.007$), with young animals excreting more than their older counterparts. Older rats had a significantly greater variability in their urinary CORT excretion than did the young (Levene's test: $F_{(3,44)}=4.07$, $p=0.012$).

3.2. Body weight and energy intake

In a repeated measures ANOVA we found a significant effect of the CORT treatment on body weight ($F_{(1,44)}=8.73$, $p=0.005$), with significantly lower body weights from day eight in the Y-CORT vs. the Y-VHC group and from day eleven in the O-CORT vs. the O-VHC group ($p < 0.05$) (Fig. 2A). Accordingly, there was a significant effect of treatment and age on the total weight change during the CORT administration period (age \times treatment interaction: $F_{(1,44)}=6.51$, $p < 0.05$), where both CORT-treated groups lost weight, O-VHC retained their weight, and Y-VHC gained weight (Fig. 2B). This was paralleled by a significant effect of treatment on the total 24 h energy intake, as measured during metabolism cage housing on day 22 ($F_{(1,44)}=10.07$, $p < 0.05$) (Fig. 2C), with a significantly reduced energy intake in the Y-CORT vs. the Y-VHC group ($p=0.004$), and a similar trend in old animals ($p=0.095$) (Fig. 2C). When factoring in the weights of the individual animals, however, only a significant effect of age was evident ($F_{(1,44)}=156.4$, $p < 0.001$), with the old animals consuming less feed in relation to their weights.

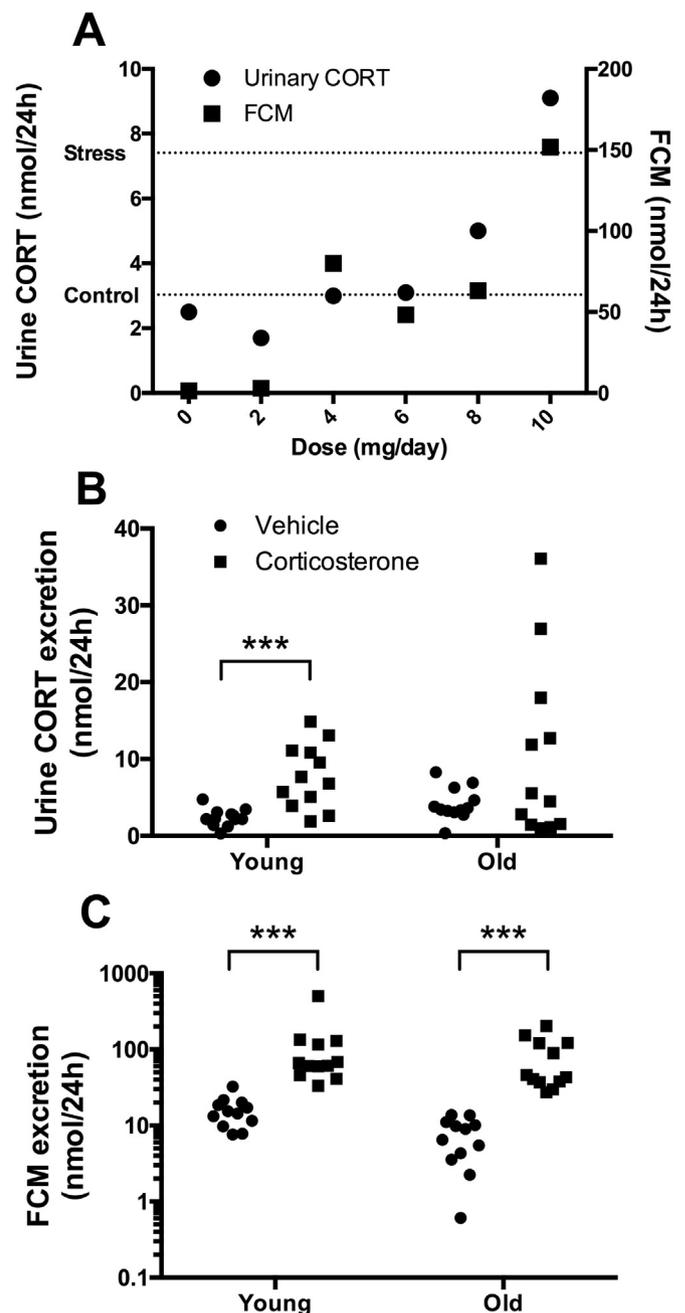


Fig. 1. Validation of the corticosterone administration paradigm. **A:** Dose titration study. The observations for each dose indicate the 24 h fecal corticosterone (FCM, squares) and urinary corticosterone (CORT, circles) excretion for one animal treated with that dose for 5 days. The horizontal lines indicate the mean levels of 24 h urinary CORT excretion after experimental stress, as observed in a previous study [30]. The dose that yields the stress-physiological level is 10 mg/day, corresponding to 25 mg/kg animal weight/day. This dose was used in the subsequent experiments **B:** 24 h urinary excretion of CORT after 3 weeks of CORT administration (25 mg/kg/day) in young and old animals. **C:** 24 h FCM excretion after 3 weeks of CORT administration (25 mg/kg/day) in young and old animals. Data are presented as individual data points and analyzed (B and C) with one-way analysis of variance, with treatment and age as fixed factors, after log-transformation. Post-hoc comparisons of groups were done by independent samples *t*-test. *** $p < 0.001$.

3.3. Expression of hippocampal glucocorticoid receptor mRNA

In the CA1 area, we found a significant effect of age ($F_{(1,43)}=5,039$, $p=0.03$) and treatment ($F_{(1,43)}=4,503$, $p=0.04$), reflecting reductions in GluR mRNA in CORT treated animals (Fig. 3B). This effect was most pronounced in old animals (though only borderline significant in the

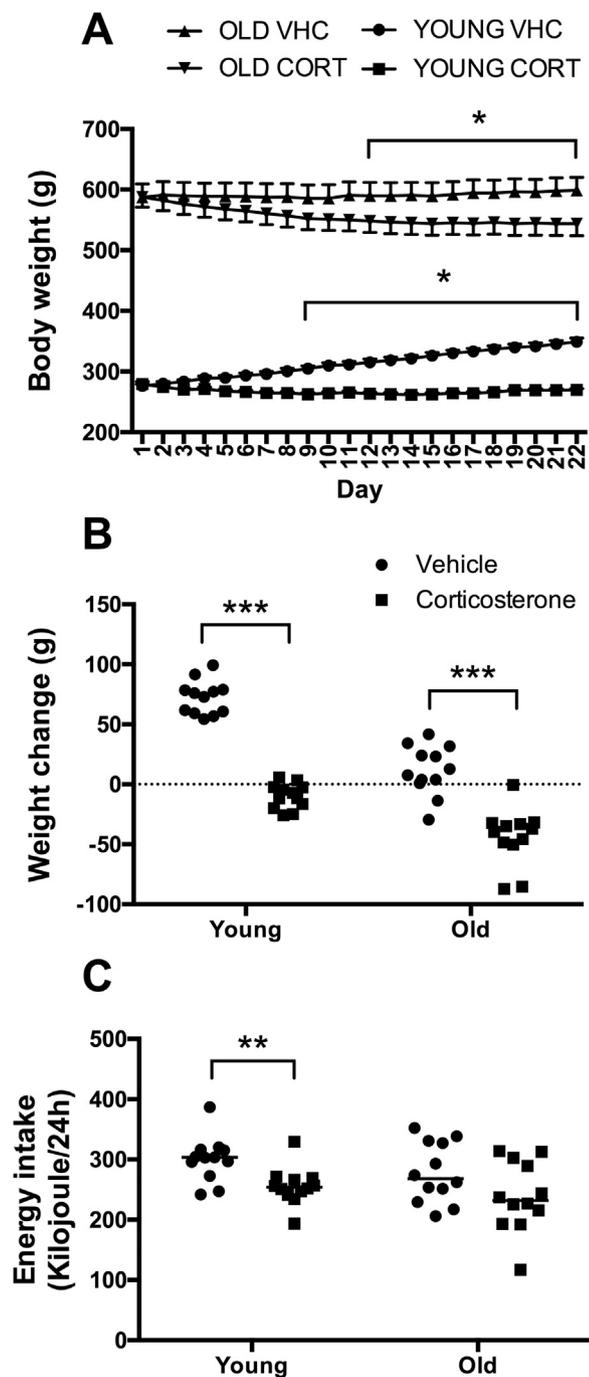


Fig. 2. Effects of corticosterone treatment on body weight and food intake. **A:** Day 1–22 body weights of young and old animals treated with vehicle (VHC) or corticosterone (CORT, 25 mg/kg/day), respectively. **B:** Body weight change from day 1–22 in young and old animals treated with vehicle or CORT, respectively. **C:** Energy intake on day 22 of the study. Data are presented as individual data points and were analyzed with analysis of variance (ANOVA) or repeated measures ANOVA with treatment and age as fixed factors. Post-hoc comparisons of groups were done by independent samples *t*-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

post hoc comparison ($p = 0.07$)). A similar but nonsignificant pattern was observed in the other hippocampal areas of interest (Fig. 3A and C).

3.4. Spatial memory

In the object location test, there were no significant differences in the total exploration time at T1 and T2 trials, demonstrating that CORT

did not influence overall mobility, exploratory behavior or other domains which might bias the results of the spatial memory test (Fig. 4A + B). The discrimination index was significantly influenced by age ($F_{(1,40)} = 5.97$, $p = 0.019$) and treatment ($F_{(1,40)} = 4.06$, $p = 0.05$). As expected, young animals (Y-VHC + Y-CORT) showed a significant preference for the moved object (deviation from zero in one-sample *t*-test, $p = 0.01$). Compared to Y-VHC, O-VHC animals showed a reduced preference for the moved object ($p = 0.02$), with a trend towards a preference for the familiar object (deviation from zero in one-sample *t*-test, $p = 0.07$). This effect was reversed by CORT towards the behavior of the young animals ($p = 0.02$) (Fig. 4C).

3.5. Markers of oxidatively generated DNA/RNA damage in urine and CSF

24 h urinary excretion of 8-oxodG was significantly higher in old animals ($F_{(1,44)} = 47.70$, $p < 0.001$), and significantly decreased by the CORT treatment ($F_{(1,44)} = 18.31$, $p < 0.001$). There was a significant interaction between age and treatment ($F_{(1,44)} = 4.15$, $p < 0.05$), with post hoc comparisons showing significantly lower levels in Y-CORT vs. Y-VHC animals ($p < 0.001$) and no differences in old animals (Fig. 5A). The 8-oxoGuo excretion showed a significant effect of age ($F_{(1,44)} = 19.59$, $p < 0.001$), but not CORT treatment ($F_{(1,44)} = 2.26$, $p = 0.12$), although there was a similar trend for reduced levels in Y-CORT animals (Fig. 5B). There was a tendency of CORT treatment also decreasing CSF 8-oxoGuo concentrations, particularly in old animals; however, the effect was not statistically significant ($F_{(1,40)} = 3.37$, $p = 0.074$) (Fig. 5C). We found no significant correlations between urinary and CSF 8-oxoGuo, neither when analyzing individual groups or the population as a whole (results not presented). The concentrations of 8-oxodG in CSF were too low for reliable detection and are not reported.

To further characterize the relationship between CORT treatment and oxidatively generated DNA/RNA damage, we performed an exploratory correlation analysis of the 24 h urinary corticosterone excretion levels vs. 8-oxodG/8-oxoGuo excretion in urine and 8-oxoGuo concentration in CSF. To conserve power, data from young and old animals were pooled in the CORT vs. the VHC group, respectively. In the VHC group, we found a highly significant positive correlation between the 24 h urinary excretion of CORT and both the 8-oxodG (Spearman's $\rho = 0.52$, $p = 0.009$, $n = 24$) and the 8-oxoGuo marker (Spearman's $\rho = 0.56$, $p = 0.005$, $n = 24$) (Fig. 6A + B). Both of these findings persisted after adjustment for age group in a linear regression model (8-oxodG: $\beta = 0.59$, $t = 4.45$, $p < 0.001$. 8-oxoGuo: $\beta = 0.46$, $t = 2.54$, $p = 0.019$). In the CORT treated animals, the 24 h urinary excretion of CORT and 8-oxodG/8-oxoGuo were not correlated (Fig. 6C + D). We found no correlations between the 24 h urinary excretion of CORT and CSF 8-oxoGuo in either treatment group (results not presented).

4. Discussion

In this study, we found that stress-associated levels of systemic CORT, administered non-invasively for three weeks, significantly reduced systemic oxidatively generated damage to DNA, as measured by the 24 h urinary excretion of 8-oxodG. There was a trend towards reduced systemic oxidatively generated damage to RNA, as measured by 8-oxoGuo excretion. These effects were age-dependent, with young animals being more sensitive than old animals. CORT caused a borderline significant reduction in CSF 8-oxoGuo concentration, which was most prominent in old animals. This effect was paralleled by a normalization of performance in an object location memory test in old animals towards the performance of their younger counterparts.

4.1. Validity of the CORT-treatment paradigm

The non-invasive administration of the selected CORT dose by voluntary ingestion yielded a urinary CORT excretion comparable to

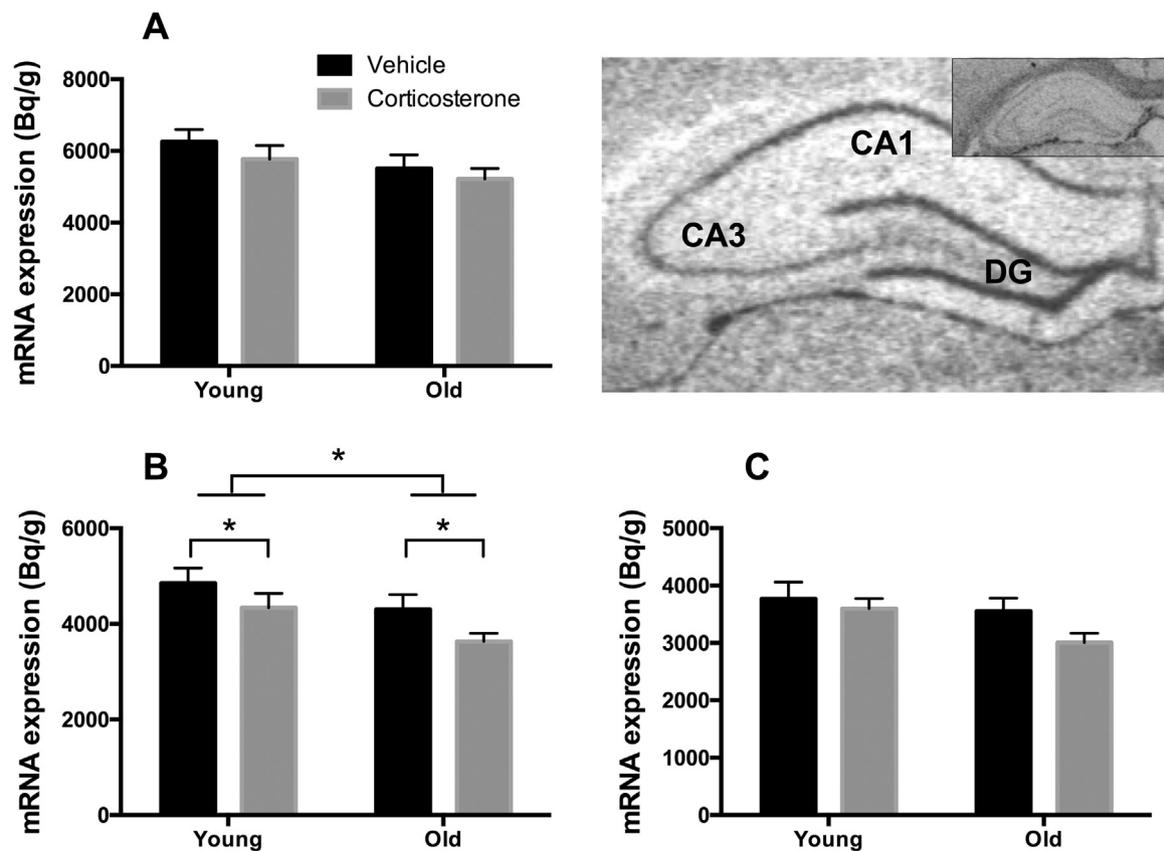


Fig. 3. In situ hybridization of the glucocorticoid receptor in hippocampus. Data show the GluR mRNA expression levels (as measured by Bq/g) in the dentate gyrus (A), CA1 (B) and CA3 (C) areas. In CA1, there is a significant effect of age and treatment. A representative micrograph of the investigated hippocampal areas is depicted (top right panel). Insert: Unlabelled probe competitive control, showing minimal hippocampal binding. Data are presented as individual data points and were analyzed with analysis of variance with treatment and age as fixed factors. * Significant effect of age group and CORT, respectively ($p < 0.05$).

that previously observed in rats subjected to severe psychological stress [30]. Using this methodology and dosing strategy for chronic administration, we have demonstrated a significant increase in FCM excretion in the CORT-treated vs. the vehicle group, showing that the animals have reliably ingested the CORT-containing nut paste. Correspondingly, we found an increase in the 24 h urinary excretion of CORT in the young CORT-treated animals comparable to the level observed in animals subjected to severe psychological stress [30]. In the old animals, while the FCM was clearly increased by CORT-administration, there was a substantial overlap in the 24 h CORT excretion between the CORT vs. the vehicle-treated animals on day 22, suggesting a larger variation in the metabolism of CORT in old animals. Considering the fact that the groups exhibited clear differences with respect to weight change and behavior, it is likely that the CORT-treated old animals have in fact sufficiently absorbed CORT during the three-week administration period. In support of this, GluR mRNA levels in the CA1 area of the hippocampus was reduced by the intervention, likely mediated by negative feedback inhibition of the receptor expression by CORT.

4.2. Effects of CORT on systemic levels of DNA/RNA damage from oxidation

Using this paradigm for CORT administration and a highly sensitive and precise method for mass spectrometric detection of oxidized nucleosides, we found that young rats treated with CORT had a reduced level of systemic oxidatively generated DNA damage (and borderline significantly reduced RNA damage) compared to VHC treated animals, as measured by urinary 8-oxodG/8-oxoGuo excretion, respectively. The reduction in 8-oxodG excretion was substantial ($\approx 45\%$). Old animals

had higher excretion levels of both markers, and this is in line with evidence that the levels of DNA damage from oxidation increases in various tissues with age [20], but could also be related to the larger body weight of the old animals. Surprisingly, old animals showed no significant changes with CORT treatment. These findings are in contrast to our hypothesis, in which we predicted that CORT would increase 8-oxodG/8-oxoGuo excretion, particularly in old animals. This prediction was based on 1) the evidence that human stress and depression are associated with increased systemic oxidative stress and telomere attrition [11–13] (however, for a recent negative report see also [41]); 2) our previous study in aged humans, in which we found a strong positive correlation between the 24 h excretion of cortisol and the 8-oxodG/8-oxoGuo markers (even after adjusting for other potential sources of oxidative stress such as obesity, diabetes, inflammation and smoking) [15], and 3) an experimental study of young rats subjected to chronic restraint stress, in which we did not identify any changes in systemic or brain levels of oxidatively generated nucleic acid damage, in spite of a substantial and sustained increase in urinary glucocorticoid levels [30]. Hence, our present findings challenge the hypothesis that psychological stress exerts its effects on cellular and organismal aging directly through CORT-induced oxidative stress on nucleic acids.

The reasons for these discrepancies are unclear. It could be speculated that the positive correlation between cortisol, stress, and markers of cellular aging observed in humans [11,12,15,16] are mediated by intermediate metabolic factors, which are also associated with stress and HPA-axis activity (e.g. obesity, inflammation, medication use, other stress hormones etc.), rather than cortisol *per se*. Although attempts are usually made to correct for such factors in human studies by statistical methods, it is difficult to completely rule

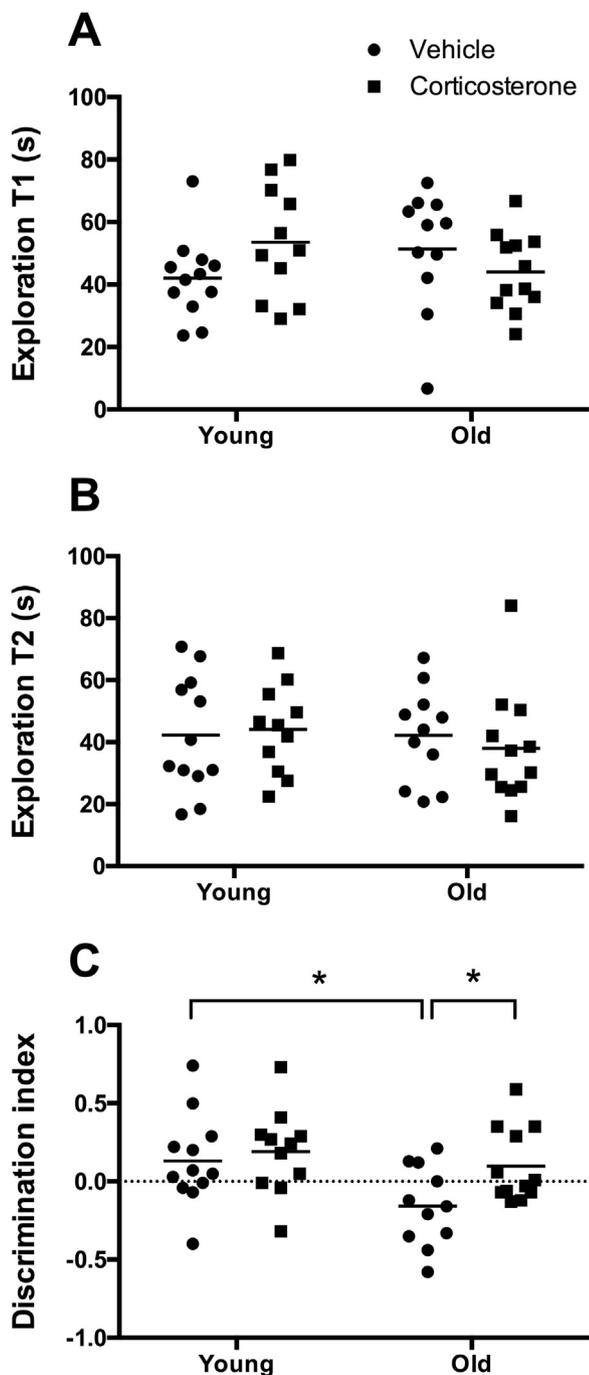


Fig. 4. Effects of corticosterone treatment on performance in the object location test. A + B: Time spent at the two objects at T1 (before one object was moved) and T2 (after one of the objects was moved) by young and old rats treated with vehicle (VHC) or corticosterone (CORT, 25 mg/kg/day), respectively. There were no differences in total exploration time between the groups at neither T1 nor T2, indicating that neither age or CORT influenced motor activity or overall exploration. C: Discrimination index (preference for the object that was moved), showing that compared to young animals, old VHC treated animals showed no preference for the moved object, and that this effect was reversed by CORT to the levels observed in young animals. Data are presented as individual data points and were analyzed with analysis of variance with treatment and age as fixed factors. Post-hoc comparisons of groups were done by independent samples *t*-test. * *p* < 0.05.

out their potential biological influence. Alternatively, some researchers have suggested that it is changes in the reactivity of the HPA-axis, rather than stable and chronic increases of cortisol, that lead to accelerated cellular aging [42].

A meta-analysis on the relation between CORT administration and

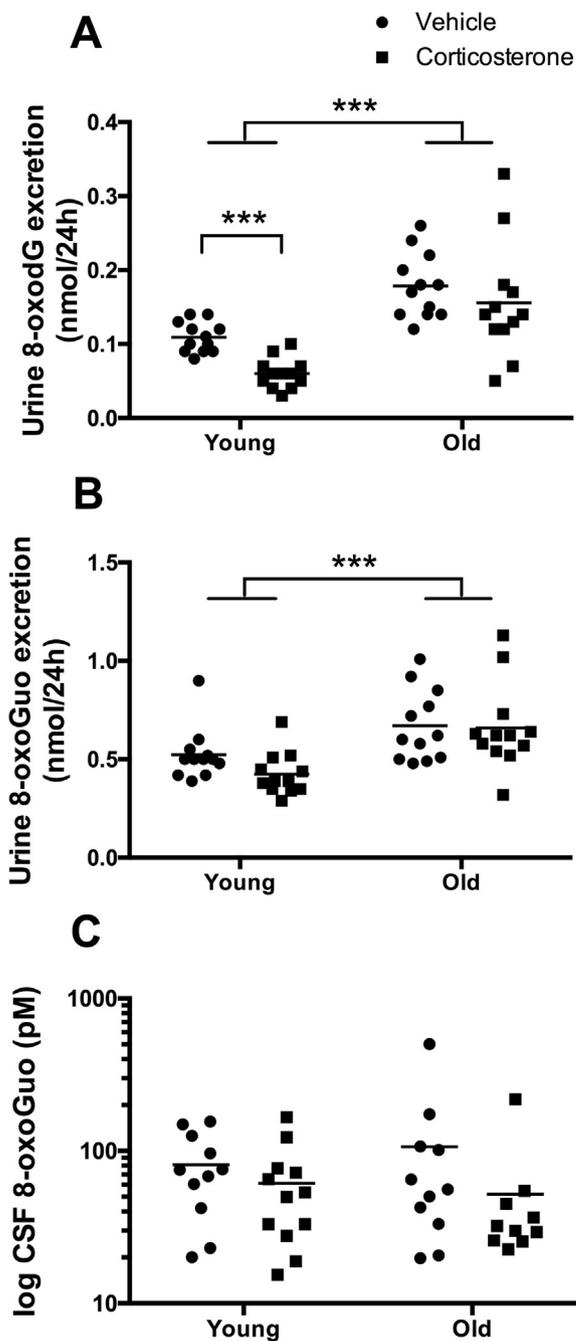


Fig. 5. Effects of corticosterone treatment on markers of systemic and CNS oxidative stress on DNA and RNA. A + B: 24 h urinary excretion of 8-oxodG and 8-oxoGuo after 3 weeks of CORT administration (25 mg/kg/day) in young and old animals, showing a significant reduction in 8-oxodG excretion, and a borderline significant reduction in 8-oxoGuo excretion, in young animals treated with CORT, as well as higher 8-oxodG/8-oxoGuo excretion levels in older animals. C: Cerebrospinal fluid concentration of 8-oxoGuo after CORT (log values on y-axis). There is a borderline significant reduction in 8-oxoGuo excretion in the old CORT-treated animals (*p* = 0.074). Data are presented as individual data points, and were analyzed with analysis of variance with treatment and age as fixed factors. Post-hoc comparisons of groups were done by independent samples *t*-test. *** *p* < 0.001.

oxidative stress in various animal species *in vivo* concluded that CORT was associated with increased markers of oxidative stress and reduced antioxidant defenses [43]. This effect was most pronounced with chronic treatment (i.e. 21 days), which is similar to the period used in our study. Hence, it is unlikely that the duration of treatment in our study explains the finding of reduced nucleic acid damage from oxidation. Furthermore, the meta-analysis indicated that females are

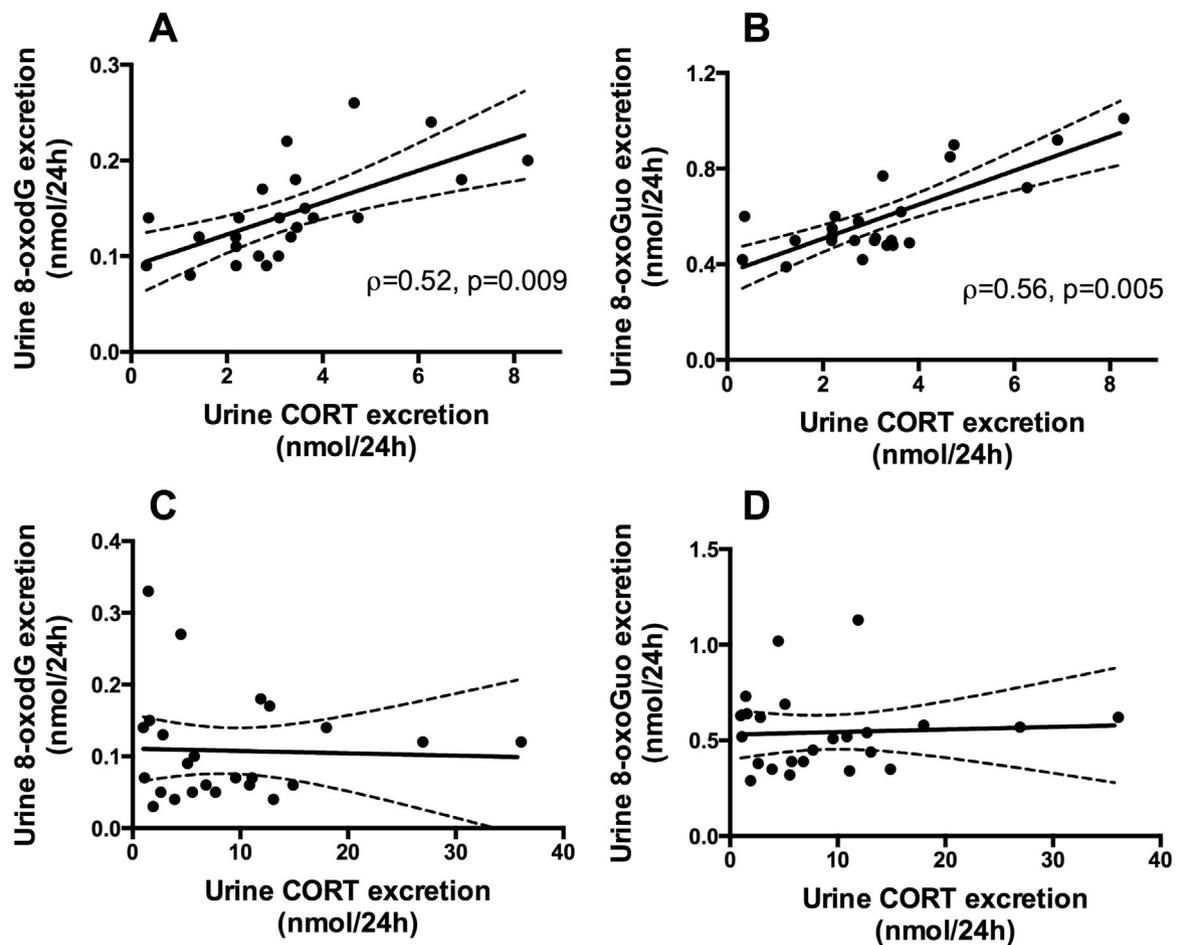


Fig. 6. Correlations between the 24 h urinary CORT vs. the 8-oxodG/8-oxoGuo excretion in VHC (A + B) and CORT treated (C + D) animals. Data from young and old animals are pooled in both groups. The significant positive correlations between the 24 h urinary excretion of CORT and 8-oxodG/8-oxoGuo (A + B) persist after adjustment for age group in a linear regression model (8-oxodG: $\beta=0.31$, $t=2.12$, $p=0.046$. 8-oxoGuo: $\beta=0.47$, $t=2.57$, $p=0.018$). There are no significant correlations in the CORT treated animals (C + D). Data are presented as individual values and regression lines with 95% confidence intervals (using log-transformed CORT values).

more susceptible to pro-oxidant effects of CORT, and hence we cannot rule out that the use of male animals in our study have influenced the finding, although it is seems unlike that a significantly different (i.e. opposite) finding would have been obtained with female animals. The studies included in the meta-analysis were almost exclusively on markers of lipid peroxidation (rather than other target molecules of oxidative stress), raising the intriguing possibility that CORT has opposite effects on lipid vs. nucleic acid oxidation. This could be speculated to be due to the subcellular localization of these molecules. For example, the increase of plasma glucose induced by CORT could increase extracellular oxidative stress on cell membranes (leading to increased lipid peroxidation), while concomitantly reduce nuclear oxidative stress levels. Because we did not include markers of lipid peroxidation, this question cannot be answered within the framework of the present study.

Interestingly, in contrast to the CORT treated animals, we observed a strong positive correlation between the 8-oxodG/8-oxoGuo markers and urinary CORT in the VHC-treated animals. This is in line with our two previous findings, where the two were positively correlated in healthy humans, but not in patients with schizophrenia [15,39]. Although the results were obtained in exploratory correlation analyses and therefore should be interpreted with caution, they do suggest that under healthy, unstressed conditions, there is a positive association between systemic levels of oxidative stress on DNA/RNA and circulating CORT in both rats and humans.

4.3. Effects of CORT on spatial memory and CNS RNA damage from oxidation

In line with recent evidence [44], we found that aging negatively influenced short-term spatial memory, as measured by a significantly reduced preference for the moved object in an object location test. However, in contrast to our hypothesis, this effect was reversed by CORT. There is ample evidence that chronic (21 days) experimental stress impairs spatial memory in young animals, and that shorter periods of stress are not sufficient to induce these deficits [45]. With CORT treatment in physiological doses without psychological stress exposure, even longer periods of treatment may be necessary for spatial memory deficits to occur [46]. In contrast, supraphysiological doses of glucocorticoids, such as those used in glucocorticoid treatment for human medical disorders, are associated with substantial adverse effects on mood and cognitive function, including memory [47]. Some evidence suggests that predictable, chronic mild stress *improves* spatial and object recognition memory and increases hippocampal neurogenesis [48]. Collectively, these data illustrate that differing durations and intensities of stress and/or CORT-treatment may yield opposite effects on hippocampus-dependent memory. They also illustrate the importance of differentiating between simple CORT administration and the neuroendocrinological complexity of psychological stress, which involves many other hormones and cerebral receptor systems.

In parallel with the improvement of spatial memory performance in

old animals, we found a borderline significant effect of CORT on CSF 8-oxoGuo concentration. The oxidation of mRNA species can negatively influence protein synthesis and folding [22,23], and thus it could be speculated that lower levels of RNA oxidation in the brain would facilitate the synthesis of functional new synaptic proteins, thereby improving spatial memory. However, the T1-T2 interval was only one hour, thereby testing the strength of short-term spatial memory, which would not be expected to require the synthesis of new proteins. Hence, if there is a causal relationship between reduced RNA oxidation in the brain and improved spatial memory, this more likely involves longer-term neurobiological changes occurring before the test itself (e.g. protein synthesis involved in synapto- or neuroneogenesis), which in turn promotes short-term spatial memory retention in the test.

As recently summarized by Spiers et al. [49], some studies have found localized hippocampal increases in hippocampal markers of oxidative stress. Our study did not include markers of cellular oxidative stress in the hippocampus, and it is therefore possible that although overall CNS oxidative stress on nucleic acid damage (as measured by CSF 8-oxoGuo concentration) tended to be unaltered or reduced, oxidative stress locally in the hippocampus specifically could be increased. However, if this is the case, then it has not led to impaired hippocampal spatial memory function, because we did not find negative effects of CORT in the object location test.

Although total exploration time did not differ between groups at neither T1 nor T2, we found that the O-VHC animals had a discrimination index below zero. While this did not reach statistical significance, it could be speculated that O-VHC animals did not simply have complete amnesia for the spatial environment in T1, but to a certain extent showed a preference for the familiar object, i.e. a neophobia related to older age. Thus, it is possible that some of the apparent restoration of spatial memory by CORT in old animals relies on an increase in novelty-seeking behavior and not on an improvement of spatial memory performance alone.

4.4. Mechanisms by which CORT could reduce systemic and CNS nucleic acid damage from oxidation

What mediates the reduction of systemic and CNS nucleic acid damage after CORT treatment observed in our study? The CORT treated animals exhibited a stalling of weight gain or outright weight loss in both age groups. This is in line with evidence that CORT released during experimental stress causes weight loss through activation of Corticotrophin Releasing Factor (CRF) receptors in the hypothalamus [50]. The weight changes observed were paralleled, and likely mediated, by a reduction in calorie intake. However, when adjusting for animal weight on day 22, the effect of CORT on calorie intake was no longer significant. Hence, CORT could cause body weight changes through a centrally mediated reduction of calorie intake; a peripheral catabolic state, or a combination of both.

There is ample evidence that calorie restriction (CR) reduces mitochondrial ROS production and oxidatively generated damage to a range of macromolecules, including mitochondrial DNA, with a corresponding increase in life span in rodents [51]. Furthermore, both CR and exercise improve insulin sensitivity and hippocampus-dependent cognition, despite the fact that both interventions are associated with CORT increase [52]. Conversely, various measures of DNA damage are positively correlated with high-calorie diets in rodents and humans [53,54].

Collectively, these findings give us cause to speculate that *in vivo*, the potential negative effects of a chronic CORT increase are outweighed by beneficial effects of a concomitant CORT-induced CR. This hypothesis would also account for the apparent discrepancy that a short-term *in vitro* exposure of murine 3T3 cells to physiological doses of CORT caused a substantial increase in DNA damage, as measured by the comet assay [29], because *in vitro*, possible beneficial effects of a reduction in calorie intake mediated by hypothalamic CRF signaling are

not recorded.

Overall, young animals were sensitive to the effects of CORT on the peripheral markers of oxidative DNA and RNA damage only, whereas they showed no significant changes in CNS 8-oxoGuo concentration or memory upon CORT treatment. The opposite was observed in the old animals. From a clinical point of view, it is interesting that older individuals are apparently more responsive to the CNS effects of CORT treatment and the ensuing weight loss. This finding could bear relevance to neuroprotective interventions in older humans.

4.5. Limitations

Because we aimed to keep the intervention as psychologically and physiologically stress-free as possible, the animals were not adrenalectomized, and although the administration of CORT must be expected to have suppressed endogenous HPA-axis activity, some degree of activity would likely persist. This is a potential cause for variation in the actual levels of circulating CORT in each animal. Also, the exogenous administration by two daily doses would be expected to interrupt the physiological diurnal rhythm of CORT secretion, which could possibly have influenced e.g. sleep patterns or feeding behavior in the animals. We cannot rule out that the vehicle used for CORT administration (nut paste and sesame oil) could have antioxidant properties; however, control animals were also administered this vehicle, meaning that the effects observed in the study can only be ascribed to CORT. The focus of the study was on nucleic acid damage from oxidation, and we cannot rule out that CORT could have differential effects on alternative markers of oxidative stress, as discussed above. Finally, we were unable to measure 8-oxodG in CSF, which was below the quantification limit of the UPLC-MS/MS, and thus we cannot conclude on CNS levels of oxidatively generated DNA damage in this study.

4.6. Conclusion

To our knowledge, this is the first demonstration that a chronic *in vivo* physiological increase of CORT reduces nucleic acid damage from oxidation. This was observed in parallel with reduced weight gain/weight loss; a trend towards lower RNA oxidation in CNS and a normalization of performance in an object location memory test in old animals. These findings do not suggest that chronically elevated glucocorticoid *per se* is a mediator of the increased oxidative stress observed in stress and depression. We consider this finding to provide an important extension of the existing mechanistic insight into the connections between psychological stress states and their associated signs of accelerated aging.

Conflict of interest

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

Contributors

AJ, OK, JH, GW, HEP and MBJ planned the study. AJ, OK, KF, AW, TH, KB and GW performed the experiments. AJ, OK, and GW performed the data analyses. AJ and OK wrote the first drafts of the manuscript. All authors contributed to and approved the final version of the manuscript.

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