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Chronic restraint stress in rats causes sustained increase in urinary corticosterone excretion without affecting cerebral or systemic oxidatively generated DNA/RNA damage

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ABSTRACT

Increased oxidatively generated damage to nucleic acids (DNA/RNA) may be a common mechanism underlying accelerated aging in psychological stress states and mental disorders. In the present study, we measured the urinary excretion of corticosterone and markers of systemic oxidative stress on nucleic acids, 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) and 8-oxo-7,8-dihydroguanosine (8-oxoGuo), respectively, in rats subjected to chronic restraint stress. To reliably collect 24 h urine samples, the full 3-week restraint stress paradigm was performed in metabolism cages. We further determined frontal cortex and hippocampal levels of oxidatively generated nuclear DNA damage, as measured by oxoguanine DNA glycosylase and formamidopyrimidine DNA glycosylase sensitive sites detected by the comet assay, as well as the expression of genes involved in DNA repair (*Ogg1* and *Nudt1*) and inflammation (*Ccl2* and *Tnf*). The metabolism cage housing in itself did not significantly influence a range of biological stress markers. In the restraint stress group, there was a sustained 2.5 fold increase in 24 h corticosterone excretion from day 2 after stress initiation. However, neither whole-body nor cerebral measures of nucleic acid damage from oxidation were affected by stress. In contrast, cerebral DNA repair enzymes exhibited a general trend towards an induction, which was significant for hippocampal *Nudt1*. The results and their implications for stress sensitivity and resilience are discussed.

1. Introduction

Accumulating evidence has substantiated that psychological stress states are detrimental to health and accelerate aging. Stress is associated with an increased risk of various age-related medical disorders, in particular cardiovascular disease (Brotman et al., 2007). In the brain, experimental chronic stress leads to neuronal remodeling and dysfunctions of the prefrontal cortex and the hippocampus (Goldwater et al., 2009; Watanabe et al., 1992). Correspondingly, epidemiological evidence suggests that stress and mental disorders such as depression and schizophrenia increase the risk of cognitive dysfunction or out-right dementia (Jeste et al., 2011; Johansson et al., 2010; Saczynski et al., 2010).

Attempts have been made to identify common mechanisms underlying a stress-induced syndrome of accelerated aging. One important finding was that telomere length – a biochemical correlate of aging – is reduced in psychological stress and mental disease (Epel et al., 2004; Kao et al., 2008; Simon et al., 2006). A related candidate phenomenon is oxidative stress, in which the generation of reactive oxygen species (ROS) exceeds the antioxidant potential of the cell, thereby causing damage to proteins, lipids or nucleic acids. The continuous ROS attacks on DNA are considered to be a key mediator of aging (Chen et al., 2007; Finkel and Holbrook, 2000). Specifically,

Abbreviations: 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; 8-oxoGuo, 8-oxo-7,8-dihydroguanosine; ROS, reactive oxygen species; CORT, corticosterone; FCM, fecal corticosterone metabolites; OGG1, 8-oxoguanine DNA glycosylase; NUDT1, Nudix (nucleoside diphosphate linked moiety X)-type motif 1; TNF, tumor necrosis factor; CCL2, C–C motif ligand 2.

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genotoxic stress from oxidation accelerates telomere shortening and activates DNA damage signaling pathways, thereby increasing the risk of cellular senescence or apoptosis, which are known to be key events in tissue aging processes (Sahin and DePinho, 2010). Indeed, a recent meta-analysis showed that an age-dependent accumulation of oxidatively damaged DNA occurs in various organs of rodents (Moller et al., 2010). Finally, oxidative stress has been implicated as a pathogenic event in age-related medical disorders such as cardiovascular disease and dementia (Bossy-Wetzel et al., 2004; Harrison et al., 2003).

Various measures of cerebral oxidative stress, *e.g.* protein or lipid oxidation markers, have been found to be increased in animal models of psychological stress (Fontella et al., 2005; Madrigal et al., 2003). In an early study, DNA oxidation levels were increased in the cerebral cortex of rats after acute restraint stress (Liu et al., 1996). In clinical studies peripheral markers of oxidative stress have been found to be associated with occupational stress (Irie et al., 2001), caregiver stress (Epel et al., 2004) and depression (Forlenza and Miller, 2006). Several post-mortem studies have found signs of mitochondrial dysfunction and oxidative stress in the brains of depressed (Gawryluk et al., 2011), bipolar disorder (Andreazza et al., 2010), and schizophrenia patients (Prabakaran et al., 2004). However, a recent post-mortem study found no activation of several oxidative stress-response genes in depressed subjects (Teyssier et al., 2011). Hence, questions regarding the specificity of oxidative stress to various psychiatric disorders, the temporal relationship between stress exposure and oxidative stress induction, and the relationship between peripheral biomarkers and cerebral oxidative stress levels, remain largely unanswered.

The aim of the present study was to investigate the urinary excretion of markers of oxidatively generated DNA/RNA damage in rats subjected to chronic restraint stress, and to relate these to post-stress brain levels of oxidatively generated nuclear DNA damage, as well as DNA repair enzyme and inflammation marker expression in the same animals. The restraint stress paradigm was chosen for its ability to reliably induce a range of the biological hallmarks of stress: increased glucocorticoid output and relative adrenal gland weight, reduced expression of the cerebral glucocorticoid receptor, as well as dendritic retractions in both the prefrontal cortex and the hippocampus (Goldwater et al., 2009; Hageman et al., 2008, 2009; Watanabe et al., 1992). Furthermore, the model is associated with depression-like behavioral changes (*e.g.* in the Forced Swim Test), thus mimicking the human depressive state (Hageman et al., 2009). Finally, the biological and behavioral consequences of restraint stress are reversible with antidepressive treatments such as electroconvulsive stimulations (Hageman et al., 2008, 2009; Maigaard et al., 2012).

We hypothesized an increased urinary excretion of the oxidation markers during stress, an increased level of DNA damage from oxidation in the brain of stressed animals, and an induction of the repair enzymes.

2. Methods

2.1. Experimental groups and general procedure

Male Sprague–Dawley rats (N = 36, weight 180–200 g, Charles-River, Germany) were randomly distributed in standard group cages (Macrolon type III cages (Tecniplast, Varese, Italy), 2 rats per cage), and left to acclimatize for one week. They were fed with food pellets (Altromin 1319; Brogaarden, Gentofte, Denmark) and acidified tap water provided *ad libitum*. Wooden chips (Tapvei Oy, Kortteinen, Finland) were used as bedding. Room temperature was maintained at 20 ± 2 °C, air humidity was 30–60% and the light regimen was a 12/12 h dark/artificial light cycle with lighting period starting at 7:00 AM.

We performed the full three-week restraint stress paradigm in metabolism cages (MC) (Tecniplast, Varese, Italy), which allowed for a continuous and non-invasive monitoring of urinary biomarkers. The day before the experiment began, animals were matched by weight and assigned to experimental groups. The three experimental groups (N = 12 per group) were 1) group cage control (GCC), 2) metabolism cage control (MCC) and 3) metabolism cage stress (MCS). The GCC group was included to allow for an evaluation of the stress effects of MC housing in itself. MCS and GCC/MCC were kept in separate rooms with similar conditions throughout the experiment to avoid sonic and ultrasonic influence between the groups.

One week after arrival at our facility, animals in the MCC and MCS groups were placed individually in the MCs for a further four days of acclimatization (day-3 through 0), while the GCC animals were left in the group cages throughout the experiment. The MCs were arranged in two racks with twelve cages each. The metabolism cages measured 26 cm in diameter, and had a grid floor to allow for the passage of excretions into a collection system. In the collection system, urine and feces are separated into two removable containers from which the samples were obtained. The MC animals had a plastic shelter (as did the GCCs) inside the cage, but no other forms of enrichment which could potentially interfere with the collection of urine and feces. A food trough and water dispenser were attached to the cage for *ad libitum* access (except during restraint). All animals received the same diet, which in the case of the MC rats was ground to avoid contamination of the collection system. Twenty-four hour urine and feces samples were collected daily at 9 AM, weighed and stored at −20 °C until analysis. The body weight of all animals was recorded daily.

2.2. Restraint stress

On day 1–21 MCS animals were subjected to restraint stress. At the initiation of restraint, the animals weighed on average 285 g, corresponding to an approximate age of postnatal day 64. The chronic restraint stress paradigm consists of 6 h of daily immobilization for 21 consecutive days. Every morning, the rats voluntarily entered wire mesh restrainers that were subsequently closed with wire clips. The restrainers were designed to tightly fit the animal without restricting breathing, interfering with thermoregulation, or causing pain. During restraint, animals remained in their metabolism cages to allow collection of the urine and feces produced while in the restrainer. The urine and feces samples from the previous 24 h were always collected before restraint stress began.

2.3. Isolation of brain tissue

On day 22 at 9 AM (*i.e.* the day after the last restraint stress session), rats from the two MC groups were sacrificed. To reduce diurnal variation in the time of death between the groups, GCC rats were sacrificed on day 23, also at 9 AM. The animals were deeply anesthetized by an intraperitoneal injection of pentobarbital (100–150 mg). Before blood circulation ceased, the animals were thoracotomized, the descending aorta was clamped, and the animals were perfused transcardially with cold 0.9% saline. Both adrenal glands were removed by manual dissection; adjacent fat and connective tissue were carefully removed, and the glands were weighed separately. The relative adrenal gland weight was defined as: (left + right adrenal gland weight (g) / total body weight (g)) × 1000‰.

The brains were removed and manually dissected on an ice-cooled metal plate. We isolated frontal cortex and hippocampal tissue for DNA oxidation and gene expression analysis. These regions where chosen for their established role in HPA-axis inhibition, sensitivity to glucocorticoids, and stress-associated plasticity (Ulrich-Lai and Herman, 2009). Frontal cortex was isolated by an incision in the coronal plane approximately 4 mm dorsal to the frontal pole, snap frozen on dry ice and stored at −80 °C. The remaining brain was snap frozen in isopentane, placed on dry ice and stored at −80 °C. To isolate hippocampal tissue for RT-PCR and comet analysis, the frozen brains were placed in an ice-cooled matrix, and consecutive 2 mm

coronal sections were cut. The sections were placed on an ice-cooled metal plate, and the hippocampus was visually identified. Each hippocampal sample for comet analysis consisted of two 3.5 mm punches, obtained from two adjacent sections. These samples were immediately processed for determination of DNA damage by the comet analysis. Similar samples of the contralateral hippocampus were isolated for RT-PCR and re-frozen at −80 °C until analysis.

2.4. Determination of urinary and fecal biomarkers

The urinary content of the 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, were assayed using ultraperformance liquid chromatography and tandem mass spectrometry (UPLC MS/MS), as described by (Henriksen et al., 2009). Before analysis and standard sample preparation, samples were filtered (VectaSpin 3 centrifuge filters, 10 K molecular weight cut off, Whatman, Kent, UK) and diluted 1:5 with water. The chromatographic separation was performed on an Acquity UPLC system (Waters, Milford, MA, USA). The column used was an Acquity UPLC BEH Shield RP18 column (1.7 μm, 2.1×100 mm) protected with in-line filter (4×2 mm, 0.2 μm) both obtained from Waters. The MS detection was performed on an API 3000 triple quadrupole mass spectrometer (Sciex, Toronto, Canada) equipped with an ESI ion source (Turbospray) operated in the positive mode.

Corticosterone (CORT) was quantified with a commercially available ELISA kit following the manufacturer's instructions (DRG Instruments, Germany). The intra- and interassay variation of the kit was 2.8 and 6.1%, respectively, according to the manufacturer. In all urinary markers, 24 h excretion was calculated as concentration (nmol/L)×urinary volume (L).

The total 24 h fecal corticosterone metabolite (FCM) excretion was quantified using a previously described method (Kalliokoski et al. , 2012). Briefly, FCM was extracted by incubating feces in 96% ethanol (5 mL/g feces) overnight. Corticosterone levels were analyzed in duplicate (EIA4164, DRG Instruments, Marburg, Germany) in accordance with the manufacturer's instructions. Standards included in the kit were replaced with a custom 9-point standard curve prepared in 96% ethanol from analytical grade corticosterone (catalog no. 46148, Sigma-Aldrich, St Louis, MO) in concentrations from 50 to 0.19 ng/mL. 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. The absorbencies were recorded at 450 nm (reference wavelength, 650 nm; Thermo Fisher Scientific, Waltham, MA).

2.5. Determination of frontal cortex and hippocampal levels of oxidatively damaged DNA

The level of DNA damage in the frontal cortex and hippocampus was determined by single-cell gel electrophoresis (comet) assay, using a previously described method adapted for the present experiment (Folkmann et al., 2007). For each day of analysis, we included samples from each experimental group. Single brain cell nuclei were isolated by placement of the sample in a stainless steel cylinder, using a plunger to force the tissue through a sieve (0.5 cm in diameter, mesh size 0.4 mm), while the cylinder was submerged in 700 μL Merchants buffer (0.14 M NaCl, 1.47 mM KH_2PO_4, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 10 mM Na_2EDTA, pH 74). The cell extract was filtered through a nylon mesh to remove cell debris. One-hundred microliters of the tissue extract was mixed with 800 μL 0.75% low melting point agarose (dissolved in phosphate-buffered saline [PBS]) and 120 μL was applied onto a GelBond® film (Cambrex, Medinova Scientific A/S, Hellerup, Denmark). The GelBonds were placed in lysis solution (1% Triton X-100, 2.5 mM NaCl, 100 mM Na_2EDTA, pH 10) overnight. The GelBond films were washed 3×5 min in a buffer containing 40 mM HEPES, 0.1 M KCl, 0.5 mM Na_2EDTA, 0.2 μg/mL BSA, pH 8. Sixty microliters of OGG1 (0.16 units/gel, P/N M0241L, Medinova Scientific A/S, Hellerup, Denmark), formamidopyrimidine DNA glycosylase (FPG, 1 mg/mL, gift from Prof. Andrew Collins, University of Oslo, Norway) or buffer were applied on separate agarose gels and were incubated for 45 min at 37 °C. OGG1 specifically excises the 8-oxodG lesion, while FPG breaks DNA at sites of oxidized purines in general. After rinsing with water, the nuclei were immersed in an alkaline solution (300 mM NaOH, 1 mM Na_2EDTA, pH>13.0) for 40 min and electrophoresed for 20 min in the same solution (1.1 V/cm across the platform and 300 mA). The electrophoresis solution was recycled at a rate of 3.6 L/h and maintained at 4 °C. After electrophoresis, the nuclei were washed 3×5 min in Tris buffer (0.4 M Tris–HCl, pH 7.5), rinsed with water and placed in 96% ethanol for 1.5 h or overnight. The nuclei were visualized in an Olympus fluorescence microscope at ×40 magnification after staining with YOYO-1 iodide (P/N 491/509, Molecular Probes, The Netherlands) in PBS buffer. The level of DNA damage was scored according to five classes of damage (0–4) in 100 randomly selected nuclei from each sample and two slides were examined for each sample. OGG1- and FPG-sensitive sites were defined as the difference in scores of parallel slides incubated with and without enzymes. The investigating technician was blind to the experimental status of the samples. The score was converted to lesions per 10^6 base pairs by use of an individual calibration factor for the investigator who analyzed the slides, as previously described (Forchhammer et al., 2010).

2.6. Brain expression of repair enzymes and inflammation markers by RT-PCR

We used RT-PCR to analyze the gene expression of repair enzymes central to the removal of oxidated nucleotides from DNA (8-oxoguanine DNA glycosylase (*Ogg1*)) and the nucleotide pool (Nudix (nucleoside diphosphate linked moiety X)-type motif 1 (*Nudt1*)), as well as markers of inflammation, which could potentially underlie a state of increased oxidative stress (tumor necrosis factor (*Tnf*) and chemokine (C–C motif) ligand 2 (*Ccl2*, also known as monocyte chemoattractant protein-1)). RT-PCR was performed as previously described (Vesterdal et al., 2010). Briefly, RNA was extracted from the brain samples using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The extracted RNA samples were DNase treated using the RQ1 RNase-Free DNase kit (Promega, Madison, WI, USA). The total RNA was converted into cDNA by reverse transcriptase, using the High Capacity cDNA Transcription Kit (Applied Biosystems, Life Technologies, Calsbad, CA, USA). The quantification of gene expression was determined by real-time PCR using the Taqman® gene expression assay. Quantitative PCRs were carried out in an ABI Prism 7900HT (Applied Biosystems). The primers used (all from Applied Biosystems) were: Rn00578409_m1 (*Ogg1*), Rn00589097_m1 (*Nudt1*), Rn00562055_m1 (*Tnf*), Rn00580555_m1 (*Ccl2*), and eukaryotic 18S rRNA (P/N 4352930E) as endogenous control. The probes were designed to be cDNA specific, *i.e.*, they did not include intron sequences. All samples were determined in triplicate. The standard curves for the primers were generated with 10 steps of 2× dilutions starting from 10 μL cDNA. The level of the target cDNA was expressed as the difference in Ct values between the average of the triplicate for *Ogg1*, *Nudt1*, *Tnf* or *Ccl2*, respectively, and the average of the triplicate for 18S in the parallel samples. The relative amount of target mRNA normalized to 18S mRNA was calculated as $2^{-\Delta Ct}$. All mRNA signals including 18S were linear over a 100-fold dilution.

2.7. Ethics

The animal experiments performed in this study were approved by the Animal Experiments Inspectorate under the Danish Ministry of Justice (license number 2007/561-1309). All procedures were performed in accordance with the European Communities Council

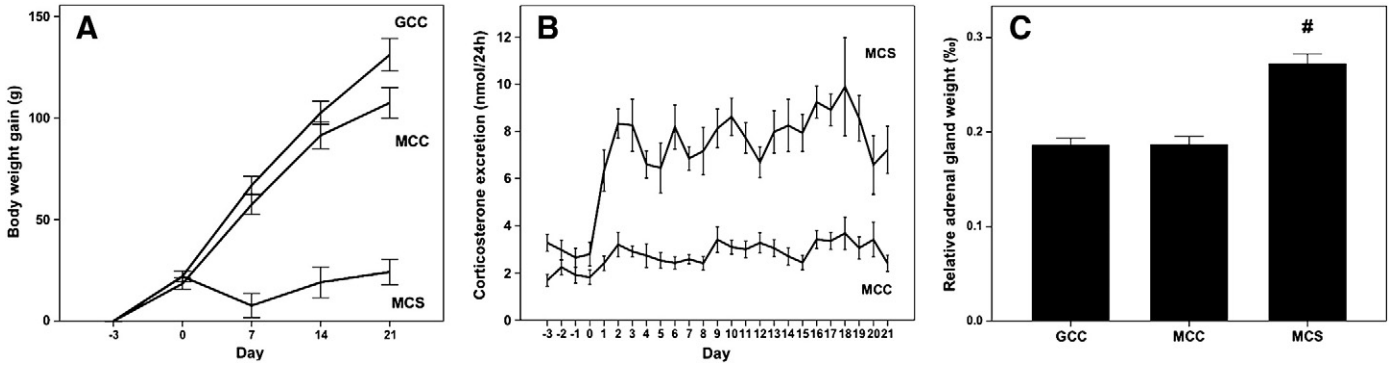

Fig. 1. Biological markers of psychological stress in stressed and control animals. Restraint stress began on day 1. A: body weight gain during the experiment. Day -3-day 21 change, GCC *vs.* MCC: $P=0.07$, MCS *vs.* GCC or MCC: $P<0.001$, one-way ANOVA with post-hoc Tukey test. B: urinary corticosterone excretion (nmol/24 h) during the experiment. Area under the curve, day 0–21 (arbitrary units): MCC = 65.2 ± 13.7, MCS = 156.7 ± 38.6, $P<0.001$, independent samples t-test. C: relative adrenal gland weight. #Different from MCC and GCC ($P<0.001$), one-way ANOVA with post-hoc Tukey test. GCC = group cage controls, MCC = metabolism cage controls, MCS = metabolism cage stress. Error bars = SEM.

Directive of 24 November 1986 (86/609/EEC) and the Guide for the Care and Use of Laboratory Animals (2011) in a fully AAALAC accredited facility. All efforts were made to minimize pain or discomfort as well as the number of animals used during the experiment.

2.8. Statistics

Data were analyzed with independent samples t-test, one-way analysis of variance (ANOVA) with post-hoc Tukey tests, or repeated measures ANOVA (with time as within-subject factor and group as between-subject factor), as appropriate. For the analysis of gene expression data, which were non-normally distributed and differentially distributed across groups, a Mood's median test was applied. If significant, post-hoc comparisons of GCC *vs.* MCC/MCS were performed. Extreme values (>3 times the interquartile range of the data) were systematically omitted from the urinary 8-oxodG/8-oxoGuo data set (4 out of a total of 192 observations) and the RT-PCR data set (9 out of a total of 288 observations). All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) software version 20.0 (IBM Corporation, NY, USA). Statistical significance was defined as $P<0.05$. All statistical tests were two sided.

3. Results

3.1. Stress and housing conditions vs. biological stress markers

Restraint stress markedly reduced weight gain, while MC housing alone only caused a slight reduction in weight gain (GCC = 131 ± 28 g, MCC = 107 ± 26 g, MCS = 24 ± 22 g. Anova $F(2,33)=59.1$, $P<0.001$, post-hoc Tukey's test, MCS *vs.* MCC or GCC: $P<0.001$, MCC *vs.* GCC: $P=0.07$). From the first day of restraint stress, the 24 h urinary excretion of corticosterone increased substantially in the MCS group, and retained a level approximately 2.5 times higher than the MCC group throughout the experiment (area under the curve, day 0–21 (arbitrary units): MCC = 65.2 ± 13.7, MCS = 156.7 ± 38.6, t-test $t=-7.58$, $dF=22$, $P<0.001$). The relative adrenal gland weight was significantly higher in the MCS group *vs.* the control groups (Anova $F(2,33)=31.0$, $P<0.001$, post-hoc Tukey's test, MCS *vs.* MCC or GCC: $P<0.001$). The control groups did not differ (GCC *vs.* MCC: $P=0.98$) (Fig. 1A–C). There was no overall group effect in FCM excretion, but a significant time × group interaction (repeated measures Anova $F(24,480)=3.1$, $P<0.001$), with a higher excretion of FCM in the MCS group on day 1 and 2 after stress (day 1: t-test $t=-3.3$, $dF=22$, $P=0.004$, day 2: $t=-3.3$, $dF=22$, $P=0.003$) (Fig. 2). Collectively these data did not point to metabolism cage housing as a significant stressor in itself.

3.2. Urinary markers of oxidatively generated DNA and RNA damage

The 24 h urinary excretion of 8-oxodG and 8-oxoGuo in the MCC and MCS groups were analyzed at baseline (day-3), and at day 2, 8 and 21 after restraint. There was no effect of group status in neither marker (8-oxodG: repeated measures Anova $F(1,19)=0.7$, $P=0.41$, 8-oxoGuo: $F(1,15)=1.0$, $P=0.33$) (Fig. 3). A significant effect of time (8-oxodG: $F(3,57)=12.9$, $P<0.001$, 8-oxoGuo: $F(3,45)=18.5$,

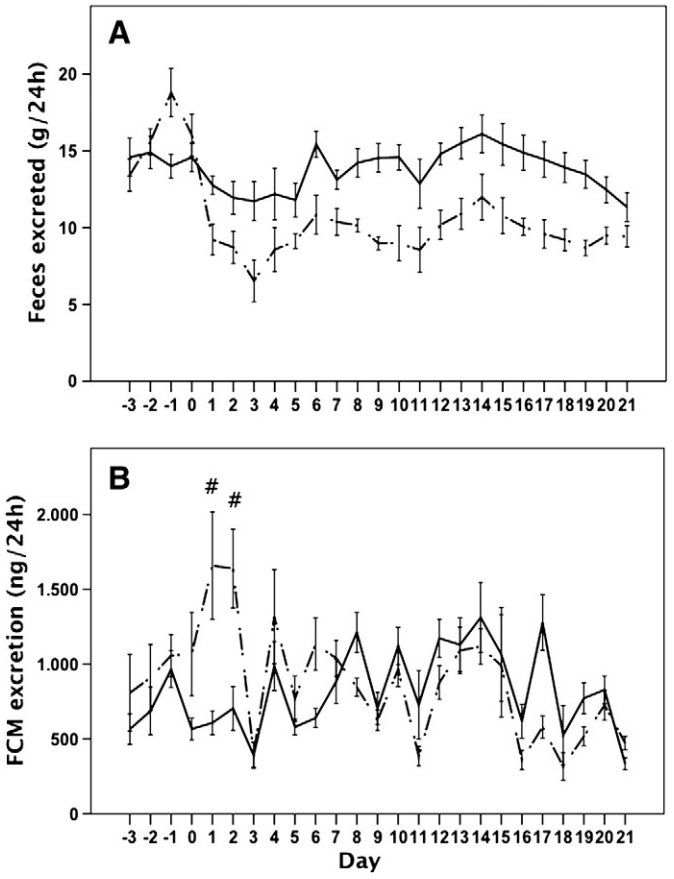

Fig. 2. Fecal corticosterone excretion during restraint stress. A. Feces excreted (g/24 h) in stressed *vs.* control animals. Restraint stress begins on day 1. There is a significant effect of group ($P<0.001$, repeated measures ANOVA). B. Fecal corticosterone metabolites (FCM) excreted (ng/24 h) in MCS and MCC animals. There is no overall effect of group ($P=0.5$), but a significant time × group interaction ($P<0.001$), with a significantly increased excretion in stressed animals on day 1 and 2 after stress initiation (#$P<0.05$). Solid line: metabolism cage control. Dashed line: metabolism cage stress. Error bars = SEM.

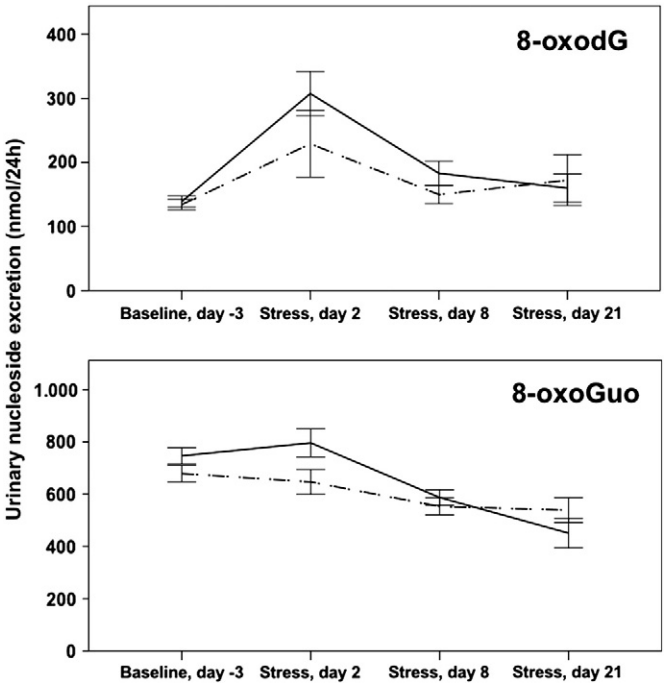

Fig. 3. 24h urinary excretion of 8-oxodG (upper panel) and 8-oxoGuo (lower panel) at baseline (day −3), and day 2, 8 and 21 after restraint stress initiation. There is a significant effect of time ($P<0.001$ for both markers) and no effect of group status in neither marker ($P=0.41$ and 0.33, respectively). Solid line: Metabolism Cage Control. Dashed line: Metabolism Cage Stress. Error bars = SEM.

$P<0.001$) with peak levels at day 2 was observed, indicating an effect of metabolism cage housing in itself. After 21 days of restraint, there was no difference between the groups in neither marker (8-oxodG: t-test t = 0.17, dF = 22, $P=0.87$, 8-oxoGuo: t = −1.55, dF = 22, $P=0.14$).

3.3. Brain levels of oxidatively damaged DNA and gene expression of repair enzymes and inflammation markers

There were no differences between the groups in the levels of oxidatively generated DNA damage in either brain region. This was the case for the number of both FPG- and OGG1-sensitive sites, *i.e.* the amount of oxidatively generated modifications of purines in general and the specific 8-oxodG lesion, respectively (Fig. 4). In contrast, the hippocampal expression of *Nudt1* showed a statistically significant

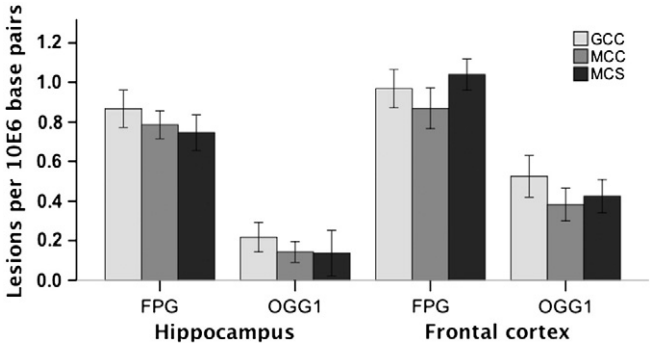

Fig. 4. Hippocampal and frontal cortex DNA damage from oxidation as measured by the comet assay in stressed *vs.* control animals. Data represents number of lesions per 10^6 base pairs generated by the FPG and OGG1 enzymes, reflecting levels of oxidatively generated purine lesions in general and the 8-oxodG lesion, respectively. There are no significant differences among the groups in neither brain region ($P>0.05$, one-way ANOVA). GCC = group cage control, MCC = metabolism cage control, MCS = metabolism cage stress. Error bars = SEM.

increase in MCS compared to GCC animals (median test, chi-square = 8.7, dF = 2, $P=0.01$, post-hoc comparison of MCS *vs.* GCC: $P=0.04$) (Fig. 5). In the frontal cortex, there was a significant difference between the groups in the expression of *Nudt1*, with a significantly reduced expression in MCC and a trend towards an increased expression in MCS compared to GCC (chi-square = 8.4, dF = 2, $P=0.02$, post-hoc comparison of GCC *vs.* MCC: $P=0.04$, GCC *vs.* MCS: $P=0.1$). A non-significant trend towards increased hippocampal expression of *Ogg1* in MCS and MCC compared to the GCC was also observed (chi-square = 8.7, dF = 2, $P=0.1$). Finally, a reduction in frontal cortex *Ogg1* ($P=0.003$), and *Ccl2* ($P=0.003$) in the MCC *vs.* GCC group was observed (median tests with post-hoc comparison of GCC *vs.* MCC after significant main test). There were no other significant differences in the expression of the inflammation markers. There were no significant correlations between the individual levels of damage in the brain and the urinary 8-oxodG/8-oxoGuo excretions on day 21.

4. Discussion

To our knowledge, this is the first demonstration of the validity of the chronic restraint stress paradigm in metabolism cages. We found that in spite of a 2.5 fold sustained increase in the urinary excretion of corticosterone during chronic restraint stress, neither cerebral nor continuously monitored markers of systemic oxidatively generated nucleic acid damage were affected. These findings are surprising in light of evidence of glucocorticoid-induced increased DNA strand breaks *in vitro* (Flint et al., 2007) and increased 8-oxodG levels in mitochondrial DNA *in vivo* (Caro et al., 2007), as well as the association between the 24 hour urinary excretion of cortisol and 8-oxodG/8-oxoGuo excretion in a large human cohort of elderly individuals (Joergensen et al., 2011). We regard our finding as robust as both systemic and cerebral levels of oxidative stress to nucleic acids were measured by two independent methods (UPLC–MS/MS and the comet assay with two different repair enzymes for oxidized purines, respectively).

There are a number of compensatory mechanisms which may account for this finding. Firstly, as previously observed by us and others, restraint stress was associated with a marked lack of weight gain. (Goldwater et al., 2009; Hageman et al., 2008, 2009). Although food intake was not monitored in the study, this growth stagnation is likely mediated by a stress-induced reduction in calorie intake. Caloric restriction is known to reduce oxidative stress and promote longevity (Gredilla and Barja, 2005), and thus a reduced calorie intake could counteract pro-oxidant effects of increased corticosterone. In that context it is interesting that while palatable, calorie-rich foods ameliorate the behavioral effects of stress through an influence on brain reward systems, they possibly promote detrimental metabolic effects, including oxidative stress (Krolow et al., 2010; Ulrich-Lai et al., 2010).

In the brain, DNA may be protected from oxidation damage by a number of factors. A key mechanism underlying the dendritic remodeling observed after stress is glucocorticoid-induced glutamatergic excitotoxicity (Popoli et al., 2012). It has been demonstrated that activation of the glutamatergic NMDA-receptor boosts antioxidant defense systems and promotes resistance to oxidative stress (Papadia et al., 2008). Furthermore, glutamate signaling has been shown to increase the capacity for DNA repair through an induction of the apurinic/apyrimidinic endonuclease (APE) 1 enzyme (Yang et al., 2011). We found a significant increase in hippocampal *Nudt1* (although only in comparison to the GCC group), and a trend towards an increase in frontal cortex *Nudt1* and hippocampal *Ogg1* expression. The NUDT1 enzyme hydrolyzes 8-oxodG triphosphate to 8-oxodG monophosphate, thereby preventing the incorporation of 8-oxodG into DNA, and NUDT1 is up-regulated by oxidative stress *in vitro* (Hah et al., 2007). Hence, in combination these events may constitute a powerful and multifaceted countermeasure to keep oxidatively

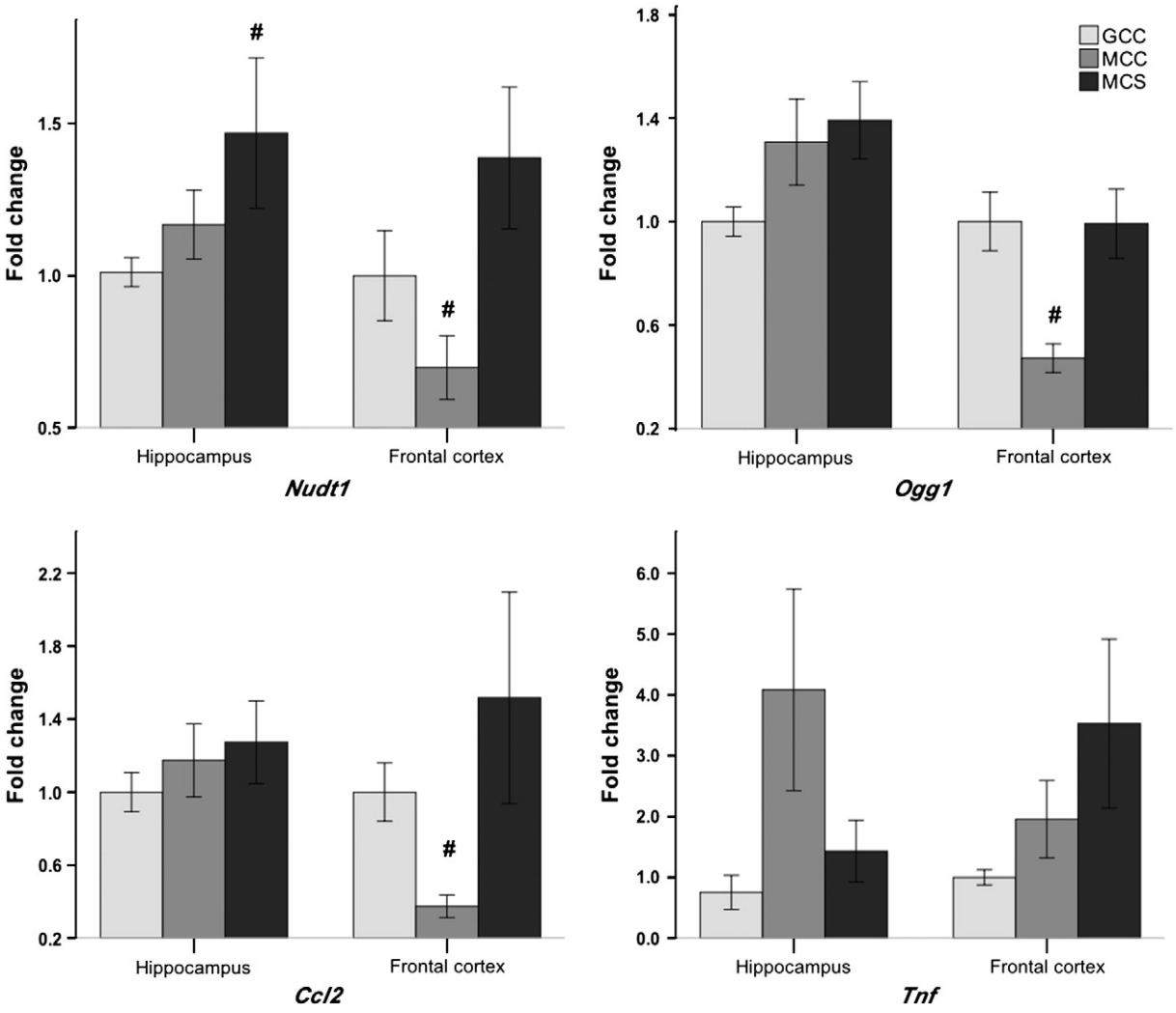

Fig. 5. Gene expression of nucleotide pool and DNA repair enzymes *Nudt1* and *Ogg1* (upper panel) and inflammation markers *Ccl2* and *Tnf* (lower panel) in the hippocampus and frontal cortex of stressed *vs.* control animals. Data are presented as fold change relative to group cage control. #Different from GCC ($P<0.05$, median test). GCC = group cage control, MCC = metabolism cage control, MCS = metabolism cage stress. Error bars = SEM.

generated genomic damage low during stress. The indication of an induction of DNA repair pathways in the brain after restraint stress should be further addressed in an independent study.

The animals used in the study were young adults. Given that both antioxidant defenses and DNA repair capacity may decrease with age (Imam et al., 2006; Sfar et al., 2009; Shih and Yen, 2007), it is possible that older animals would have exhibited more sensitivity to pro-oxidant effects of stress. In keeping with this notion, a recent study found that while restraint stress-induced changes in neuronal morphology were completely reversible after a recovery period in young animals, aged animals failed to reverse the same morphological changes after recovery (Bloss et al., 2010).

The study brings into question the mechanisms by which stress (as well as stress-related mental disorders such as depression) influences human morbidity and mortality. Based on our findings, it might be speculated that the age-accelerating effects of stress not only require increases in circulating glucocorticoids or other stress hormones, but also stress-induced life-style changes such as a more calorie-rich diet, less exercise and increased alcohol, tobacco and drug intake. Furthermore, biological variation in DNA repair capacity could be a mechanism underlying human sensitivity and resilience to stress and its associated detrimental somatic effects. In support of this hypothesis, recent evidence from a large human cohort indicates that single nucleotide polymorphisms in the APE1 and OGG1 gene influence the cognitive decline during normal aging (Lillenes et al., 2011).

Although the continuous monitoring of the urinary excretion of 8-oxodG/8-oxoGuo did not show differences between stressed and control animals, it is still possible that cerebral oxidative stress occurs as a transient phenomenon in the early phase of restraint stress exposure. In a recent study also applying the comet assay, levels of DNA strand breaks were elevated after the first day of restraint stress in the frontal cortex, amygdala and hippocampus, whereas after 1 week only the hippocampus showed elevated levels (Consiglio et al., 2010). Thus, after 21 days of restraint stress, the levels of DNA damage may have normalized, possibly due to DNA repair. In an animal model of Parkinson's disease, neuronal death induced by 6-hydroxydopamine resulted in a transient doubling of urinary 8-oxodG excretion (Kikuchi et al., 2011). Hence, if cerebral oxidative stress on nucleic acids is transiently elevated in the early phases after restraint stress, which involves much more subtle neuronal insults (Czeh and Lucassen, 2007), the amounts of free oxidized nucleosides generated might be too small to be detectable in the urine.

In contrast to the finding of others (Madrigal et al. , 2010), we found no signs of inflammation in terms of significant changes in the expression of *Ccl2*, except for a reduced level in the frontal cortex of the MCC's. With respect to the expression of *Tnf* we found that while the frontal cortex levels tended to be increased in the MCS group, there

was a remarkable but non-significant trend (median test, chi-square = 4.8, dF = 2, $P = 0.09$) towards the highest levels in the MCC group in the hippocampus. It could be speculated that the shift to the new environment of the metabolism cage stimulates an increase in hippocampal TNF, which is attenuated by the stress-induced corticosterone increase (Liao et al., 1995).

The sustained increase in urinary corticosterone contradicts the observation of a normalization of the blood levels of corticosterone after repeated stress exposure (Luine et al., 1996; Watanabe et al., 1992). This raises the possibility that while singular plasma corticosterone determinations may show normalization after repeated stress, the overall 24 h secretion of corticosterone – and thereby the general exposure to the hormone – remains elevated. This may explain the paradox that dendritic retraction in the CA3 pyramidal cells requires 3 weeks of stress in spite of a habituation of blood levels of corticosterone after 2 weeks (Luine et al., 1996; Watanabe et al., 1992). For the present experiment we deliberately did not introduce any invasive sampling methods, and therefore we are not able to further address the possible discrepancy between urinary and blood levels of corticosterone during restraint stress within the context of this study.

In contrast to urinary corticosterone, FCM excretion was only elevated on day 1 and 2 after stress initiation. A previous study in mice showed that a range of intravenously injected corticosterone doses were fully excreted in urine and feces, with a dose-independent 60/40% distribution, respectively (Kalliokoski et al., 2010). It is likely that the stress-induced alterations in food intake and reduction in fecal production influence the relative amount of corticosterone recovered in feces.

There are several limitations to the study: 1) the dissection of brain tissue allows only a relatively crude delineation of brain areas, and no distinction between cell types can be made. Consequently, levels of DNA damage and gene expression should be viewed only as a profile of a general biochemical milieu in the selected brain regions. 2) RT-PCR only provides information on mRNA levels, and does not necessarily imply alterations at the protein or activity level. 3) The fact that the restraint stress model appears to be valid and applicable when performed in metabolism cages opens new opportunities for the preclinical research in metabolic changes associated with stress and mental disorders. However, it should be noted that in many of the oxidative stress parameters measured in the study, an effect of metabolism cage housing in itself was observed. Furthermore, although non-significant, the MCC animals did exhibit a reduced weight gain during the course of the study compared to the GCC animals. Both of these observations could be explained by an increased energy expenditure due to the lack of cold protection from the litter and the cage-mate. 4) The study focused on oxidative stress on nucleic acids, which bears direct relevance to cellular aging processes and can be determined by valid, reliable and sensitive methods. However, we can not rule out that other markers of oxidative stress, such as 4-hydroxynonenal or malondialdehyde, would have exhibited changes after chronic stress, as previously observed by others (Madrigal et al., 2001, 2003). 5) The determination of the urinary nucleosides was limited to 4 time points, and although stress had no effect on marker excretion on any of these, we cannot completely rule out that differences between the groups could have occurred in the intervening time points.

5. Conclusion

In conclusion, we found that restraint stress performed in metabolism cages lead to a significant and sustained increase in the urinary excretion of corticosterone, whereas neither urinary nucleoside markers of systemic nucleic acid oxidation, nor hippocampal and frontal cortex levels of oxidatively generated DNA damage, were affected. The expression of DNA and nucleotide pool repair enzyme genes indicated an up-regulation in stressed animals (significant for hippocampal *Nudt1* in comparison to the GCC group), possibly reflecting a compensatory countermeasure to increased levels of genomic stress. The study questions whether it is an increased glucocorticoids *per se* that underlie signs of oxidative stress and telomere attrition observed in human psychological stress states. It further introduces cerebral enzymatic DNA repair as a potential component of the allostatic adaptation to stressful conditions.

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