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# Brain region-specific effects of long-term caloric restriction on redox balance of the aging rat

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ABSTRACT

Caloric restriction (CR) is the most effective intervention to improve health span and extend lifespan in pre-clinical models. This anti-aging effect of CR is related to attenuation of oxidative damage in various tissues, with divergent results in the brain. We addressed how brain oxidoreductive balance would be modulated in male Sprague-Dawley (SD) rats submitted to a 40% CR from 8 to 19 months of age, by reference to *ad libitum*-fed (AL) rats at 2 and 19 months of age. Four brain structures were compared: hippocampus, striatum, parietal cortex, cerebellum. Our CR diet elicits significant prevention of oxidative damages with the upregulation of antioxidant defenses (levels of glutathione [GSH], mRNAs of clusterin and of three key antioxidant enzymes) as compared to age-matched AL controls, in a strikingly region-specific pattern. CR also prevented a drastic rise of the glial fibrillary acidic protein in the hippocampus of old AL rats. Besides, the CR effects at age 19 months mainly consist in improving endogenous defenses before the onset of age-related redox alterations. These effects are more prominent in the hippocampus.

## 1. Introduction

Aging can be defined as a progressive accumulation of cell and tissue damages which increase the risk of chronic diseases and death (Harman, 2001). In the central nervous system, aging is associated with a progressive decline in cognitive performances, even in the absence of any neurodegenerative disease (Boyle et al., 2013; Verny et al., 2015). The free radical theory of aging proposes a causal role of the redox imbalance due to an overproduction of reactive oxygen species (ROS) / reactive nitrogen species (RNS) and a weakening of ROS/RNS-detoxifying systems (Harman, 2001; Pastore et al., 2015; Liochev, 2013; Walsh et al., 2014). Oxidative damage to macromolecules is indeed involved in most of the nine "hallmarks of aging" i.e.: mitochondrial dysfunction, genomic instability, loss of proteostasis, telomere attrition, epigenetic alterations, cellular senescence, stem cell exhaustion, deregulated nutrient-sensing, and altered intercellular communication (Lopez-Otin et al., 2013). The brain is known to be particularly vulnerable to oxidative damage considering its high metabolic activity, high oxygen consumption, high levels of oxidation-sensitive polyunsaturated fatty acids and lower levels of non-enzymatic and enzymatic antioxidants than in other tissues (Ahmed et al., 2015; Moyse et al., 2015).

Caloric restriction (CR) without malnutrition is recognized as a successful anti-aging strategy to prolong lifespan and health span, and slows down aging processes in yeasts, worms, flies, fish, rodents and non-human primates (Redman and Ravussin, 2011; Fontana and Partridge, 2015; Spindler, 2010; Anderson and Weindruch, 2012; Colman et al., 2014; Bédard et al., 2010, 2013, Moyse et al., 2012). In non-human primates though, the CR beneficial effect is still controversial (Mattison et al., 2012). In humans, CR was also reported to prevent obesity, diabetes, hypertension and cardiovascular diseases, and to diminish cancer. morbidity and mortality (Omodei and Fontana,

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2011). Moreover, CR can prevent brain alterations in a variety of animal models of cognitive decline and neurodegenerative disorders (Schafer et al., 2015; Parikh et al., 2016). The mechanisms underlying the beneficial effects of CR have been investigated (Anderson and Weindruch, 2012), particularly in the brain (Fusco and Pani, 2013) but with heterogeneous and partially discrepant results. These studies have focused on either one or few parameters, using diverse species or rodent strains, of either sex and on tissue extracts from one among various brain structures or even whole brain (Walsh et al., 2014). Therefore, the aim of the present study was to investigate whether CR-induced successful aging involves maintenance of antioxidant capacities and/or limitation of molecular oxidative damages in four brain regions, three of which are associated with human neurodegenerative disorders (*e.g.* hippocampus, striatum, parietal cortex) in comparison to the unrelated cerebellum. Implementation of CR was performed in the rat because this species displays higher beneficial responses to CR than the mouse (Swindell, 2012). A 40% CR was imposed to 8-month-old male Sprague Dawley (SD) rats for 11 months, without changing vitamins and mineral content. These conditions meet the reliability-enhancing criteria for experimental CR design in rodents (Spindler, 2012) and offers a significant maintenance of cognitive performances in comparison to *ad libitum*-fed (AL) 19-month-old male rats (Ménard et al., 2014). We characterized the region-specific effects of this long-term CR (LTCR) paradigm on key markers of oxidative damage, antioxidant defense and neural phenotypes in four brain structures with graded propensity to neuronal loss: hippocampus, striatum, parietal cortex and cerebellum.

## 2. Material and methods

### 2.1. Animals and diets

Six- to eight-week-old male SD rats were purchased from Charles River Canada (St-Constant, QC, CA). They were housed individually in standard plastic cages in temperature- (22°C), humidity- (65%) and lighting-controlled rooms (12:12-h light-dark cycles; lights on at 07:00), at the University of Montreal animal facilities dedicated to the Rat Colony Platform of the Quebec Network for Research on Aging (www.rqrv.com). They were fed AL a chow diet prepared according to Teklad control diet TD04088 (Harlan Teklad, Madison, WI), consisting of 499 g sucrose, 220 g casein (vitamin-free), 122 g corn starch, 57.5 g corn oil, 59 g cellulose, 35 g mineral mix, 1 g vitamin mix, 3 g L-methionine and 2 g choline bitartrate per kg chow. At 8 months, the rats were randomly assigned to one of two groups. The first group continued to be fed AL until euthanasy at 19 months of age while the second one was submitted to CR for the same duration. The mean body weight (BW) of each group was not significantly different from each other. This long-term CR was implemented at 8 months of age in two steps: 20% restriction for 2 weeks and 40% thereafter (Girard et al., 1998). Daily ration of each rat was adjusted to 60% of its AL intake at 8 months and no further adjustment was made. Rats submitted to CR were fed a Teklad chow TD04089 containing equivalent amounts of each energetic substrate (sucrose, casein, corn starch, corn oil) per kg chow as in the control diet, but enriched in micronutrients (vitamin mix, mineral mix, L-methionine, choline bitartrate) and containing half the cellulose load of the control diet. Consequently, CR rats received 40% less calories daily but equivalent amounts of micronutrients from the TD04089 fortified diet (Harlan Teklad) compared to AL rats. AL and CR rats were euthanized at 19 months of age by rapid decapitation, between 08:30 and 11:30 h, in a block-design fashion, along with 2-month-old AL-fed rats that stayed no longer than three weeks in our animal facilities. At the time of euthanasia, BW were 976 ± 25 g, 575 ± 11 g and 339 ± 5 g for the old AL, old CR and young AL groups, respectively. Brains were rapidly removed and transferred on ice to macrodissect the hippocampus, striatum, parietal cortex and cerebellum. Tissues were snap-frozen in liquid nitrogen and stored at −80 °C. Post-mortem macroscopic evaluation of old animals revealed no gross pathology or tumors. Large abdominal fat pads were observed in old AL rats. Additional information is available in previous publications with this model (Girard et al., 1998; Bédard et al., 2010; Moyse et al., 2012; Ménard et al., 2014). The animal protocols were approved by the *Centre Hospitalier de l'Université de Montréal* Research Center Animal Care Committee in compliance with guidelines of the Canadian Council on Animal Care.

### 2.2. Tissue preparation

A first series of frozen brain tissues from the three experimental groups described above (2 A L, 19 A L, 19 CR; n = 14 per group) was used for protein extractions. Frozen tissues were homogenized in phosphate buffer containing 1 mM ethylene-diamine-tetra-acetic acid (EDTA) and a cocktail of protease inhibitors (1/100; Sigma-Aldrich Canada, Oakville, ON, CA), using a glass/teflon potter tissue grinder. Homogenates were centrifuged and supernatants removed and stored at −80 °C. Proteins contents were quantified using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA) (Moyse et al., 2012). A second series of brain tissues from the three rat groups (n = 6 for each) was used for RNA extractions. Each frozen brain sample was homogenized in 400 µL TRIzol (Invitrogen Canada Inc., Burlington, ON, CA) as previously described (Moyse et al., 2012). Purity and concentration were assessed by spectrometry: 260/280 nm ratios were all above 1.9 and 20–100 µg of total RNA was obtain for each sample. Quality control of RNA was also assessed using a 2100 Bioanalyzer and the RNA 6000 Nano LabChip kit (Agilent Technologies, Mississauga, ON, CA). The RNA integrity number of each sample was > 9.0. Purified RNA pellets were resuspended in 10 µL RNAse-free distilled water (Ambion, Streetsville, ON, CA) and frozen at −80 °C.

### 2.3. Total thiol assay

Forty-µg samples of brain protein homogenates were added to 40 µL of 1 mM 5,5′-dithiobis-(2)-nitrobenzoic acid (DTNB) dissolved in phosphate buffer, followed by addition of 140 µL of 0.3 mM nicotinamide adenine dinucleotide phosphate (NADPH), solubilized in 0.1% NaHCO3 (Ellman, 1959). After a 20-min incubation at 37 °C, sample absorbance was measured at 405 nm using a microplate reader MRX TC Revelation from Dynex Technologies (Chantilly, VA, USA). Assays were performed in duplicate and the intra- and inter-assay CV was < 2% and 3%, respectively.

### 2.4. Reduced glutathione assay

Fifty-µL samples of brain homogenates containing fifty-µg of proteins were added to 100 µL of 100 µM monochlorobimane (MCB) as previously described (Moyse et al., 2015). The reaction was started with 1 U/mL glutathione S-transferase. After a 30-min incubation at room temperature (RT), fluorescence was measured using a Synergy HT multi-detection microplate reader from BioTek (Winooski, VT, USA) with excitation and emission at 360 nm and 460 nm, respectively. Raw data were compared to a standard curve performed with reduced glutathione (GSH) for quantification. Assays were performed in duplicate and the intra- and inter-assay CV was < 9 and < 3%, respectively.

### 2.5. Western blotting

For the quantification of γ-glutamyl-cysteine-synthetase (γ-GCS), Trx-1, astrocytic glial fibrillary acidic protein (GFAP) and neuronal nuclear antigen (NeuN), 30- or 50-µg protein homogenates were subjected to 10% or 12% SDS-PAGE gel electrophoresis and transferred onto PVDF membranes as previously reported (Moyse et al., 2015). Membranes were blocked for 1 h in Tris-buffered saline (TBS) containing 5% skim milk, rinsed and incubated overnight with a primary antibody at optimized dilution in TBS: γ-GCS (Millipore, 1:500), GFAP

(1/200) or NeuN (Millipore, 1:2000). Immunolabelled membranes were then rinsed and further incubated with an appropriate HRP-conjugated secondary antibody, either goat anti-rabbit (Santa-Cruz Biotechnologies, CA, USA; 1:10,000) or rabbit anti-mouse (Promega, Madison, WI, USA; 1:10,000) for 1 h at RT with each antibody. Detection was performed using the Immobilon Western Chemiluminescent horseradish peroxidase (HRP) substrate from Millipore (Mississauga, ON, CA). Bands were visualized and quantified using the chemoluminescent imaging system FluorChem HD2 (Alpha Innotech, San Leandro, CA, USA). Background labeling was assessed on membranes incubated in the absence of the primary antibody (Moyse et al., 2015). For determination of protein loading, all blotted membranes were stripped and further incubated 20 min at RT with an anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (1:20,000) followed by the HRP-conjugated secondary anti-mouse (1:10,000) antibody for 1 h at RT (Promega, Madison, WI, USA).

For the protein carbonyl assay, 20-µg samples of brain protein homogenates were denatured in 6% SDS and incubated with an equal volume of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2 N HCl 15 min at RT. Loading buffer was added and samples were separated by electrophoresis on 10% SDS-PAGE gels and transferred onto PVDF membranes (PerkinElmer, Montreal, QC, CA). Membranes were blocked for 1 h in TBS containing 5% skim milk and rinsed. DNP-derivatized protein carbonyls were labeled with a primary mouse monoclonal anti-DNP antibody (Sigma-Aldrich; 1:2000) followed after rinsing by HRP-conjugated anti-mouse secondary antibody (Promega, Madison, WI, USA; 1:10,000) for 1 h at RT. Detection and quantification of labelled bands were performed as described above. Background labeling was assessed on membranes incubated in the absence of the primary antibody (Singh et al., 2010).

### 2.6. GSH-peroxidase and GSH-reductase activity assays

Oxidized glutathione formation was measured in brain protein homogenates in the presence of *tert*-butyl-hydroxyperoxide (TBH) and NADPH consumption, in the presence of exogenously added GSH-reductase to ensure a constant level of GSH (Moyse et al., 2015). Fifty-µg sample of brain protein homogenates were preincubated (3 min, 37 °C) in 200 µL of 50 mM potassium phosphate buffer at pH 7.0, containing 1 mM GSH, 0.25 mM NADPH, 0.5 U GSH-reductase and 1 mM EDTA. The reaction was started by addition of 50 µL TBH (5 mM) and the rate of NADPH utilization was monitored at 340 nm during 5 min at 37 °C. Control values were taken as 100% and data presented GSH-peroxidase activity in % of control.

GSH-reductase activity was measured on 50-µg samples of brain protein homogenates in 150 □L of 50 mM potassium phosphate buffer at pH 7.5, containing 1 mM of oxidized L-glutathione, 0.2 mM NADPH and 1 mM EDTA (Singh et al., 2010). The decrease of absorbance at 340 nm due to NADPH oxidation was monitored at RT. The control values were taken as 100%, and data expressed as GSH-reductase activity in % of control. Assays were performed in triplicate and the intra- and inter-assay CV was ≤9% and ≤3%, respectively.

### 2.7. Total antioxidant capacity assay

The ability of tissue homogenates to degrade hydrogen peroxide ($H_2O_2$) was measured by electrochemical detection of H2O2, using the Free Radical Analyzer Apollo 4000 (World precision instrument, Sarasota, FL, USA). The analysis was conducted as previously described (Moyse et al., 2015) in 3 mL phosphate buffered saline (PBS) with continuous stirring at RT. Once the output signal was stabilized (around 10 min), increasing volumes (2.5, 5.0, 7.5, 10.0, 12.5 µl) of 0.3 mM $H_2O_2$ were added to the PBS solution containing 60 µg of brain protein homogenates. The output signal was allowed to stabilize after each addition. The results were analyzed by comparing the slopes obtained for each group of rats. Assays were performed in duplicate and intra- and inter-assay coefficient of variation (CV) were ≤2% and ≤4%, respectively.

### 2.8. RT-qPCR

Three µg of total RNA from each brain tissue sample were reversed-transcribed using the SuperScriptTM II RT kit (Invitrogen) and Oligo-dT(12–18) random primers (Invitrogen), in a total volume of 20 µL. Primer specificity of PCR products was established previously (supplementary table S1; Moyse et al., 2015). qPCR was performed using the QuantitectTM SYBR® Green PCR kit (Qiagen, Mississauga, ON, CA) in a Rotor-Gene 3000 real-time thermal cycler (Montreal Biotech Inc., Dorval, QC, CA). The results were analyzed with Rotor-Gene application software (version 6.0). A five-point standard curve was performed for each gene tested, using serial 1: 5 dilutions of RT-cDNA (1: 5 to 1: 3125) from the four brain regions of 2-month-old male rats. Normalization of mRNA levels was performed with β-actin as the reference gene. Relative mRNA levels were then determined in the old rat groups in comparison to those of 2-month-old rats (Pfaffl, 2001). The intra-assay CV of the various cycle threshold values was ≤2.5% in all experiments.

### 2.9. Statistical analysis

The results are expressed as means + SEM. Data sets from the three age groups were compared by one-way ANOVA, followed by the Tukey's multiple comparison test.

## 3. Results

### 3.1. Differential effects of caloric restriction on the tissue concentrations of total thiols, reduced glutathione and γ-glutamyl-cysteine-synthetase

Modifications of thiol-containing proteins by ROS represent an important aspect of redox dysregulation of signal transduction due to the presence on SH groups in the catalytic sites of numerous kinases and phosphatases. We therefore studied the impact of CR during aging on tissue concentrations of thiols and on GSH, the most abundant endogenous antioxidant in the brain. In the four brain structures of old rats, thiol levels were 20–38 % lower than those of young AL rats (Fig. 1). CR had no effect on age-induced decrease of thiol levels in striatum, but prevented their diminution in the three other regions examined (Fig. 1).

Tissue concentrations of GSH were not altered in any of the four brain structures of old AL rats in comparison to those of young AL rats (Fig. 2). Interestingly, CR was associated with a 60–80% increase of GSH concentrations in the hippocampus, striatum and cerebellum of old CR rats compared to those of young and old AL animals while they remained similar in the parietal cortex of the three rat groups (Fig. 2).

Since CR has a differential effect on GSH concentrations, we analyzed the levels of γ-GCS, the rate-limiting enzyme of *de novo* GSH biosynthesis. Differences were observed in basal immunoreactive levels of γ -GCS of the four brain structures under study but not with age and diet (Supplementary Fig. 1).

### 3.2. Glutathione peroxydase and reductase activities

None of the two GSH enzymatic activities differed between the brain structures of young and old rats (Fig. 3A, B). However, the GSH peroxidase activity was 287% and 122% higher in the hippocampus and parietal cortex of 19-month-old CR compared to old AL rats, respectively (Fig.3A).

The GSH reductase activity was 68% higher in the hippocampus of 19-month-old CR rats compared to that of young AL rats. It was also 37% and 81% higher in the striatum and cerebellum of old CR compared to old AL rats, respectively (Fig. 3B).

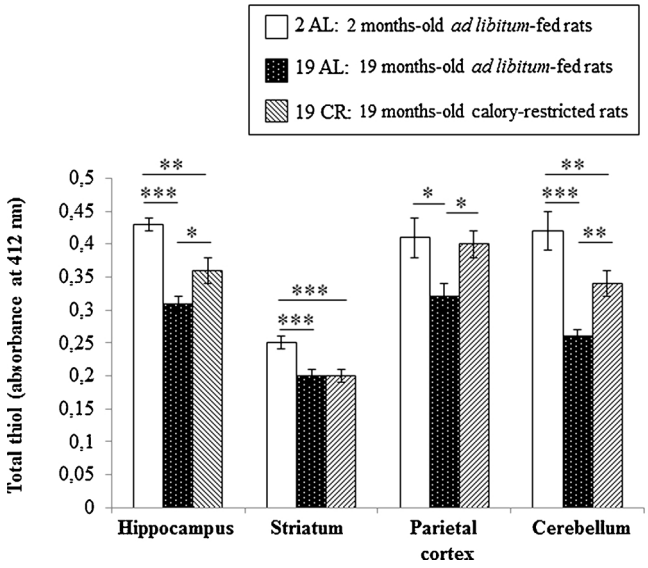


**Fig. 1.** Effects of age and CR on total thiol concentrations in hippocampus, striatum, cortex and cerebellum from male SD rats.
Thiol concentrations were quantified in brain tissue homogenates from calory-restricted (CR) and *ad libitum*-fed (AL) rats using DTNB. Thiol concentrations were lower in the four brain structures from old AL rats than in those from young ones. CR partially (hippocampus, cerebellum) or totally (parietal cortex) prevented this decrease. Data are means ± SEM of 4–6 rats for each brain structure, age and diet with *: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.001$.

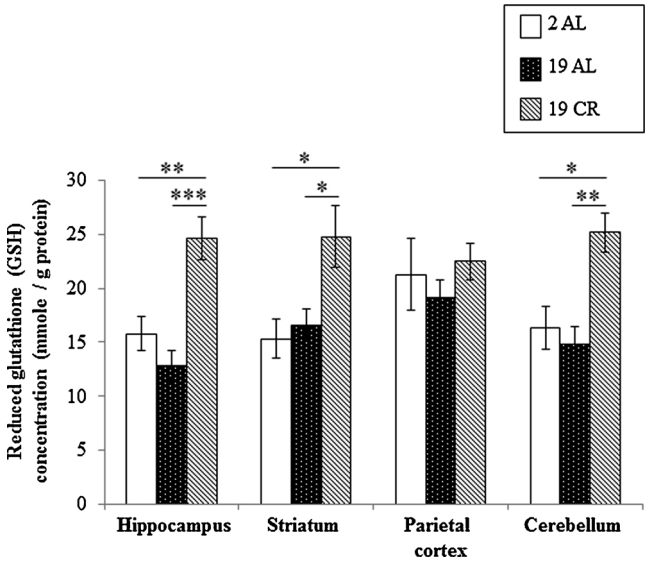


**Fig. 2.** Effects of age and CR on reduced GSH concentrations in male SD rat brain.
GSH levels were analyzed on proteins from tissue homogenates using the GSH-specific fluorescent probe MCB. GSH concentrations were significantly higher in old rats as compared to the two AL groups in hippocampus, striatum and cerebellum. Data are means ± SEM from 4 to 6 rats for each brain structure, rat age and diet, with *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$. Abbreviations for rat groups are the same as in Fig.1.

### 3.3. Total antioxidant capacity

The total antioxidant capacity was measured by the electrochemical slope of time-dependent H2O2 conversion by tissue homogenates. No age or diet-related difference was observed in the four brain regions studied (Table S2 and Fig. S2).

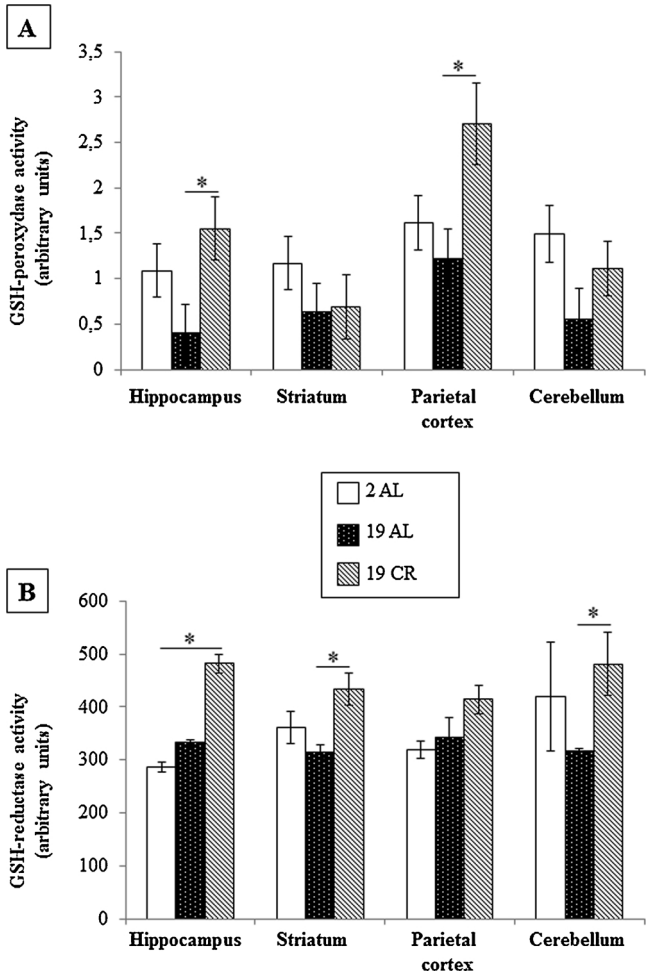


**Fig. 3.** Effects of age and CR on antioxidant enzyme activities in male SD rat brain.
GSH-peroxidase (A) and GSH-reductase (B) activities were quantified on protein extracts by substrate reduction colorimetric assay. Data are means ± SEM from 4 to 6 rats for each brain structure, rat age and diet, with *$P < 0.05$. Abbreviations for rat groups are the same as in Fig.1.

### 3.4. Total protein carbonyl content

The protein carbonyl content of the four brain structures was measured to compare the overall level of oxidative damage to proteins between the three rat groups. No significant effect of age or diet was observed (data not shown).

### 3.5. Thioredoxin-1 and glutaredoxin-1 mRNA levels

Trx-1 and glutaredoxin-1 (Glrx-1) enzymes play crucial roles in the preservation of reduced thiol-disulfide and GSH balance in the brain. Trx-1 facilitates the reduction of proteins by cysteine thiol-disulfide exchange while Glrx-1 specifically catalyzes their deglutathionylation. Trx-1 mRNA levels were 25% and 18% higher in the hippocampus of 19-month-old AL and CR rats, respectively, compared to young AL rats (Fig.4A). In the three other structures assayed, Trx-1 mRNA levels were not significantly different between the three rat groups (Fig. 4A).

Glrx-1 mRNA levels were 10–20% higher in the hippocampus, striatum, cerebellum of old AL rats compared to those of young AL rats. This increase was prevented in the striatum by CR (Fig. 4B). No difference of Glrx-1 mRNA levels was observed in the parietal cortex of the three rat groups (Fig. 4B).

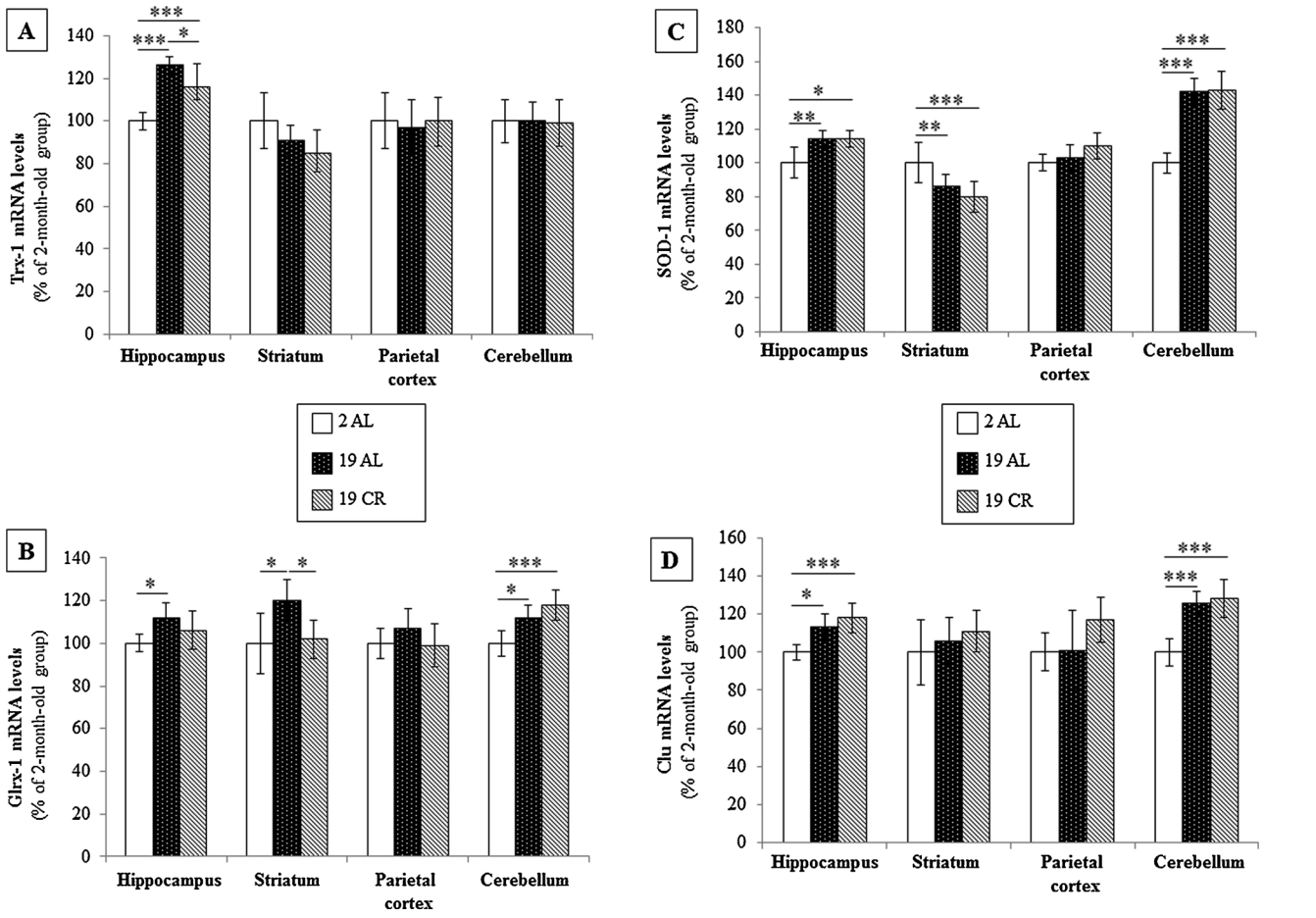


**Fig. 4.** Effects of age and diet on Trx-1 (A), Glrx-1 (B), SOD-1 (C), Clusterin (Clu, D) mRNA levels in brain structures from SD rats.
mRNA levels were analyzed by qPCR with normalization on age-independent β-actin expression. Statistical comparisons among ages for each structure were analyzed by ANOVA and Tukey's multiple comparison test. Data are means ± SEM from 4 to 6 rats for each group with: *, $p < 0.05$, **, $p < 0.01$ and ***, $p < 0.001$. Abbreviations for rat groups are the same as in Fig.1.

### 3.6. Superoxide dismutase-1 and Clusterin/apolipoprotein-J mRNA levels

SOD-1 mRNA levels were 15% and 40% higher in the hippocampus and cerebellum of old AL and CR rats compared to those of young AL rats, respectively, and 15% lower in the striatum of old AL and CR rats compared to those of young rats (Fig. 4C). They remained stable in the parietal cortex of the three groups of animals (Fig.4C) and were not different in the four brain structures of AL and CR old rats (Fig.4C).

Clu has emerged as an antioxidant and a neuroprotective protein (Lee et al., 2012). Clu mRNA levels were 15–25% higher in the hippocampus and cerebellum of old AL and CR rats compared to those of young AL rats. Age and CR did not affect their levels in the striatum and parietal cortex (Fig.4D).

### 3.7. Glial and neuronal immunoreactive levels

Since astrogliosis and neuronal loss are associated with brain aging, they were assessed using GFAP and NeuN immunoreactivity. GFAP immunoreactive levels increased by more than 300% in the hippocampus of old AL rats compared to those of young AL and old CR rats (Fig. 5). GFAP immunoreactivity was not altered by age or CR in the striatum and cerebellum (Fig. 5). In contrast, immunoreactive levels of NeuN were not regulated by age or CR in any of the four brain structures assessed (data not shown).

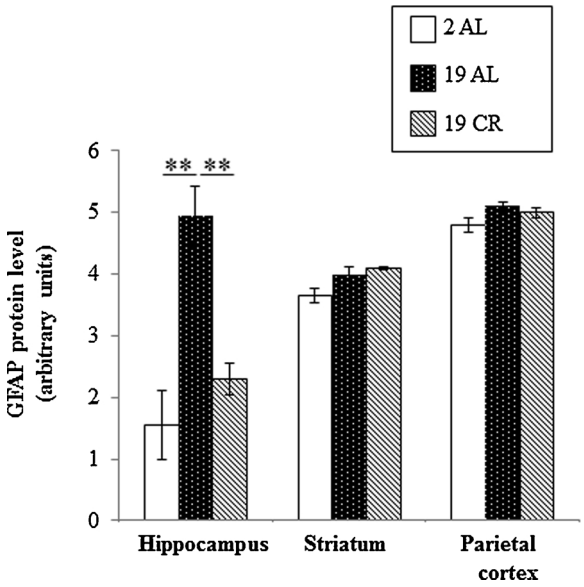


**Fig. 5.** Effects of age and diet on GFAP immunoreactive protein level in three rat brain structures, by Western-Nlot on protein extracts.
Statistical comparisons among ages for each structure were analyzed by ANOVA and Tukey's multiple comparison test. Data are means ± SEM from 4 to 6 rats for each group with **, $p < 0.01$. Abbreviations for rat groups are the same as in Fig.1.

## 4. Discussion

CR without malnutrition is recognized as a powerful manoeuvre to delay aging and extend lifespan in various phylogenic groups. The present comprehensive study shows that the RQRV paradigm of CR in adult SD male rat (http://www.rqrv.com) elicits some significant up-regulation of endogenous antioxidant defenses, in a strikingly region-specific pattern within the brain.

The experimental model species of the present study was the rat because in a recent meta-analysis, dietary restriction-induced lifespan extension averaged 14–45% in rat *vs* 4–27% in mouse (Swindell, 2012). Our CR paradigm is 40% caloric restriction without malnutrition, this diet being implemented at 8 months of age in male SD rats which corresponds to the mid-30 s age in humans, in order to avoid the period of rapid growth with persistence of growth cartilages and ongoing maturation of central nervous system (Kilborn et al., 2002). Our CR diet is maintained for 11 months duration, *i.e.* on the long-term, up to euthanasia in parallel with age-matched AL-fed rats for direct comparative evaluation of the CR impacts. This CR paradigm has been validated and shown to improve several health indexes since 20 years of colony-raising in the RQRV animal facilities (Girard et al., 1998; Bédard et al., 2010; Moyse et al., 2012; Ménard et al., 2014). At the brain level, LTCR prevents and/or largely minimizes the cognitive deficits observed in old male SD rats fed *ad libitum* using the novel object recognition test for the episodic and reference memory and the Morris water maze test of spatial learning (Menard et al., 2014). In addition, LTCR prevents the development of anxious behavior in the elevated-plus maze and open field tests (Menard et al., 2014) which has been confirmed (Parikh et al., 2016). Euthanasy and tissue sampling of old rats were performed at the age 19 months, which is less old than in most anterior studies of age-related oxidative damages in rat (Supplementary Tables S3, S4). Our aim was indeed to investigate early, putatively protective, effects of CR on oxidative status in the brain; consistently, oxidative damage markers in our AL old rats are not significantly different from young AL rats.

The four brain regions that were compared in the present study (*i.e.* hippocampus, striatum, parietal cortex, cerebellum) are known for their differential vulnerabilities to oxidative stress and neuronal degeneration. Hippocampus is considered as a most neurodegeneration-prone structure (Fan et al., 2017), especially with regard to oxidative stress and other insults (Abd El Mohsen et al., 2005; Perluigi et al., 2014). Hippocampal degeneration is involved in Alzheimer's disease and in other age-related cognitive deficits (Fan et al., 2017). An aging-related increase of the redox potential was reported in the CA1 and CA3 regions (Stebbings et al., 2016). The higher vulnerability of CA1 pyramidal neurons is related to a higher level of superoxide anions (Wang et al., 2005) and a higher release of mitochondrial ROS than in other brain regions (Mattiasson et al., 2003). This redox imbalance could be associated with the early decrease of hippocampal volume and memory and susceptibility to neurotoxic insults by glutamate or ß-amyloid peptides (Stebbings et al., 2016). In the striatum, dopaminergic neurons are especially prone to oxidative damage accumulation due to the auto-oxidation of dopamine releasing reactive quinone radicals in addition to its degradation by H2O2–producing monoamine oxidase. Such chronic oxidative stress facilitates Parkinson's disease-related neurodegeneration. In the parietal cortex, selective accumulation of β-amyloid deposits and oxidative damage leads to Alzheimer-like neurodegeneration (Hartley et al., 2017). In contrast, the cerebellum was reported to exhibit a greater resistance to oxidative stress (Elahi et al., 2016).

The present CR paradigm strikingly enhances the endogenous ROS-scavengers: protein thiols and/or reduced glutathione, in all four brain regions studied (Supplementary Table S3). Our data are consistent with previous reports showing that ROS concentrations were lower in the cerebrum, cerebellum (Baek et al., 1999), hippocampus and cerebral cortex (Ribeiro et al., 2012) of old Wistar rats submitted to CR in comparison to AL controls. Within the cellular "redoxome", protein cysteine thiols are used to sense changes of the redox environment (Thamsen and Jakob, 2011) as they represent a very important component of oxidoreductive homeostasis. They can undergo a range of redox modifications which include sulfenylation (*i.e.* formation of SOH by direct SH oxidation), protein thiyl radical formation (*e.g.* modification of a cysteine residue by the superoxide anion radical) or protein S-glutathionylation (Comini, 2016). In the present study, aging with AL diet consistently decreased thiol levels in all four brain regions assessed in comparison to young control rats (Supplementary Table S4). Oxidant-induced metabolites of thiols were indeed observed to be increased in previous studies of aging (Supplementary Table S4) and in some neurodegenerative disorders. For example, an increase of protein S-glutathionylation was identified in the striatum and parietal cortex of patients suffering from Huntington and Alzheimer's diseases, respectively, which may contribute to neuronal loss (Hong et al., 2015; Newman et al., 2007). In the present study, the CR intervention significantly prevented the age-induced thiols diminution in the brain structures assessed except for striatum (Supplementary Tables S3, S4).

Reduced glutathione (GSH) is the most important among direct ROS scavenger, in nervous cells as in other tissues, and it is raised by our LTCR paradigm, except in parietal cortex, with a striking effect in hippocampus and cerebellum (Supplementary Table S3). It is interesting to note that, in our age-matched AL controls, GSH levels were not decreased in any of the structures (Supplementary Table S4). A decrease of GSH with aging has been previously described in rats but it appears at older ages, around 30–35 months old (Supplementary Table S4), which indicates that our study addresses an early status of redox dysfunction in various regions of the brain. Secondly, this result demonstrates that LTCR induces a preventive mechanism (increase of GSH level) in the rat brain against subsequent age-related oxidative insults, which consists in specific biological response(s) to this regimen. Our observations thus reinforce the physiological relevance of comparing CR to AL feeding for the field of aging biology, which has been debated (Sohal and Forster, 2014).

Such preventive inductions of protective antioxidant effectors by CR, prior to age-related deficits onset, were also observed in the present study for two other antioxidant defenses: the two main GSH-regenerating enzymes, GSH-peroxidase and GSH-reductase, both and all the most in hippocampus (Supplementary Tables S3, S4). The elevation of GSH system in hippocampus could explain the maintenance of cognitive performance in LTCR rats previously described (Ménard et al., 2014) as elevation of GSH in hippocampus can improve memory deficits (Yabuki and Fukunaga, 2013).

The levels of brain oxidative damage were assessed using protein carbonyls that are produced by a wide range of oxidizing species (Amici et al., 1989) and are thus considered a robust marker of oxidative stress. No effect of age or CR was observed in the four brain structures studied. Data on brain protein carbonylation remain inconsistent in the literature since their levels depend on several different factors (*i.e.* rat strain, brain structure, age of euthanasia, methods of quantification; Supplementary Table S4). It is worth noting that many studies have assessed protein carbonyl tissue levels using the classical spectrophotometric approach. This method is widely used to estimate protein carbonyls but several drawbacks and pitfalls have been identified (Rogowska-Wrzesinska et al., 2014) such as non-protein carbonyl derivatives (Luo et al., 2009). The lack of change in brain carbonyl content with age or CR is observed concurrently with unaltered total antioxidant capacity and γ-GCS activity, and further suggests that we are analyzing an early phase of redox dysfunction in the present study.

Additional regulatory levels are the expression (mRNA) levels of the enzymes thioredoxine-1 (Trx1), glutaredoxin-1 (Glrx), superoxidismutase-1 (SOD1), keeping protein thiols in the reduced state and low levels of superoxide anions (Miao and St Clair, 2009; Fukai and Ushio-Fukai, 2011). Thioredoxins (Trx) are key enzymes regulating the cellular redox state and are involved in a variety of redox-dependent

pathways to reduce intramolecular disulfide bonds (Cunningham et al., 2015). The expression level of Trx-1, the major and cytosolic isoform of Trx, was shown to correlate with organismal lifespan and age-associated tissue deterioration (Mitsui et al., 2002). Glutaredoxin (Glrx-1) maintains neuronal cell viability under oxidative stress by restoring the steady-state function of oxidized proteins at cysteine residues (Lillig et al., 2008). Our results on Glrx-1 mRNA levels suggest an enhancement of protein oxidation with age in the hippocampus, striatum and cerebellum. and CR could prevent these oxidative modifications in the striatum. SOD-1, *i.e.* the cytosolic and Cu/Zn-dependent superoxide dismutase, is known to remove 90% of superoxide anions produced in neurons (Gandhi and Abramov, 2012), but its expression and activity in the aging brain are controversial. The activity of SOD-1 was reported to be unchanged in the whole rat brain (Sahoo and Chainy, 1997) as well as in specific brain structures (*e.g.* cortex, hippocampus, striatum, hypothalamus and cerebellum) of old Wistar (Danh et al., 1983) and Fisher 344 rats (Tian et al., 1998) and its expression was shown to vary with the rat strain. Our data show that SOD-1 mRNA levels were elevated in hippocampus and cerebellum and decreased in striatum through aging of male SD rats, but were unaffected by CR. The present data suggest that the beneficial effects of CR on redox homeostasis are mainly mediated by a more chemically-reduced GSH pool, rather than by enzymatically decreasing mitochondrial superoxide anions production (Walsh et al., 2014). However, it must be reminded that many enzyme activities are modulated at the post-traductional level, which leads to soften interpretation of transcriptomic data.

Clusterin (Clu) is up-regulated following exposure to oxidative stress (Michel et al., 1997; Rohne et al., 2014; Strocchi et al., 2006) which is in keeping with its present age-related overexpression in the hippocampus and cerebellum of AL rats. Clu overexpression is associated with higher tolerance to oxidative stress while its suppression makes cells more sensitive to oxidative stress (Viard et al.,1999), in line with the existence of several putative binding sites for redox-responsive transcription factors in its DNA sequence (Sen and Packer, 2006). If Clu may represent a central molecule in cell homeostasis under oxidative stress conditions (Trougakos, 2013), our data indicate that it would not mediate CR beneficial effect in the brain structures assessed here.

Among the four structures investigated in the present study, the most important effects of LTCR were observed in hippocampus (Supplementary Table S3) i.e.: prevention of age-related decrease of thiol concentration, higher tissue concentration of aging-independent GSH, strikingly higher levels of GSH-peroxydase and GSH-reductase activities, prevention of the drastic age-related increase of GFAP content. In addition, our results show that astrogliosis *i.e.* increase in GFAP expression, occurs specifically in the hippocampus of 19-month-old AL rats, in line with what has been observed by immunohistochemistry in the CA1 region (Hayakawa et al., 2007). We also show that this age-induced increase of hippocampal GFAP content is prevented by LTCR, which has been reported at the mRNA level (Major et al., 1997). It is interesting to note that hippocampal astrogliosis occurred in the absence of neuronal loss, as evidenced here by stable immunoreactive NeuN levels. The astrogliosis observed in hippocampus of 19-month-old AL rats may represent an early process preceding neurodegeneration, and the neuroprotective effects of CR could involve a modulation of astrocytic functions likely by reducing inflammation and oxidative stress.

Based on current knowledge, all the present LTCR-induced alterations, when compared to AL brain aging, were putatively beneficial with regards to both oxidative damages and cognitive function, and further strengthen LTCR as a “successful aging model” in rat. It is therefore interesting to compare the present results with the age-dependent variations of redox biomarkers in the same brain regions of another model of successful aging: the LOU/C rat (Moyse et al., 2015). The LOU/C rat is an obesity-resistant strain with higher longevity and health span than common rats, displaying maintenance of the cognitive functions with age (Bédard et al., 2010; Ménard et al., 2014). As summarized in Supplementary Table 5, the main difference between the models is the elevation of GSH under CR in hippocampus, striatum and cerebellum by comparison to young SD rats. In contrast, in the hippocampus of the male LOU/C and CR rat, thiols and mRNA Glrx1 levels remain comparable to those of young animals while Trx1 mRNA levels increase. Given the extent of changes observed in different oxidative markers in both models, it is clear that a reductive redox environment operates in both CR and LOU/C rat brains, which could contribute to the preservation of their cognitive functions in old age. However the oxidative metabolic pathways are differentially regulated in the brains of both rat models.

In a translational perspective, the present data further document the global health span-increasing effect of CR that has emerged from numerous studies in rodents and nonhuman primates. As recently reviewed (Balasubramanian et al., 2017) CR delays aging and major age-related diseases *i.e.* diabetes, atherosclerosis and cardiovascular disease, sarcopenia, arthritis, osteoporosis, incidence and progression of cancer, inflammation, immune deficiency, brain atrophy and cognitive decline. Moreover, serum from humans on long-term CR was shown to enhance *in vitro* stress resistance of cell cultures (Omodei et al., 2013). The discovery of underlying biological mechanisms has powerful translational potential for preventive health span extension of humans (Balasubramanian et al., 2017). CR without malnutrition seems difficult to achieve nowadays in developed countries but several chemical compounds such as resveratrol, rapamycin or metformin are being proposed as “caloric restriction mimetics” (Fontana and Partridge, 2015; Balasubramanian et al., 2017). Consistent with the present study, these CR mimetics were reported to decrease oxidative damage and increase antioxidant defence in adult rat brain (Garg et al., 2017). In the case of brain and oxidative damage, the translational significance of our result as regards the lack of glutathione decrease in the brain of 19-month-old AL rats, is supported by similar findings observed in a human cohort up to 99 years of age (Tong et al., 2016). The present study thus prompts to confirm the positive impact of CR-mimicking drugs on human cognition *via* clinical studies.

## 5. Conclusions

By comparison between the 19 month-old and young AL-fed rat groups, our comprehensive multiparameter assay revealed heterogeneity of age impact on oxidoreductive balance among four unrelated brain structures. Our CR diet elicits significant decrease of oxidative damages and upregulation of antioxidant defenses as compared to age-matched AL controls, in a strikingly region-specific pattern. These CR effects are most prominent in the hippocampus, which is the most prone to age-related dysfunction among the four structures studied. The beneficial effects of CR on oxidoreductive balance fall into two categories: CR prevention of AL-fed aging-induced alterations of the brain redox state, and CR-induced protective alterations of aging-insensitive parameters. Our study thus demonstrates that long-term caloric restriction actively triggers some specific physiological mechanisms to counteract deleterious cellular dysfunctions prior to their onset in the aging brain.

## Conflict of interest

The authors declare that they have no competing interests.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.mad.2019.01.002.

## References


Abd El Mohsen, M.M., Iravani, M.M., Spencer, J.P., Rose, S., Fahim, A.T., Motawi, T.M., Ismail, N.A., Jenner, P., 2005. Age-associated changes in protein oxidation and proteasome activities in rat brain: modulation by antioxidants. Biochem. Biophys. Res. Commun. 336 (2), 386–391.

Ahmed, T., Gilani, A.U., Abdollahi, M., Daglia, M., Nabavi, S.F., Nabavi, S.M., 2015. Berberine and neurodegeneration: a review of literature. Pharmacol. Rep. 67 (5), 970–979. https://doi.org/10.1016/j.pharep.2015.03.002.

Amici, A., Levine, R.L., Tsai, L., Stadtman, E.R., 1989. Conversion of amino acid residues in proteins and amino acid homopolymers to carbonyl derivatives by metal-catalyzed oxidation reactions. J. Biol. Chem. 264 (6), 3341–3346.

Anderson, R.M., Weindruch, R., 2012. The caloric restriction paradigm: implications for healthy human aging. Am. J. Hum. Biol. 24 (2), 101–106. https://doi.org/10.1002/ajhb.22243.

Baek, B.S., Kwon, H.J., Lee, K.H., Yoo, M.A., Kim, K.W., Ikeno, Y., Yu, B.P., Chung, H.Y., 1999. Regional difference of ROS generation, lipid peroxidation, and antioxidant enzyme activity in rat brain and their dietary modulation. Archiv. Pharm. Res. 22, 361–366.

Balasubramanian, P., Howell, P.R., Anderson, R.M., 2017. Aging and caloric restriction research : a biological perspective with translational potential. EBioMedicine 21, 37–44. https://doi.org/10.1016/j.ebiom.2017.06.015.

Bédard, K., Robinette, K., Ferland, G., Gaudreau, P., 2010. Effects of long-term dietary interventions on pituitary growth hormone-releasing hormone receptor in aging rats and potential mechanisms of action. Mech. Ageing Dev. 131 (3), 169–178.

Bédard, K., Bédard, J., Rocheleau, G., Ferland, G., Gaudreau, P., 2013. Aging and diets regulate the rat anterior pituitary transcriptome. Neuroendocrinology 97 (2), 146–159. https://doi.org/10.1159/000338411.

Boyle, P.A., Yu, L., Wilson, R.S., Schneider, J.A., Bennett, D.A., 2013. Relation of neuropathology with cognitive decline among older persons without dementia. Front. Aging Neurosci. 5, 50. https://doi.org/10.3389/fnagi.2013.00050.

Colman, R.J., Beasley, T.M., Kemnitz, J.W., Johnson, S.C., Weindruch, R., Anderson, R.M., 2014. Caloric restriction reduces age-related and all-cause mortality in rhesus monkeys. Nat. Commun. 5, 3557. https://doi.org/10.1038/ncomms4557.

Comini, M.A., 2016. Measurement and meaning of cellular thiol:disulfide redox status. Free Radic. Res. 50 (7), 246–271. https://doi.org/10.3109/10715762.2015.1110241.

Cunningham, G.M., Roman, M.G., Flores, L.C., Hubbard, G.B., Salmon, A.B., Zhang, Y., Gelfond, J., Ikeno, Y., 2015. The paradoxical role of thioredoxin on oxidative stress and aging. Arch. Biochem. Biophys. 576, 32–38. https://doi.org/10.1016/j.abb.2015.02.025.

Danh, H.C., Benedetti, M.S., Dostert, P., 1983. Differential changes in superoxide dismutase activity in brain and liver of old rats and mice. J. Neurochem. 40, 1003–1007.

Elahi, M., Hasan, Z., Motoi, Y., Matsumoto, S.E., Ishiguro, K., Hattori, N., 2016. Region-specific vulnerability to oxidative stress, neuroinflammation, and tau hyperphosphorylation in experimental diabetes mellitus mice. J. Alzheimers Dis. 51 (4), 1209–1224.

Ellman, G.L., 1959. Tissue sulfhydryl groups. Arch. Biochem. Biophys. 82, 70–77.

Fan, X., Wheatley, E.G., Villeda, S.A., 2017. Mechanisms of hippocampal aging and the potential for rejuvenation. Annu. Rev. Neurosci. 40, 251–272. https://doi.org/10.1146/annurev-neuro-072116-031357.

Fontana, L., Partridge, L., 2015. Promoting health and longevity through diet: from model organisms to humans. Cell 161, 106–118. https://doi.org/10.1016/j.cell.2015.02.020.

Fukai, T., Ushio-Fukai, M., 2011. Superoxide dismutases: role in redox signaling, vascular function, and diseases. Antioxid. Redox Signal. 15 (6), 1583–1606. https://doi.org/10.1089/ars.2011.3999.

Fusco, S., Pani, G., 2013. Brain response to calorie restriction. Cell. Mol. Life Sci. 70 (17), 3157–3170. https://doi.org/10.1007/s00018-012-1223-y.

Gandhi, S., Abramov, A.Y., 2012. Mechanism of oxidative stress in neurodegeneration. Oxid. Med. Cell. Longev. 2012, 428010. https://doi.org/10.1155/2012/428010.

Garg, G., Singh, S., Singh, A.K., Rizvi, S.I., 2017. Antiaging effect of metformin on brain in naturally aged and accelerated senescence model of rat. Rejuvenation Res. 20 (3), 173–182. https://doi.org/10.1089/rej.2016.1883.

Girard, N., Ferland, G., Boulanger, L., Gaudreau, P., 1998. Long-term calorie restriction protects rat pituitary growth hormone-releasing hormone binding sites from age-related alterations. Neuroendocrinology 68, 21–29.

Harman, D., 2001. Aging: overview. Ann. N. Y. Acad. Sci. 928, 1–21.

Hartley, S.L., Handen, B.L., Devenny, D., Mihaila, I., Hardison, R., Lao, P.J., Klunk, W.E., Bulova, P., Johnson, S.C., Christian, B.T., 2017. Cognitive decline and brain amyloid-β accumulation across 3 years in adults with down syndrome. Neurobiol. Aging 58, 68–76.

Hayakawa, N., Kato, H., Araki, T., 2007. Age-related changes of astorocytes, oligodendrocytes and microglia in the mouse hippocampal CA1 sector. Mech. Ageing Dev. 128, 311–316. https://doi.org/10.1016/j.mad.2007.01.005.

Kilborn, S.H., Trudel, G., Uhthoff, H., 2002. Review of growth plate closure compared with age at sexual maturity and lifespan in laboratory animals. Contemp. Top. Lab. Anim. Sci. 41 (5), 21–26.

Lee, Y.N., Shim, Y.J., Kang, B.H., Park, J.J., Min, B.H., 2012. Over-expression of human clusterin increases stress resistance and extends lifespan in Drosophila melanogaster. Biochem. Biophys. Res. Commun. 420 (4), 851–856.

Lillig, C.H., Berndt, C., Holmgren, A., 2008. Glutaredoxin systems. Biochim. Biophys. Acta 1780, 1304–1317.

Liochev, S.I., 2013. Reactive oxygen species and the free radical theory of aging. Free Radic. Biol. Med. 60, 1–4.

Lopez-Otin, C., Blasco, M.A., Partridge, L., Serrano, M., Kroemer, G., 2013. The hallmarks of aging. Cell 153, 1194–1217.

Major, D.E., Kesslak, J.P., Cotman, C.W., Finch, C.E., Day, J.R., 1997. Life-long dietary restriction attenuates age-related increases in hippocampal glial fibrillary acidic protein mRNA. Neurobiol. Aging 18, 523–526.

Mattiasson, G., Friberg, H., Hansson, M., Elmér, E., Wieloch, T., 2003. Flow cytometric analysis of mitochondria from CA1 and CA3 regions of rat hippocampus reveals differences in permeability transition pore activation. J. Neurochem. 87 (2), 532–544.

Mattison, J.A., Roth, G.S., Beasley, T.M., Tilmont, E.M., Handy, A.M., Herbert, R.L., Longo, D.L., Allison, D.B., Young, J.E., Bryant, M., Barnard, D., Ward, W.F., Qi, W., Ingram, D.K., de Cabo, R., 2012. Impact of caloric restriction on health and survival in rhesus monkeys from the NIA study. Nature 489, 318–321. https://doi.org/10.1038/nature11432.

Ménard, C., Quirion, R., Bouchard, S., Ferland, G., Gaudreau, P., 2014. Glutamatergic signaling and low prodynorphin expression are associated with intact memory and reduced anxiety in rat models of healthy aging. Front. Aging Neurosci. 6 (81), 1–21. https://doi.org/10.3389/fnagi.2014.00081.

Miao, L., St Clair, D.K., 2009. Regulation of superoxide dismutase genes: implications in disease. Free Radic. Biol. Med. 47 (4), 344–356.

Michel, D., Chatelain, G., North, S., Brun, G., 1997. Stress-induced transcription of the clusterin/apoJ gene. Biochem. J. 328 (Pt. 1), 45–50.

Mitsui, A., Hamuro, J., Nakamura, H., Kondo, N., Hirabayashi, Y., Ishizaki-Koizumi, S., Hirakawa, T., Inoue, T., Yodoi, J., 2002. Overexpression of human thioredoxin in transgenic mice controls oxidative stress and life span. Antioxid. Redox Signal. 4, 693–696. https://doi.org/10.1089/15230860260220201.

Moyse, E., Bédard, K., Segura, S., Mahaut, S., Tardivel, C., Ferland, G., Lebrun, B., Gaudreau, P., 2012. Effects of aging and caloric restriction on brainstem satiety center signals in rats. Mech. Ageing Dev. 133 (2-3), 83–91. https://doi.org/10.1016/j.mad.2012.01.004.

Moyse, E., Arseneault, M., Gaudreau, P., Ferland, G., Ramassamy, C., 2015. Gender- and region-dependent changes of redox biomarkers in the brain of successfully aging LOU/C rats. Mech. Ageing Dev. 149, 19–30.

Omodei, D., Fontana, L., 2011. Calorie restriction and prevention of age-associated chronic disease. FEBS Lett. 585, 1537–1542. https://doi.org/10.1016/j.febslet.2011.03.015.

Omodei, D., Licastro, D., Salvatore, F., Crosby, S.D., Fontana, L., 2013. Serum from humans on long-term calorie restriction enhances stress resistance in cell culture. Aging (Albany NY) 5 (8), 599–606.

Parikh, I., Guo, J., Chuang, K.H., Zhong, Y., Rempe, R.G., Hoffman, J.D., Armstrong, R., Bauer, B., Hartz, A.M., Lin, A.L., 2016. Caloric restriction preserves memory and reduces anxiety of aging mice with early enhancement of neurovascular functions. Aging 8 (11), 2814–2826.

Pastore, A., Petrillo, S., Piermarini, E., Piemonte, F., 2015. Systemic redox biomarkers in neurodegenerative diseases. Curr. Drug Metab. 16 (1), 46–70.

Perluigi, M., Swomley, A.M., Butterfield, D.A., 2014. Redox proteomics and the dynamic molecular landscape of the aging brain. Ageing Res. Rev. 13, 75–89. https://doi.org/10.1016/j.arr.2013.12.005.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, e45.

Redman, L.M., Ravussin, E., 2011. Caloric restriction in humans: impact on physiological, psychological and behavioral outcomes. Antioxid. Redox Signal. 14 (2), 275–287. https://doi.org/10.1089/ars.2010.3253.

Ribeiro, L.C., Rodrigues, L., Quincozes-Santos, A., Tramontina, A.C., Bambini-Junior, V., Zanotto, C., Diehl, L.A., Biasibetti, R., Kleinkauf-Rocha, J., Dalmaz, C., Goncalves, C.A., Gottfried, C., 2012. Caloric restriction improves basal redox parameters in hippocampus and cerebral cortex of Wistar rats. Brain Res. 1472, 11–19. https://doi.org/10.1016/j.brainres.2012.07.021.

Rogowska-Wrzesinska, A., Wojdyla, K., Nedić, O., Baron, C.P., Griffiths, H.R., 2014. Analysis of protein carbonylation - pitfalls and promise in commonly used methods. Free Radic. Res. 48 (10), 1145–1162.

Rohne, P., Prochnow, H., Wolf, S., Renner, B., Koch-Brandt, C., 2014. The chaperone activity of clusterin is dependent on glycosylation and redox environment. Cell. Physiol. Biochem. 34 (5), 1626–1639. https://doi.org/10.1159/000366365.

Sahoo, A., Chainy, G.B., 1997. Alterations in the activities of cerebral antioxidant enzymes of rat are related to aging. Int. J. Dev. Neurosci. 15, 939–948.

Schafer, M.J., Alldred, M.J., Lee, S.H., Calhoun, M.E., Petkova, E., Mathews, P.M., Ginsberg, S.D., 2015. Reduction of β-amyloid and γ-secretase by calorie restriction in female Tg2576 mice. Neurobiol. Aging 36 (3), 1293–1302.

Singh, M., Murthy, V., Ramassamy, C., 2010. Modulation of hydrogen peroxide and acrolein-induced oxidative stress, mitochondrial dysfunctions and redox regulated pathways by the bacopa monniera extract: potential implication in Alzheimer's disease. J. Alzheimers Dis. 21, 229–247.

Sohal, R.S., Forster, M.J., 2014. Caloric restriction and the aging process: a critique. Free Radic. Biol. Med. 73, 366–382.

Spindler, S.R., 2010. Caloric restriction: from soup to nuts. Ageing Res. Rev. 9 (3), 324–353.

Spindler, S.R., 2012. Review of the literature and suggestions for the design of rodent survival studies for the identification of compounds that increase health and life span. Age(Dordr) 34 (1), 111–120.
Stebbings, K.A., Choi, H.W., Ravindra, A., Llano, D.A., 2016. The impact of aging, hearing loss, and body weight on mouse hippocampal redox state, measured in brain slices using fluorescence imaging. Neurobiol. Aging 42, 101–109.
Strocchi, P., Smith, M.A., Perry, G., Tamagno, E., Danni, O., Pession, A., Gaiba, A., Dozza, B., 2006. Clusterin up-regulation following sub-lethal oxidative stress and lipid peroxidation in human neuroblastoma cells. Neurobiol. Aging 27 (11), 1588–1594.
Swindell, W.R., 2012. Dietary restriction in rats and mice: a meta-analysis and review of the evidence for genotype-dependent effects on lifespan. Ageing Res. Rev. 11, 254–270.
Thamsen, M., Jakob, U., 2011. The redoxome: proteomic analysis of cellular redox networks. Curr. Opin. Chem. Biol. 15, 113–119.
Tian, L., Cai, Q., Wei, H., 1998. Alterations of antioxidant enzymes and oxidative damage to macromolecules in different organs of rats during aging. Free Radic. Biol. Med. 24, 1477–1484.
Tong, J., Fitzmaurine, P.S., Moszczynska, A., Mattina, K., Ang, L.C., Boileau, I., Furukawa, Y., Sailasuta, N., Kish, S.J., 2016. Do glutathione levels decline in aging human brain? Free Radic. Biol. Med. 93, 110–117. https://doi.org/10.1016/j.freeradbiomed.2016.01.029.
Trougakos, I.P., 2013. The molecular chaperone Apolipoprotein J/Clusterin as a sensor of oxidative stress: implications in therapeutic approaches – a mini-review. Gerontology 59, 514–523.
Verny, M., Moyse, E., Krantic, S., 2015. Successful cognitive aging: beween functional decline and failure of compensatory mechanisms. Biomed Res. Int. 2015, 367407.
Viard, I., Wehrli, P., Jornot, L., Bullani, R., Vechietti, J.L., Schifferli, J.A., Tschopp, J., French, L.E., 1999. Clusterin gene expression mediates resistance to apoptotic cell death induced by heat shock and oxidative stress. J. Invest. Dermatol. 112, 290–296.
Walsh, M.E., Shi, Y., Van Remmen, H., 2014. The effects of dietary restriction on oxidative stress in rodents. Free Radic. Biol. Med. 66, 88–99.
Wang, X., Pal, R., Chen, X.W., Limpeanchob, N., Kumar, K.N., Michaelis, E.K., 2005. High intrinsic oxidative stress may underlie selective vulnerability of the hippocampal CA1 region. Brain Res. Mol. Brain Res. 140 (1-2), 120–126.
Yabuki, Y., Fukunaga, K., 2013. Oral administration of glutathione improves memory deficits following transient brain ischemia by reducing brain oxidative stress. Neuroscience 250, 394–407. https://doi.org/10.1016/j.neuroscience.2013.07.017.